Published in final edited form as:

Ecol Entomol. 2010 June 1; 35(3): 329–340. doi:10.1111/j.1365-2311.2010.01183.x.

Senescent leaf exudate increases mosquito survival and microbial activity

K. S. PELZ-STELINSKI^{1,*}, E. D. WALKER^{2,3}, and M. G. KAUFMAN³

¹Department of Entomology and Nematology, Citrus Research and Education Center, University of Florida, 700 Experiment Station Rd, Lake Alfred, FL 33850

²Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, MI 48824

³Department of Entomology, Michigan State University, East Lansing, MI 48824

Abstract

1. We conducted experiments to evaluate the effects of soluble components in senescent leaf material on the growth and development of the eastern tree hole mosquito, *Aedes triseriatus* (Say). Oak leaves that were either leached for three days to remove the labile nutrient fraction, or were not leached, served as basal nutrient inputs in each experiment.
2. Mosquito performance in microcosms containing leachate only was significantly worse compared with microcosms containing leaf material in combination with either leachate or water, indicating the importance of leaf substrates to mosquito production.
3. Adult mosquito biomass, emergence, and development time were significantly higher in microcosms containing unleached leaves compared with leached leaf material. Additions of leachate to leached leaf treatments enhanced adult production, but not to the level observed in unleached leaf treatments.
4. Filtered and unfiltered leachate added substantial nitrogen and phosphorus to microcosms and significantly affected mosquito growth responses. Bacterial productivity and abundance were also significantly affected by leachate additions and filtering.
5. Taken together, these results suggest that while leaves decline with respect to nutritional value during decomposition, they remain important components of the habitat as substrates for microbial growth and mosquito feeding, particularly when nutrients (here, leachate) enter the system. Our results also illustrate the importance of soluble leaf material, which enhances mosquito production through effects on microbial community dynamics.

Keywords

Aedes triseriatus; microbial ecology; detritus; bacteria; microcosm; larval nutrition

Introduction

Production of container-breeding mosquitoes from tree holes depends on larval sustenance derived from allochthonous detrital inputs. These inputs consist primarily of plant material,

* Address correspondence to: Dr. Kirsten Pelz-Stelinski Department of Entomology University of Florida Citrus Research and Education Center 700 Experiment Station Rd Lake Alfred, FL 33850 Ph. (863) 956-4311 ext.1244 Fax: (863) 956-4631.

with leaf litter as the major source of coarse particulate organic matter (CPOM), fine particulate organic matter (FPOM), and dissolved organic matter (DOM) (Fish & Carpenter, 1982; Leonard & Juliano, 1995; Walker *et al.*, 1997). In addition to plant detritus, stemflow (Kitching, 1971; Fish, 1983; Carpenter, 1982b; Walker *et al.*, 1991; Kaufman *et al.*, 1999, 2002; Kaufman & Walker, 2006) and animal detritus (Daugherty *et al.*, 2000; Yee & Juliano, 2006; Harshaw *et al.*, 2007) contribute to allochthonous nutrient pools. Water runoff during rain events collects in tree holes, introducing critical soluble nutrients (e.g. nitrogen, phosphorus and other inorganic nutrients) and carbon to the nutrient-poor ecosystem. Macroinvertebrate carcasses and fecal material also accumulate in tree holes, although the presence of these materials is ephemeral compared to leaves as a result of their more rapid decay rate (Yee & Juliano, 2006).

Mosquito production is directly and indirectly, through microbial intermediates, linked to detrital inputs (Kaufman & Walker, 2006). Detritus such as leaf material serves both structural and dietary functions that promote microbial colonization. Microorganisms utilize the detritus as a growth surface while contributing to its breakdown through microbial metabolism. Thus, microorganisms afford a bridge between the nutrients trapped in the leaf matrix and mosquito larvae (Fish & Carpenter, 1982). Analyses of larval gut content and feeding behavior indicate mosquitoes obtain food by browsing the microbial biofilm associated with leaf and container wall surfaces or by filtering small particles such as planktonic bacteria as well as FPOM from the water column (Cummins & Klug, 1979; Fish & Carpenter, 1982; Merritt *et al.*, 1992; Walker & Merritt, 1991). The tree hole-associated microbial milieu consists of a diverse community of heterotrophic bacteria, fungi, and protozoans (Kaufman *et al.*, 2001, 2002; Kaufman & Walker, 2006). These microorganisms contribute to carbon and nutrient cycles through the recycling of detritus and stemflow inputs for the production of microbial biomass, thus increasing the availability of detritus-derived nutrients to higher trophic levels in tree hole communities (Kaufman & Walker, 2006).

