Effects of combination of leaf resources on competition in container mosquito larvae

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Abstract

Resource diversity is critical to fitness in many insect species, and may determine the coexistence of competitive species and the function of ecosystems. Plant material provides the nutritional base for numerous aquatic systems, yet the consequences of diversity of plant material have not been studied in aquatic container systems important for the production of mosquitoes. To address how diversity in leaf detritus affects container-inhabiting mosquitoes, we examined how leaf species affect competition between two container inhabiting mosquito larvae, *Aedes aegypti* and *Aedes albopictus*, that co-occur in many parts of the world. We tested the hypotheses that leaf species changes the outcome of intra- and interspecific competition between these mosquito species, and that combinations of leaf species affect competition in a manner not predictable based upon the response to each leaf species alone (i.e. the response to leaf combinations is non-additive). We find support for our first hypothesis that leaf species can affect competition, evidence that, in general, leaf combination alters competitive interactions, and no support that leaf combination impacts interspecific competition differently than intraspecific competition. We conclude that combinations of leaves increase mosquito production non-additively such that combinations of leaves act synergistically, in general, and result in higher total yield of adult mosquitoes in most cases, although certain leaf combinations for *A. albopictus* are antagonistic. We also conclude that leaf diversity does not have a different effect on interspecific competition between *A. aegypti* and *A. albopictus*, relative to intraspecific competition for each mosquito.

Keywords: *Aedes*, detritus, invasive species, diversity-productivity relationship

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Introduction

Resource diversity can affect animal fitness (reviewed in Pyke, 1984), and the importance of acquiring a diverse and complimentary diet for increased fitness has been well known for terrestrial arthropods (Greenstone, 1979; Waldbauer &

Friedman, 1991; Eubanks & Denno, 1999; Behmer, 2009). Furthermore, resource diversity may contribute to the coexistence of insects with similar, or seemingly similar, nutritional niches (Behmer & Joern, 2008). Resource diversity is often acquired by consumer choice (Greenstone, 1979; Behmer & Joern, 2008); however, many terrestrial and aquatic detritus-based environments may not allow for obvious, active behavioral choices by consumers, especially constrained systems like the small, lentic, aquatic habitats found in tree holes, rock pools and natural and artificial containers dominated by mosquitoes. The consequences of resource

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diversity on insect fitness in these systems may be profound because consumers confined to a delimited habitat may not have the freedom of choice available in less circumscribed systems. Resource diversity in these 'container' habitats may help address two fundamental questions in community ecology: the coexistence of ecologically similar species (Hutchinson, 1959), and the relationship of biodiversity and productivity (Tilman *et al.*, 1996; Loreau *et al.*, 2001; Mittelbach *et al.*, 2001; Cardinale *et al.*, 2009; Srivastava *et al.*, 2009). One explanation for the coexistence of ecologically similar species is their utilization of slightly different resources, suggesting resource diversity alone may lead to the coexistence of competitive species (Behmer & Joern, 2008). This, in turn, may allow for increased productivity, e.g. biomass yield, of an ecosystem containing those competing species when provided with diverse resources (Tilman, 1980). This occurs because diverse resources decrease interspecific competition and can lead to lower competition overall, resulting in greater production of both competitors.

In detritus-based ecosystems, the co-occurrence of multiple leaf species may promote resource diversity, and the effects of different leaf species in various combinations on the decomposition of litter are well studied in terrestrial and lotic aquatic environments (Hattenschwiler *et al.*, 2005; Ball *et al.*, 2008; Srivastava *et al.*, 2009; Swan *et al.*, 2009). Although the focus of both terrestrial and aquatic studies has been on the decay of leaf material, recent work has examined the consequences of detrital leaf combinations as a resource for aquatic insect detritivores or filter-feeders. In general, combinations of leaves yield better growth of macroinvertebrates than expected, based upon responses to each leaf species individually for both detritivores and filter feeders, although the synergistic effects of leaf combination are small relative to the variation in invertebrate response to individual leaf species (Swan & Palmer, 2006; Reiskind *et al.*, 2009). These studies have focused on a single consumer and have not examined how combinations of resources affect interactions between consumers. Specifically, the effect of leaf combination on interspecific competition between invertebrate consumers in aquatic systems remains unexplored.

The larval stages of mosquitoes that occur in artificial or natural containers, such as discarded tires or phytotelmata, compete intra-and interspecifically (Juliano, 2009). With few exceptions, most container mosquitoes are filter-feeders and surface browsers and, therefore, do not consume leaf material *per se* (unless broken into fine particulates), but the microbial flora that consumes the leaf material, for which larvae compete (Walker *et al.*, 1988, 1991; Yee *et al.*, 2007). Very diverse resources, e.g. of leaf material and invertebrate carcasses, favor coexistence of larvae of mosquito species that otherwise might competitively exclude one another (Daugherty *et al.*, 2000; Murrell & Juliano, 2008), and leaf resources from different plants differentially affect mosquito larval competition (Fish & Carpenter, 1982; Sota, 1993; Yanoviak, 1999; Dieng *et al.*, 2002; Murrell & Juliano, 2008; Reiskind *et al.*, 2009, 2010). However, the effects of combinations of leaves have not been examined in the context of interspecific competition, in spite of the fact that plant species may affect the distribution and abundance of mosquito species (Barrera *et al.*, 2006; Reiskind *et al.*, 2010).