Adult mosquito production is conditionally dependent on the available leaf ration per larva (Fish & Carpenter, 1982; Hard *et al.*, 1989; Leonard & Juliano, 1995; Walker *et al.*, 1997), and the interaction thereof with variables such as larval density, presence of other macroinvertebrates, and stemflow inputs. The quality of leaf material is also of critical importance, varying with tree species (Carpenter, 1983; Fish & Carpenter, 1982; Reiskind *et al.*, 2009) and indicated in part by observations of greater mosquito production from microcosms stocked with fresh leaves compared with senescent leaves (Walker *et al.*, 1997). Decomposition of leaf material is associated with its availability for microbial degradation and for macroinvertebrate consumption, as both processes are governed by the presence of lignin and nitrogen content (Bärlacher, 1985; Moorehead & Sinsabaugh, 2006). Indeed, the presence of carbohydrates and other nutrients, particularly N, has a positive effect on fungal productivity and mosquito growth parameters (Kaufman & Walker, 2006).

Leaf decomposition occurs in two distinct stages: initial leaching of labile components over a short time period and a long-term breakdown of refractory components (Carpenter, 1982a). Release of soluble leaf components occurs early in the decay process, approximately within 24 hours of entering the system (Gessner & Schwoerbel, 1989; Webster & Benfield, 1986). Concentration of phenolic compounds, C:N ratios, and physical characteristics vary among leaf species, resulting in relative differences in the portions of labile and refractile leaf components and subsequently, differential breakdown among leaf types (Cornelissen, 1996). The labile material, or leachate, is rich in nutrients that are critical for microbial and mosquito productivity (Walker *et al.*, 1997). Refractile material, on the other hand, has a high C:N ratio, low phosphorus content, and is likely unavailable directly to developing larvae (Carpenter, 1982a; Webster & Benfield, 1986).

We describe here three experiments designed to elucidate the individual effects of labile and refractile leaf components on tree hole community processes, in particular growth and production of the eastern tree hole mosquito, *Aedes triseriatus* (Say) (Craig, 1983). The objectives of this study were to determine: 1) if the nutrients in leached senescent leaves are sufficient for mosquito growth; 2) whether leachate alone can support larval development; 3) whether fresh leachate stimulates bacterial abundance and/or productivity; and 4) if the positive effect of unleached leaves on mosquito development can be restored by returning labile leaf components to microcosms. Leaching has dampening effects on the growth responses of mosquitoes due to the consequent reduction in nutrient content (Walker *et al.*, 1997); therefore, we hypothesized that the labile leaf fraction comprises the most important contribution of nutrients to the tree hole environment of larval mosquitoes. Because this material disappears rapidly from the leaf matrix, we predict that leached leaf litter in tree holes is of lower quality than unleached leaves, contributes relatively little to mosquito growth, and may physically impede inputs of higher quality leaves. Thus, leached leaves alone should be insufficient for larval growth while the leached fraction, containing labile substrates and nutrients that initiate high microbial activity, should support larval growth and development comparable to that observed in response to fresh leaves (objectives 1 and 2). This prediction follows from the results of previous studies showing enhanced mosquito growth in response to nutrient supplementation via the microbial loop (Kaufman *et al.*, 2002; Kaufman & Walker, 2006). Thus, the positive effect of high nutrient concentration in leachate should manifest itself via the increased production of microorganisms (objective 3). Finally, we predict that additions of soluble material obtained from an equivalent leaf mass and added back to leached leaves should mitigate the negative effects of poor leaf quality on mosquito developmental success (objective 4).

Materials and Methods

Microcosm construction

For each of the following experiments, tree hole-based microcosms were stocked with senescent red oak leaves (*Quercus rubra* L.) collected at Michigan State University's Kellogg Forest (Augusta, MI, USA). Leaves were dried at 45°C for 48 h and added as 1 g quantities to microcosms constructed of polyvinyl chloride (700 ml volume, 7.6 cm diameter, 15.2 cm height), as described elsewhere (Walker *et al.*, 1991; Kaufman & Walker, 2006). Microcosms also received a microbial inoculum consisting of 3 ml homogenized natural tree hole water and particulates. Each contained a final volume of 500 ml, composed of deionized, distilled water and, if applicable, leachate in the amounts described below. Water levels were maintained throughout the experiment to account for evaporative losses. Microcosms were loosely covered with nylon window screen and incubated under indirect lighting at 21 °C and 16:8 (L:D) cycle (Percival Scientific, Inc., Perry, IA). Prior to the addition of 40 newly-hatched first instar *Ae. triseriatus* larvae (day 0 for all experiments), microcosms were incubated for 3 days to allow time for microbial colonization of leaf surfaces and the water column. The larvae used in the following experiments were hatched from eggs collected from our colonies at the Insect Microbiology Laboratory at Michigan State University (East Lansing, MI, USA).

Experiment 1

In this experiment, we compared the relative contributions of labile and refractile leaf components to mosquito productivity. Senescent red oak leaves (1 g) were leached for three days in 12 replicate microcosms containing 500 ml deionized, distilled water. This period is sufficient to account for the leaching of the labile components into the water column as the majority of this fraction is lost from the leaf matrix within three days of introduction to an aquatic environment (Carpenter, 1982a). Leaching was conducted under cold conditions

(4°C) to inhibit microbial growth. The water from six of the microcosms, now containing labile leaf components (leachate), was poured into new microcosms and replaced with fresh deionized, distilled water. The resulting treatment design consisted of six replicates of each of the following treatments: leached leaf + water, leached leaf + leachate, no leaf + leachate. Microcosms were checked daily for adult mosquitoes, which were collected and stored at -80°C. At the end of the experiment (day 70), adults and any remaining larvae and pupae were lyophilized and weighed.

Experiment 2

The effects of initial leaf quality and leachate addition were assessed in a 2 × 4 multifactorial design with six replicates per treatment. Unleached senescent red oak (*Quercus rubra*) leaves (1 g) were compared to similar leaves subjected to leaching for three days as described above. Leachate was added to microcosms containing either leached or unleached leaves and 40 newly hatched first instar *Ae. triseriatus* larvae in amounts equivalent to 0, 25, 50, or 100% of that obtained from 1 g of leaf material. To account for the effect of labile nutrients alone on mosquito performance, all leachate was filter-sterilized using a 0.2 µm vacuum filter before addition to microcosms. The 0.2 µm pore size was selected as it is sufficient for the removal of bacteria; this step is necessary to control for possible augmentation of larval diets with microorganisms that would promote development. As in the previous experiment, all developmental stages of mosquitoes were collected at the end of this experiment and processed to obtain dry mass measurements. The remaining leaf mass was also determined after drying leaves for three days at 50°C.

Experiment 3

We tested the hypothesis that nutrients and microbial biota present in the labile fraction of leaf material increase mosquito performance. Two levels of leaf quality, unleached or leached for three days, were applied to replicate microcosms similar to those described above. In contrast to the previous experiment, leachate was added to microcosms equivalent to 100% of the amount obtained from 1 g of leaves in two forms: unfiltered or filtered through a 0.2 µm vacuum filter. Additionally, a control treatment of deionized, distilled water was applied to replicate microcosms for each leaf quality treatment. The resulting 2 × 3 factorial design was replicated seven times to permit the replacement of leaf material sampled from six replicate microcosms on day 0 (prior to the addition of larvae). At day -3, filtered and unfiltered bulk leachates were sampled for bacterial productivity, bacterial abundance, and nutrient analysis. Microcosm sampling was done at the onset of the experiment on day 0. Water samples were collected for bacterial productivity, bacterial abundance, and nutrient analysis (1–10 ml). Productivity subsamples (1 ml) were maintained at 20°C and abundance subsamples (5 ml) were preserved with formaldehyde at a final concentration of 3.7% until measurements could be taken. Leaf material was subsampled using a cork borer (10 mm diam) for estimates of bacterial abundance. Two discs were aseptically removed from leaf material into 5 ml filter-sterilized phosphate buffer and preserved with formaldehyde (3.7% final concentration). Larvae, pupae, adults, and leaves were treated as described above to obtain dry weights.