Two invasive container mosquito species, *Aedes albopictus* and *Aedes aegypti*, compete frequently in nature, a phenomenon that has been well studied under a variety of resource and field conditions (Barrera, 1996; Juliano, 1998; Daugherty *et al.*, 2000; Braks *et al.*, 2004; Murrell & Juliano, 2008). Juliano

(2010) performed a meta-analysis of interspecific competition between these two species and concluded that *A. albopictus* was the superior competitor under conditions of poor quality (low nitrogen) detritus, while in environments with higher nitrogen diets (either invertebrate carcasses or artificial, high-protein diets) *A. aegypti* is less competitively inferior. A diversity of leaf species, including invasive and native plants, occur in container habitats occupied by both *A. aegypti* and *A. albopictus* in south Florida (Reiskind *et al.*, 2010). How different leaf species commonly encountered in these habitats affect competition between *A. albopictus* and *A. aegypti* is not known, nor the effects of combinations of leaves on this interaction.

In this study, we address three basic hypotheses on the effects of different leaf species on competition between co-occurring container mosquitoes. First, we hypothesize that different leaf species change the outcome of intra- versus interspecific competition in *A. albopictus* and *A. aegypti* larvae. Second, we hypothesize that larvae grow better (higher survival, larger size and shorter development time) on diverse resources and combinations of leaves have a different effect on larvae relative to single leaf species. Finally, we hypothesize that combinations of leaf species have different effects on interspecific competition, and, therefore, coexistence of competing species, than single leaf species. Based upon previous experiments (Reiskind *et al.*, 2010), our first hypothesis predicts that, keeping leaf mass constant, larval habitats provisioned with Brazilian pepper (*Schinus terebinthifolious*, a common invasive plant in Florida) would alleviate the effects of both inter- and intraspecific competition, and, having been associated with superior performance at high larval densities in previous studies, may favor *A. aegypti*, relative to habitats provisioned with Australian pine (*Causarina equisetifolia*, another common invasive plant in Florida) or live oak (*Quercus virginiana*, a common native plant in Florida). Our second hypothesis predicts that larval habitats provisioned with combinations of these three leaf species at a constant total leaf mass would show reduced density effects from larval competition. This will result in higher survival, larger adult size and faster growth in habitats with multiple leaf species, relative to microcosms with single leaf species and thus result in higher overall yield of mosquito biomass in microcosm provisioned with two or three leaf species relative to the average of single leaf species provisioned microcosms. The final hypothesis predicts that interspecific competition will be alleviated relative to intraspecific competition when microcosms are provisioned with multiple leaf species relative to single leaf species.

Materials and methods

Mosquitoes

Mosquitoes of both species used in this study were F_3 progeny of field-collected larvae and eggs from Palm Beach County, Florida, USA. We made the initial collections from May to July 2008 of greater than 2000 individuals, and colonies were maintained at Oklahoma State University under the following larval conditions: 14:10 L:D photoperiod, 27°C, larvae reared on 0.3g l^{-1} 1:1 yeast:albumin at a density of 100 larvae per liter. Colonies were blood fed on a human volunteer (MHR) (maintenance of colonies exempted from Institutional Review Board for use of human subjects, 24 August 2008).

Two generations of mosquitoes were reared under these conditions before use in this experiment.

Leaf material choice and collection

We choose three leaf species based upon their abundance in south Florida container habitats and their ecological importance as dominant native and invasive species (Reiskind *et al.*, 2010). We collected leaf material during the summer of 2008 by species-specific methods in Indian River County, Florida, USA. Australian pine leaves were collected by placing tarps (1.7 m × 2.5 m) under stands of Australian pine and collecting all litter fall every other day to collect only recently fallen leaves and avoid leaves that may have already leached resources. We removed any non-Australian pine leaves. We collected Brazilian pepper by hand, only taking senesced leaves that were ready to fall or recently fallen. We collected live oak leaves by hand under large live oaks during the same collection period. We air-dried the collected leaf material in a low humidity environment (rH < 35%) for three weeks prior to use, which was sufficient time for their dry weights to stabilize. Leaf material from each species was pooled, and leaves were removed from these pools at random for use in microcosms.

Foliar chemistry

We assessed the carbon to nitrogen ratio of ten individual, dried leaves of each plant species, pulled at random from our pool of leaves to be used in microcosm experiments. To ensure complete dryness, we dried the leaves used for this analysis at 50°C for 48 h and weighed approximately 4 mg of leaf material (exact weights measured using a MP3 ultra microbalance, Sartorius Balances, Data Weighing Systems, Elk Grove, IL, USA). Carbon to nitrogen ratios per exact weight were then determined using an Elementar varioMICRO CHN analyzer (Elementar Americas Inc., Mt. Laurel, NJ, USA) as per the manufacturer's instructions.