Chemical analyses

Total nitrogen (N) and phosphorus (P) present in the samples were quantified via spectroscopy of unfiltered water samples (Kaufman & Walker, 2006). For each analysis, persulfate oxidation techniques were used to convert all forms of phosphorus and nitrogen to phosphate and nitrate, respectively (Menzel & Corwin 1965, Crumpton *et al.* 1992, Bachmann & Canfield, 1996). A colorimetric assay was used to enumerate total P (Murphy & Riley, 1962), while second derivative spectroscopy was employed for enumeration of total N (Crumpton *et al.*, 1992; Bachmann & Canfield, 1996).

Bacterial abundance

Bacterial abundance on the leaf surface and in the water column sub-samples was quantified via direct microscopic counts using the DAPI (49,6-diamidino-2-phenylindole) fluorescent staining procedure (Porter & Feig, 1980; Walker *et al.*, 1988; Kaufman *et al.*, 2001). Water column and leaf disc samples were sonicated (Aquasonic model 50T, Westchester, PA) for 12 min to reduced cell clumping and/or dislodge cells (Velgi & Albright, 1993). Samples were vortexed and diluted as necessary with filtered-sterilized Milli-Q water (Millipore, Bedford, MA). After the staining material at a final concentration of 20 µg/ml for 15 min, samples were filtered onto black filters (0.2-mm pore size; Nucleopore, Costar, Cambridge, Mass). For each subsample, filters were counted (600 cells per filter minimum) at 1000×

Bacterial productivity

Direct measurements of microbial biomass accumulation were conducted using a ³H-leucine incorporation assay (Kirchman, 2001). This technique measures of the incorporation of amino acids into protein in a bacteria-specific manner, through the use of short incubation periods and nanomolar leucine concentrations (Riemann & Azam, 1992). A 5.85 ratio of labeled:unlabeled leucine was added to water subsamples at a concentration of 25 nM to achieve saturation of uptake kinetics (Kirchman, 2001; Kaufman *et al.*, 2001). Water samples were incubated with labeled leucine (L-leucine (4,5-³H), 50 Ci/mmol- NEN, Life Science, Boston, MA) in the dark at room temperature for 30 min in 2 ml microcentrifuge tubes (Smith & Azam, 1992; Kirchman, 2001). Trichloroacetate [TCA, final concentration 10% (vol:vol)] was added to terminate reactions and precipitate protein. Two rinses of the TCA-protein precipitates were conducted with 10% TCA, followed by a single rinse with 5°C, 80% (vol:vol) ethanol. Standard liquid scintillation counting techniques were used to quantify the amount of radioactivity present in the samples.

Statistical analysis

Within each experiment, multivariate analysis of variance (MANOVA) was used to analyze mosquito performance variables (Scheiner, 2001) (Proc GLM, SAS Institute). Specifically, mosquito production variables used for MANOVA were survival, total male and female mass per microcosm, and total male and female emergence time per microcosm. Due to the lack of mosquito production from some treatments, emergence time data were omitted from the MANOVA in the first experiment because a missing value (cannot enter an infinite emergence time) in one of these categories would result in the whole case being dropped from the analysis. Since zero values for survival and mass are meaningful in that they indicate microcosm conditions are unable to support mosquito development, these were still included in the analyses. To assess the relationship of each mosquito production variable to microcosm inputs, standardized canonical coefficients (SCCs) were calculated for each MANOVA (Scheiner, 2001). Dependent mosquito variables with significant MANOVA results were subjected to individual univariate analysis of variance ('protected' ANOVA, Scheiner, 2001) followed by post-hoc comparisons (Fisher's protected LSD). Significance levels for post-hoc comparisons were adjusted using a sequential Bonferroni correction to reduce the chance of Type 1 error (Rice, 1989). Leaf mass was analyzed independently of other dependent variables in a separate ANOVA for each experiment. When necessary, data were square-root $[(x + 0.5)^{1/2}]$ or arcsine transformed prior to analysis to meet normality criteria. All values reported are non-transformed.

Canonical correlation analysis (CCA) was used to examine the relationship between mosquito and microcosm variables (microbial abundance, microbial productivity, nitrogen content, and phosphorus content) (Proc CANCELL, SAS Institute). Bacterial parameters and nutrient concentrations measured in experiment 3 were analyzed using separate repeated measures MANOVAs (also called doubly-multivariate repeated measures MANOVA) to

account for the effect of time on measurements taken on days 0 and 70 (Tabachnick & Fidell, 2001). Because these parameters dropped to equally low levels across all treatments by day 70, standard MANOVA was used to analyze nutrient and microbial levels on day 0.

Results

Experiment 1

To determine whether the labile portion of leaf detritus (e.g. leachate) was sufficient to support mosquito production, microcosms containing leachate only were compared with microcosms containing leached leaves with leachate or with distilled water. In treatments containing leaves, there was no significant difference in the amount of leaf mass remaining at the end of the experiment ($F_{1,10} = 0.64$, $P = 0.44$). Mosquito parameters were significantly affected by the type of input provided in microcosms such that the treatment main effect was influenced primarily by changes in mosquito survival (Table 1, SCC). The first, and only significant, canonical explained 93.4% of the variation. Survival was significantly lower in the absence of leaves (Fig. 1; $F_{2,15} = 8.37$, $P = 0.004$). Compared with microcosms receiving leachate only, microcosms containing leaf treatments produced more adult mosquitoes, and these developed faster and attained greater mass (Fig. 1). In addition, female mosquitoes in microcosms that received leachate in addition to leaf material developed faster than in microcosms containing leaf material and water. Mosquito performance in leachate only microcosms was poor, with only one male and zero females produced from this treatment.

Experiment 2

The main effects of leaf type and leachate had significant effects on mosquito production parameters (MANOVA, Table 2). The leaf, leachate and interaction main effects were explained mainly by changes in male emergence time, and mosquito survival, although female mass also contributed to the explanatory power of the first canonical (Table 2, SCC). Only the first canonical was significant, accounting for 78.5% of the variation. Overall, mosquitoes in microcosms containing unleached leaves were characterized by significantly increased survival ($F_{1,40} = 26.7$; $df =$; $P \leq 0.0001$), reduced development time (females: $F_{1,23} = 8.9$, $P = 0.006$; males: $F_{1,38} = 44.2$, $P < 0.0001$), and greater average mass of adult *Ae. triseriatus* (females: $F_{1,40} = 31.3$, $P < 0.0001$; males: $F_{1,40} = 23.7$, $P < 0.0001$) (Fig. 2), indicating that unleached leaf material was a better resource for mosquitoes. Indeed, unleached leaves lost a significantly greater amount of mass over the course of the experiment compared with leached leaves ($F_{7,39} = 5.13$, $P = 0.0003$). In contrast to the effect of leaf material, univariate analyses showed leachate additions were associated with significant changes in development time (Fig. 2; $F_{3,38} = 6.6$, $P = 0.001$) and body mass (Fig. 2; $F_{3,40} = 4.3$, $P = 0.01$) parameters for male mosquitoes only; male development time was shorter in microcosms containing leached leaves and any amount of leachate compared with microcosms containing no leachate and leached leaves (Fig. 2, Table 2). A significant interaction effect between leaf and leachate (Fig. 2; $F_{3,38} = 5.1$, $P = 0.005$) indicated male development time decreased with amount of leachate added to microcosms containing leached leaves (Table 2); however, the positive effect of unleached leaves on male development time was not recovered through the addition of leachate to microcosms with leached leaves. In all cases, mosquito parameters associated with additions of 100% leachate to leached leaves did not recover the level of those parameters associated with additions of 0% leachate to unleached leaves such that survival and adult body mass obtained were comparatively lower and development time was slower. Furthermore, additions of leachate to unleached leaves generally did not affect mosquito growth.