Experimental design

We conducted a modified response surface design to test the effects of leaf identity and diversity on interspecific competition (Inouye, 2001). All microcosms were provisioned with a total of 1 g of leaf material and 250 ml of water in 500 ml plastic food-grade containers (Instawares Inc., Wilmington, DE, USA). These conditions are based upon field observations of the leaf detritus contained in container habitats favored by *A. aegypti* and *A. albopictus* and numerous competition experiments (Juliano, 1998; Braks *et al.*, 2004). We tested seven leaf combinations ('leaf', all combinations of Australian pine (A), Brazilian pepper (B) and live oak (O) leaves plus each single species: A, B, O, AB, AO, BO, ABO), two initial larval densities ('density', ten (low) or 20 (high) first instar larvae) and three competition levels ('competition', all *A.aegypti*, all *A. albopictus*, 50% *A. aegypti*/50% *A. albopictus*) for a total of 42 individual treatment combinations (7 × 2 × 3). Larval densities were chosen based upon field observations of similar sized containers (O'Meara *et al.*, 1995; Braks *et al.*, 2004; Reiskind *et al.*, 2010). Each treatment had five replicates, except the high-density, all-*A. aegypti* treatments, which had three replicates, and the low-density, all-*A. aegypti* in O and BO treatments, which had four, due to insufficient numbers of neonate *A. aegypti*. Therefore, there were a total of 194 microcosms at

the beginning of the experiment. We added the appropriate number of first instar larvae to microcosms within two hours of adding water to the leaf material. Microcosms were kept covered in a single 28°C incubator (Percival Co., Perry, IA, USA) with a 14:10 L:D photoperiod and checked daily for pupation. We recorded pupation and placed all pupae collected from the same microcosm on the same day into 50 ml conical tubes with a small amount of water. We checked all tubes daily for adult emergence, which was recorded. Adults were killed by freezing 48 h after emergence.

Variables measured

We measured the weight and emergence time and determined sex and species for each individual mosquito in the study ($n = 1834$). Emergence time was calculated from the day of egg hatch to adult emergence. Weight was measured after at least 24 h in a drying oven at 45°C to the nearest μg using the same ultra-microbalance used to weigh leaves for foliar chemistry. As the experimental unit of analysis is the microcosm, we determined average weight and emergence time, and percent survival for each sex for each microcosm. Because of small starting numbers of each species in the interspecific, low-density treatments, total survival (and not sex-specific survival) was used to compare survival rates among treatments. As a measure of total mosquito productivity of a replicate, we calculated the total yield in dry weight of adult male and female mosquitoes of both species for each replicate.

Statistical analysis

We conducted all analyses in SAS 9.1.3 (SAS Corporation, Cary, NC, USA). We could not reject the hypothesis of normality for our outcome variables of C:N ratio, female weight, female days to emergence and total (male and female) percent survival. We focused our analysis on female characteristics because females are more important in determining population level effects relative to males and because males had similar responses.

Foliar chemistry

We tested the effect of leaf species on C:N ratio with a one-way ANOVA and subjected means to post-hoc comparisons after Bonferroni's correction (PROC GLM).

Effects of single leaf species

To test our first hypothesis, that different leaf species can affect the outcome of competition, we used a multivariate analysis of variance (MANOVA) model to analyze the effects of all individual leaf species (Australian pine (A), Brazilian pepper (B) and live oak (O)), density (low versus high) and competition (inter- versus intraspecific) and all interactions on total survival (males and females), female weight and female times to emergence for each mosquito species separately using type III sums of squares to account for unbalanced replication (PROC GLM). A significant competition effect means that there is a difference in the outcomes measured between inter- and intraspecific competition for that species. A significant interaction term that includes competition means that the effect of competition depends on the level of the interactive variable. We used MANOVA because multiple, potentially

correlated outcomes were measured on each replicate (Scheiner & Gurevitch, 2001). We assessed significance through the use of Pillai's trace, a statistic robust to the assumptions of MANOVA (Scheiner & Gurevitch, 2001). As we were specifically interested in the effects of leaf species on the multivariate outcomes, we examined multivariate contrasts between each leaf type for each mosquito species separately. Although several previous studies on similar subjects have used an index of performance to compare responses in container mosquitoes (e.g. λ′: Juliano 1998), we were unable to use this approach due to the combination of non-normal distributions of our calculated λ′ and our unbalanced design. We used MANOVA to generate statistically tractable artificial variables, and not necessarily biologically meaningful, so they must be interpreted with caution.

Effects of leaf combination

We tested our second and third hypotheses, the importance of leaf mixtures to mosquito competition, both intra- and interspecifically, by comparing the effect of the number of leaf species (one, two or three), density (ten or 20 initial larvae) and competition (inter- or intraspecific) on female weight, female development time and total survival (males and females) by MANOVA (PROC GLM), following an analytical methodology similar to Smith & Bradford (2003). As in the single species analysis, we assessed significance with Pillai's trace. As the experimental design includes all single leaf species and possible combinations of leaf species, the average values of the outcomes at each level of species number (one, two or three leaf species) should be identical if there is no synergy or antagonism when leaf resources are combined. After a significant leaf species number term, post-hoc tests contrasting the global, multivariate means of one versus two versus three species treatments would demonstrate if leaf combination differed generally from the assumption of additivity in one direction (synergistic or antagonistic interactions between leaf species when in combination). However, if the combination of leaf species was neutral or idiosyncratically non-additive, this first approach may not result in a significant leaf species number term, or may obscure the actual direction of responses. Therefore, our second approach was to determine the direction and magnitude of deviation from strict additivity by performing contrasts between each leaf combination and the mean response to each individual leaf species for the univariate outcomes of mean survival (male and female), female weight and female days to emergence. We used a generalized linear model with leaf combination (A, B, O, AB, AO, BO, ABO) as the only factor and contrasted each combined leaf treatment to the mean of the constituent leaves (e.g. AB was contrasted to the mean of A and B). Based upon many studies of density dependence in mosquitoes (Gilpin et al., 1976; Juliano, 2007, 2009), we assume that a synergistic response would result in higher larval survival to adult, larger adults, and shorter larval development time, while an antagonistic result would have lower survival, smaller adults and longer development time. An additive (neutral) response would result in a significant contrast.

Effects of leaf combination on total mosquito yield

We also tested the effects of leaf combination on total mosquito biomass yield, which is a further test of our second and third hypotheses. To examine this, we summed the total adult male and female body mass of both species from each replicate. As we were not able to measure the amount of mass lost as dead larvae and larval or pupal casts, we avoid the term biomass in favor of yield to denote the amount of insect biomass leaving the container microcosm. We then compared this variable, for which we could not reject the null hypothesis of normality, among different levels of leaf species number, initial larval density and whether competition was intraspecific for each species or interspecific (three levels: all *A. aegypti*, all *A. albopictus* or interspecific) with a general linear model ANOVA (PROC GLM). Post-hoc differences between means were assessed using the Tukey-Kramer method.

Results

Foliar chemistry of leaves

The three leaf species had different ratios of carbon to nitrogen (average values of ten leaves: Australian pine: 1.019% N, 49.623% C, C:N ratio = 50.2; Brazilian pepper: 0.6495% N, 49.985% C, C:N ratio = 79.47; live oak: 0.889% N, 48.305% C, C:N ratio = 57.42; $F_{2,27} = 15.77$, $P < 0.0001$). Post-hoc tests demonstrated that Brazilian pepper leaves had a significantly higher mean C:N ratio than live oak or Australian pine leaves, which did not differ.

Competition in A. albopictus *with single leaf species*

For *A. albopictus*, the main effects of density and leaf species were significant in explaining the multivariate outcomes of total survival, female weight and time to emergence, as was the interaction between leaf species and competition (table 1). Higher density microcosms produced mosquitoes of smaller size, longer development, and that had poorer survival, and had one significant canonical correlation (table 1; can1: eigenvalue = 1.2839, 100% explained variation, $F_{3,35} = 14.98$, $P < 0.0001$). The leaf by competition interaction term had one significant canonical correlation (table 1; can1: eigenvalue = 0.8896, 88.96% explained variation, $F_{6,70} = 2.45$, $P = 0.0331$). The significant interaction means that the importance of interspecific versus intraspecific competition for *A. albopictus* depended upon leaf species, and the standardized canonical coefficients suggest this difference is driven by differences in survival to adult and female weight (fig. 1 and table 1). In general, *A. albopictus* fared better when competing against *A. aegypti* relative to when competing against conspecifics in Australian pine, suggesting that interspecific competition was not as strong as intraspecific competition under these conditions. In Brazilian pepper, there was a trend for the reverse, such that interspecific competition was stronger than intraspecific competition, with lower survival and lighter weights when *A. albopictus* was competing against *A. aegypti* relative to when competing against conspecifics. In live oak, there was no difference between inter- and intraspecific competition. Competition, density by competition, density by leaf and the three-way interaction were not significant.

Competition in A. aegypti *with single leaf species*

For *A. aegypti*, only the main effects of density and leaf species were significant in explaining the multivariate outcomes of total survival, female weight and time to emergence

Table 1. MANOVA for *A. albopictus* average female weight, survival (males and females) and average female days to emergence by leaf species (leaf), density (dens), competition (comp) and all possible interactions. Contrasts are indented under the leaf × competition factor (inter- versus intraspecific competition in: A, Australian pine; B, Brazilian pepper; O, olive oak). Standardized canonical coefficients (SCCs) are given for significant factors.

| Factor	Pillai's trace	F-value	Num, Den DF	P-value	Standardized canonical coefficients		
					Female days to emergence	Survival	Female weight
Leaf	0.8139	8.23	6, 72	**<0.0001**	**0.852**	**−0.266**	**1.432**
Density	0.5622	14.98	3, 35	**<0.0001**	**0.580**	**−0.992**	**−0.765**
Competition	0.0316	0.38	3, 35	0.7675	–	–	–
Leaf × dens	0.2406	1.64	6, 72	0.1484	–	–	–
Leaf × comp	0.3301	2.37	6, 72	**0.0379**	**0.475**	**1.401**	**1.168**
A	0.1778	2.88	3, 40	**0.0475**	**0.983**	**1.413**	**1.037**
B	0.1551	2.45	3, 40	0.0708	0.303	1.196	1.384
O	0.0318	0.44	3, 40	0.7274	0.824	0.890	1.748
Dens × comp	0.0713	0.90	3, 35	0.4531	–	–	–
Leaf × dens × comp	0.0843	0.52	6, 72	0.7910	–	–	–
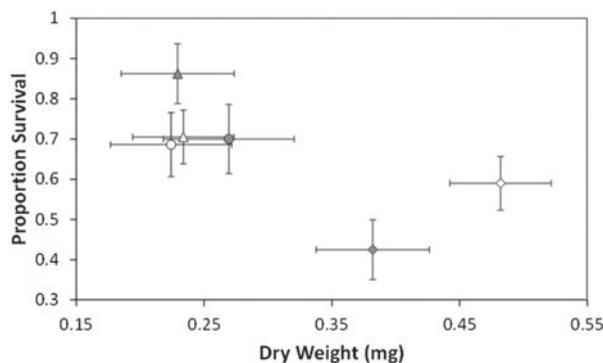
Fig. 1. Bivariate plot of *A. albopictus* average female weight and total proportion survival (males and females) in response to three leaf species (different shapes) by type of competition (grey versus white). Error bars are ±1 sem (△, Australian pine, intra; ▲, Australian pine, inter; ◊, Brazilian pepper, intra; ◆, Brazilian pepper, inter; ○, Live oak, intra; ◉, Live oak, inter).

(table 2). Density had only one canonical correlation and, as in *A. albopictus*, higher density microcosms were associated with smaller size, longer development and poorer survival (table 2; can1: eigenvalue = 0.5049, 100% explained variation, $F_{3,29} = 4.88$, $P = 0.0072$). We associated leaf treatment with two significant canonical correlations (fig. 2 and table 2; can1: eigenvalue = 1.2301, 62.78% explained variation $F_{6,58} = 9.32$, $P < 0.0001$; can2: eigenvalue = 0.7293, 37.22% explained variation, $F_{2,30} = 10.94$, $P = 0.0003$). The major difference between leaf species was in female weight, with Brazilian pepper microcosms producing much heavier females than Australian pine or live oak provisioned microcosms. Individual contrasts between leaf species could not be made with Australian pine, one treatment of which failed to produce any females in any replicates (high density, intraspecific), but multivariate contrasts revealed significant differences between live oak and Brazilian pepper (B vs O: $F_{3,29} = 11.06$, $P < 0.0001$). The competition factor was not significant for *A. aegypti*, nor were any interaction terms. Standardized canonical coefficients for both the leaf and density effects show percent survival and female weight contributed the most to the multivariate differences between leaf species and density (table 2).

Effects of leaf combination on competition

For both mosquito species, number of leaf species had a significant effect on adult weight, survival to adulthood and time to emergence, as did initial larval density (tables 3 and 4 and fig. 3a, b). In this analysis, there was no difference between inter- and intraspecific competition, nor was there a significant interaction between leaf species number and competition or density. For both species of mosquito, standardized canonical correlations demonstrated female weight and days to emergence were the most important factors for both leaf number and density effects (tables 3 and 4). Post-hoc multivariate contrasts showed one, two and three species treatments to have significantly different multivariate means for *A. albopictus* (table 3), and one species treatments to have significantly different multivariate means from two or three species treatments for *A. aegypti* (table 4). In general, two or three species in combination generated larger females in less time (compare black to grey to white symbols in fig. 3a, b).

Effects of leaf combination on larval outcomes

The significant effects of species number on all outcomes suggest synergy or antagonism between leaf species. The combination of all three leaf species was generally synergistic, except for percent survival for *Ae. albopictus*, which was additive (tables 5 and 6). The combination of Brazilian pepper and live oak had higher than expected survival for both mosquitoes and shorter days to emergence for *A. aegypti*. Only the combination of Australian pine and Brazilian pepper was antagonistic, with significantly lower survival than expected in *A. albopictus*. This combination was also synergistic with regards to weight in both species and was additive for development rate for both species and survival in *A. aegypti*. The other leaf combinations and outcomes for each species were not significantly different from the null hypothesis of additivity.

Leaf combinations and mosquito yield

When we examined total mosquito yield produced from each microcosm, we found a significant positive association between number of leaf species and total mosquito production, while initial larval density and competitive treatment were not significant in explaining variation in total yield

Table 2. MANOVA for *A. aegypti* of average female weight, survival (males and females) and average female days to emergence by leaf species (leaf), density (dens), competition (comp) and all possible interactions. Contrasts are indented under the leaf factor (A, Australian pine; B. Brazilian pepper; O, olive oak). NB. Contrasts with Australian pine could not be estimated because one whole treatment of Australian pine (high density, intraspecific competition) produced no females. Standardized canonical coefficients (SCCs) are given for significant factors.

| Factor	Pillai's trace	F-value	Num, Den DF	P-value	Standardized canonical coefficients		
					Female days to emergence	Survival	Female weight
Leaf	0.9733	9.48	6, 60	**<0.0001**	**0.512**	**0.428**	**1.503**
A vs B	–	–	–	–	–	–	–
A vs O	–	–	–	–	–	–	–
B vs O	0.5336	11.06	3, 29	**<0.0001**	**0.610**	**0.821**	**1.350**
Density	0.3355	4.88	3, 29	**0.0072**	**0.288**	**0.793**	**1.281**
Competition	0.0024	0.02	3, 29	0.9950	–	–	–
Leaf × dens	0.2303	1.30	6, 60	0.2706	–	–	–
Leaf × comp	0.0367	0.19	6, 60	0.9794	–	–	–
Dens × comp	0.0403	0.41	3, 29	0.7497	–	–	–
Leaf × dens × comp	0.0270	0.27	3, 29	0.7685	–	–	–
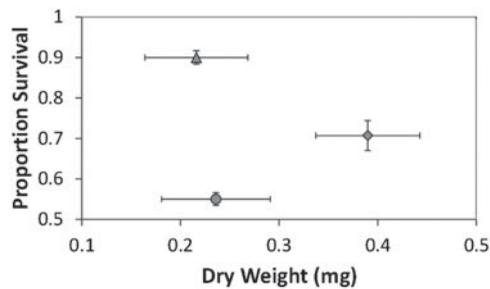
Fig. 2. Bivariate plot of *A. aegypti* average female weight and total proportion survival (males and females) in response to three leaf species (different shapes) Error bars are ±1 sem (△, Australian pine; ◇, Brazilian pepper; ●, Live oak).

(average yields: 1-species: 1.728 mg; 2-species: 2.284 mg; 3-species: 3.301 mg; ANOVA : full model $F_{17}=3.23$, $P<0.0001$; number of leaf species: $F_2=17.30$, $P<0.0001$; density: : $F_1=2.26$, $P=0.1254$; competition: $F_2=0.52$, $P=0.59660$). Post-hoc tests demonstrated significant differences between the three leaf species treatment and the two and one leaf species treatments.

Discussion

In this study, we addressed three hypotheses: leaf species changes the outcome of interspecific competition between *A. aegypti* and *A. albopictus*, the combination of leaves alleviate competition overall and leaf combination alters interspecific competition between these two species. Our findings partially support the first hypothesis, strongly support the second and provide no support for the final hypothesis. In addition to testing these hypotheses, the results can be used to address a more general question in these container habitats: does diverse leaf litter affect the overall productivity of these environments for mosquitoes?

We found partial support for our first hypothesis. For *A. albopictus*, the significant interaction between competition and leaf species suggests that the effect of intra- versus interspecific competition on growth and survival depends on leaf species. However, this trend was not strong, and weakly

significant interactions in MANOVA should be interpreted conservatively (Scheiner & Gurevitch, 2001). The examination of the pattern of performance in intraspecific versus interspecific competition for *A. albopictus* on Australian pine resources suggests better performance when in competition with *A. aegypti* than when in competition with conspecifics, but a trend towards the opposite pattern when Brazilian pepper was the resource. The effect of competition type was not different on live oak. The foliar chemistry data suggested that Brazilian pepper leaves contribute relatively more carbon per unit nitrogen than the other two leaves, which does not correspond with Juliano's (2010) general findings that high nitrogen habitats are associated with better *A. aegypti* performance. That being said, in general, mosquitoes did better with Brazilian pepper leaves than Australian pine and live oak (this study and Reiskind *et al.*, 2010), which might suggest that productivity in these container habitats is limited by carbon, a result suggested for tree hole systems (Kaufman *et al.*, 2002). However, in many aquatic systems, nitrogen or phosphorous are more likely to limit productivity (Elser *et al.*, 1990; Frost *et al.*, 2002), as is the case with most terrestrial systems (Vitousek & Howarth, 1991) and some container systems (Yee & Juliano, 2006). In addition, detritus-based systems in terrestrial settings may be sensitive to other nutrients, such as sodium or calcium (Kaspari *et al.*, 2008; Kaspari & Yanoviak, 2009). We did not measure phosphorous, calcium or sodium in these leaves, which may be more telling than the ratio of C:N. It is also possible that carbon to nitrogen ratio may not be important relative to the chemical components (e.g. secondary compounds) of the leaf material in question.

Our results suggest *A. albopictus* would be a superior competitor on Australian pine, and perhaps live oak leaves, but not Brazilian pepper leaves. Senesced live oak leaves have been commonly used as a resource for interspecific studies between *A. aegypti* and *A. albopictus* where they have been associated with *A. albopictus* superiority (Juliano, 1998; Braks *et al.*, 2004). Braks *et al.* (2004) also demonstrated *A. albopictus* to be a superior competitor when fed senesced avocado leaves. On the other hand, Murrell & Juliano (2008) showed that the outcome of competition between these two species can be altered by detritus type (insect carcasses, fresh cut grass, pine or oak leaves), with *A. aegypti* an inferior competitor on insect carcasses and pine or oak leaves, but not when provided fresh

Table 3. MANOVA for *A. albopictus* of average female weight, survival (males and females) and average female days to emergence by number of leaf species (SpNum), density (dens), competition (comp) and all possible interactions. Specific contrasts between numbers of leaf species are indented under the species number term. Standardized canonical coefficients (SCCs) are given for significant factors.

Factor	Pillai's trace	F-value	Num, Den DF	P-value	Standardized canonical coefficients		
					Female days to emergence	Survival	Female weight
Species number	0.2919	6.50	6, 228	<0.0001	−0.422	0.546	1.221
1 vs 2	0.0687	2.78	3, 113	0.044	−0.585	0.112	1.029
1 vs 3	0.2813	14.73	3, 113	<0.0001	−0.421	0.549	1.220
2 vs 3	0.1754	8.01	3, 113	<0.0001	−0.333	0.716	1.256
Density	0.3579	21.00	3, 113	<0.0001	−0.787	0.435	0.902
Competition	0.0431	1.70	3, 113	0.1715	–	–	–
SpNum × dens	0.0996	1.99	6, 228	0.0677	–	–	–
SpNum × comp	0.0283	0.55	6, 228	0.7729	–	–	–
Dens × comp	0.0619	2.49	3, 113	0.0642	–	–	–
SpNum × dens × comp	0.1019	2.04	6, 228	0.0615	–	–	–

Table 4. MANOVA for *A. aegypti* of average female weight, survival (males and females) and average female days to emergence by number of leaf species (SpNum), density (dens), competition (comp) and all possible interactions. Specific contrasts between numbers of leaf species are indented under the species number term. Standardized canonical coefficients (SCCs) are given for significant factors.

Factor	Pillai's trace	F-value	Num, Den DF	P-value	Standardized canonical coefficients		
					Female days to emergence	Survival	Female weight
Species number	0.1921	3.40	6,192	0.0033	−0.798	0.403	0.807
1 vs 2	0.1208	4.35	3,95	0.0064	−0.729	0.257	0.734
1 vs 3	0.1608	6.07	3,95	0.0008	-0.540	0.420	0.854
2 vs 3	0.0354	1.16	3,95	0.3282	–	–	–
Density	0.3372	16.11	3,95	<0.0001	−0.384	0.182	1.09
Competition	0.0509	1.70	3,95	0.1729	–	–	–
SpNum × dens	0.0895	1.50	6,192	0.1802	–	–	–
SpNum × comp	0.0662	1.10	6,192	0.3668	–	–	–
Dens × comp	0.0252	0.82	3,95	0.4863	–	–	–
SpNum × dens × comp	0.0127	0.20	6,192	0.9753	–	–	–

cut grass. They detected no difference in tree leaf species (pine or oak) on mosquito growth or survival for either species or for interspecific competition. Our study is the first to examine interspecific competition between these mosquitoes with leaves from common invasive species known to contribute to container habitats (Reiskind *et al.*, 2010), and the much higher growth in Brazilian pepper suggests it may provide more nutrients and therefore may favor *A. aegypti* as other conditions of high nutrients have (Juliano, 2010). Brazilian pepper, although associated with high larval survival in previous studies (Reiskind *et al.*, 2010), was associated with lower larval survival for *A. albopictus* and intermediate levels of survival for *A. aegypti*. We did not examine the secondary compounds in the leaves, nor did we analyze the microbial communities of these different leaf environments, which may provide some insight into the differences in growth and mortality between different leaves. Other researchers have noted different bacterial communities in container habitats provisioned with different leaf species, which may also contribute to the quality of the habitat (Ponnusamy *et al.*, 2008).

We supported our second hypothesis, that leaf combination can affect density dependent reductions in growth and survival, but did not find support for our third hypothesis, that leaf combinations would affect interspecific competition more than intraspecific competition. The number of leaf species was significant in determining growth and survival, although it did not interact with density or competitive

environment (intra- or interspecific competition), and therefore leaf combination did not change the outcome of competition between *A. aegypti* and *A. albopictus*. Leaf combinations were generally associated with higher survival, shorter development time and larger female adult weights than one would expect based upon the response to each leaf species, and we conclude that leaf resources usually act synergistically as a detrital base for mosquito larvae in containers.

Previous studies of the effects of mixed resources on aquatic invertebrates also found synergy, although the current results are generally stronger than observed in previous studies (Swan & Palmer, 2006; Reiskind *et al.*, 2009). It is tempting to generalize that resource diversity increases ecosystem function (in this case, the overall yield of adult mosquito biomass) from our results. However, the conclusion that leaf diversity increases the production of mosquitoes needs to be made with caution for four reasons. First, leaf species were not selected at random, but based upon their commonness in larval habitats (Reiskind *et al.*, 2010). Second, with only three leaf species, combinations are highly susceptible to the 'selection probability effect', wherein it is not biodiversity *per se* that allows increased mosquito production but the probability of selecting a species that is beneficial to the measured aspect of ecosystem function (Huston, 1997). Indeed, Brazilian pepper appeared to be a high-quality resource, based upon previous studies (Reiskind *et al.*, 2010), and its presence in two-thirds of the two species
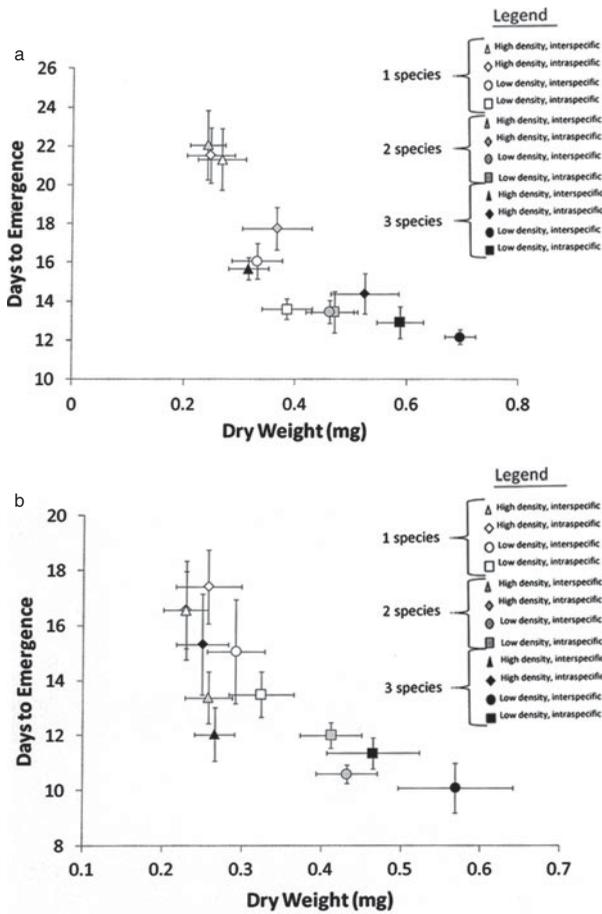
Fig. 3. (a) Bivariate plot of *A. albopictus* average female weight and days to emergence in response to number of leaf species (different colors) and different density/competition combinations (different shapes). (b) Bivariate plot of *A. aegypti* average female weight and days to emergence in response to number of leaf species (different colors) and different density/competition combinations (different shapes). Error bars are ±1 sem.

Table 5. ANOVA contrasts comparing responses of leaf combinations to mean responses to individual leaves for *Aedes albopictus*.

Outcome	Leaf combination	F-value	P-value	Effect
Percent survival	**AB**	**15.17**	**0.0002**	**antagonistic**
	AO	3.21	0.0754	additive
	BO	**7.83**	**0.0059**	**synergistic**
	ABO	0.49	0.6309	additive
Female weight	**AB**	**29.44**	**0.0001**	**synergistic**
	AO	0.97	0.3276	additive
	BO	0.33	0.5685	additive
	ABO	**37.58**	**<0.0001**	**synergistic**
Days to emerge	AB	−3.54	0.06	additive
	AO	0.46	0.5002	additive
	BO	0.21	0.6488	additive
	ABO	**8.76**	**0.0037**	**synergistic**

Table 6. ANOVA contrasts comparing responses of leaf combinations to mean responses to individual leaves for *Aedes aegypti*.

Outcome	Leaf combination	F-value	P-value	Effect
Percent survival	AB	0.09	0.7618	additive
	AO	3.87	0.0512	additive
	BO	**4.02**	**0.0471**	**synergistic**
	ABO	**4.39**	**0.0380**	**synergistic**
Female weight	**AB**	**13.66**	**0.0004**	**synergistic**
	AO	1.31	0.2553	additive
	BO	0.23	0.6350	additive
	ABO	**10.26**	**0.0018**	**synergistic**
Days to emerge	AB	2.71	0.1025	additive
	AO	3.06	0.0833	additive
	BO	**4.52**	**0.0360**	**synergistic**
	ABO	**7.19**	**0.0086**	**synergistic**

and the three species combinations may result in leaf combinations appearing to result in greater mosquito performance. Indeed, all significant deviations from additivity included Brazilian pepper, although they were not all synergistic. The effect of diversity on ecosystem function may indeed be probabilistic and not linear in nature, such that the selection of a highly nutritive species, and not simply the number of species, leads to increased ecosystem function. Third, there is the possibility that the synergy observed in leaf combinations is simply a function of superior performance at lower leaf levels, something we did not test. For example, it is possible that the mosquitoes would have higher survival and better growth on 0.5 g of Brazilian pepper than on 1 g. We cannot rule this interpretation out because we did not alter the total amount of leaf material per microcosm, only the composition. Finally, the effects of combined leaf resources, although on average synergistic for both mosquito species, had idiosyncratic effects depending upon mosquito species and leaf combination. The combination of all three leaf species was significantly better than additive for all outcomes for both species except percent survival for *Aedes albopictus*. The only antagonistic leaf combination was Australian pine and Brazilian pepper for *A. albopictus* survival but showed larger than expected weight gain. This same leaf combination was additive for *A. aegypti* survival but synergistic for *A. aegypti* weight. The overall pattern suggests synergy between leaf resources is common but somewhat idiosyncratic depending upon combination and mosquito species.

Some leaf litter has been noted to be toxic, and some species of mosquitoes seem to tolerate toxic leaf litter better than others (David *et al.*, 2000a,b). The effects of leaf litter amount on mosquito larval performance is generally positive (Walker & Merritt, 1988), but a recent study using path analysis suggested that the direct effect of leaf litter on mosquito larvae may be negative, while the effect of plant material on mosquito larvae through microbial production is positive, which may suggest that the toxic properties of leaves are important (Yee *et al.*, 2007). Toxicity of leaf material has not been explored as a contributing factor to interspecific competition between these or other larval mosquitoes. Further experiments examining individual responses to different leaf litters, along with toxicological examinations, may suggest tolerance of a toxic environment as an alternative pathway explaining the pattern of competition in mosquito larvae. Juliano's (2010)

meta-analysis, suggesting that *A. aegypti* is a superior competitor in richer environments, could also be interpreted as *A. aegypti* being more tolerant of toxicologically intense habitats, which correlates with enrichment. The mechanism by which 'richer' environments may favor one mosquito species over another remains unexplored but is worthy of investigation.

Leaf combination had a major impact on the total mosquito yield produced above that expected by the responses to individual leaf species. We conclude that diversity of resource (leaf species) increases the survival and growth of mosquito larvae of both species and, therefore, the function of the ecosystem as measured by mosquito yield. Possible mechanisms by which leaf combinations yield higher productivity could be complementary resources (*sensu* Greenstone, 1979) but also could be due to varying speeds with which leaves degrade, which may provide a more consistent source of nutrients throughout the development period, as opposed to a pulse early or late depending on the lability of the leaf material. Questions of resource diversity may be particularly critical in these container habitats because oviposition choice may occur weeks or months before hatching in these mosquitoes (Christophers, 1960; Hawley, 1988), and mosquito larvae are primarily generalist filter feeders and browsers (Merritt *et al.*, 1992). Acquiring a diverse diet is important in many taxa (Pyke, 1984; Waldbauer & Friedman, 1991; Behmer, 2009), and it is reasonable to conclude that a diverse set of leaves may benefit the mosquito larvae considered in our study by providing more diverse nutrients or a more diverse microbial community (Ponnusamy *et al.*, 2008). If there is a more diverse array of nutrients available within a constrained container habitat, mosquito larvae may be able to exert some degree of diet choice by altering gut transit times as some filter feeders do (e.g. Lehman, 1976), but this is cryptic and not well studied in mosquitoes (Dadd, 1970; David *et al.*, 2003). Although our study system is simplified, we also conclude that neither initial larval density nor type of competition (intra- or interspecific) affected total mosquito yield. This result does not corroborate the conclusion that the relationship between ecosystem function and resource diversity is dependent on both resource and consumer diversity (Cardinale *et al.*, 2009; Srivastava *et al.*, 2009), suggesting resource diversity alone can result in increased ecosystem function (in our case, yield of adult mosquito biomass) in these container habitats.

Acknowledgements

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