Experiment 3

As in the second experiment, unleached leaves were a better resource for mosquitoes, supporting significantly greater adult production in these microcosms compared with microcosms containing leached leaf material (Table 3, Fig. 3). MANOVA indicated significant main effects for leaf, leachate and their interaction, and these effects were explained mainly by changes in male mass, male development time, and mosquito survival (Table 3, SCC). The first mosquito performance canonical explained 98.0% of the variation and was positively correlated with female and male mass, female emergence time and survival, but negatively correlated with male emergence. Mean female mass and mosquito survival were significantly greater in the presence of unleached leaves (Fig. 3; female mass: $F_{1,42} = 7.4$, $P = 0.01$; survival: $F_{1,38} = 11.7$, $P < 0.0001$), while development time was unaffected. Similarly, additions of leachate to microcosms had a significantly positive effect on female mosquito growth and mosquito survival (Fig. 3; female mass: $F_{6,80} = 11.7$, $P < 0.0001$; survival: $F_{2,42} = 35.4$, $P < 0.001$). In contrast to the previous experiment, the negative effect of leached leaves on mosquito production was mitigated by the addition of leachate such that adult emergence in microcosms with this treatment combination was equal to or greater than emergence in microcosms with unleached leaves and 0% leachate (Fig. 3).

Microbial parameters—MANOVA yielded significant leaf condition and leachate effects on water column bacterial abundance and productivity and leaf surface bacterial abundance (Figs. 4 and 5; Table 4). The first microcosm canonical was positively correlated with water column microbial productivity and microbial abundance (Table 4, SCC) and accounted for 77.9% of the variation. The remaining four canonicals were not significant, each accounting for $\leq 2\%$ of the variance. Individual univariate ANOVA's revealed that water column bacterial productivity and abundance and leaf surface abundance were each significantly affected by leaf (water productivity: $F_{1,26} = 111.9$, $P < 0.0001$; water abundance: $F_{1,26} = 56.4$, $P < 0.0001$; leaf abundance $F_{1,26} = 19.7$, $P < 0.0001$) and leachate treatments (water productivity: $F_{2,26} = 37.8$, $P < 0.0001$; water abundance: $F_{2,26} = 11.2$, $P = 0.0003$; leaf abundance $F_{1,26} = 5.7$, $P = 0.01$) (Fig. 4). Production of bacteria in the water column was greatest in response to unleached leaves at day 0, prior to larval addition. Moreover, productivity was higher for this treatment combination in the presence of filtered leachate.

The effects of leachate type, and leaf condition, were significantly influenced by time, resulting in reduced bacterial abundance and productivity in microcosms across all treatments by day 70 (data not shown). In general, bacterial productivity dropped below 1×10^{-7} pmol/ml for all treatment combinations at this time, although direct microscopic counts indicate that bacteria remained present at a similar abundance over the course of the experiment (Fig. 4). Bacterial abundance in the water column and on leaf surfaces responded in a similar manner to leaf condition, with higher concentrations of cells/ml at day 0 evident in microcosms receiving unleached leaves (Fig. 4; Table 4). In the water column, bacterial abundance was also significantly affected by leachate, resulting in higher bacterial abundance and productivity in the water column of microcosms with filtered leachate when compared with those containing unfiltered leachate or water. There was also a significant interaction between leaf condition and leachate addition for water column bacterial production and abundance, with increases in these parameters occurring in the presence of unleached leaves treated with leachate. Leachate or leaf condition did not significantly affect the abundance of leaf-associated microorganisms and there was no interaction between the two factors.

Nutrient Analysis—Reductions in the total concentration of N and P were significantly affected by time, dropping to nearly zero across all treatments by day 70 (data not shown).

Prior to larval addition, total N and P concentrations (Fig. 5) were significantly affected by leaf type leachate, and their interaction (Table 4, MANOVA). The first microcosm canonical variate was positively correlated with N, but negatively correlated with P (Table 4, SCC). Microcosms receiving unleached leaves contained significantly greater amounts of N and P relative to those with leached leaves (Fig. 5; N: $F_{1,42} = 937.1$, $P < 0.0001$; P: $F_{1,42} = 94.6$; $P < 0.0001$). Similarly, microcosms with leachate contained significantly higher N levels compared with microcosms lacking leachate ($F_{2,42} = 234.0$, $P < 0.0001$). Canonical correlation analysis of mosquito and microcosm variables indicated that nitrogen level was positively correlated with mosquito survival (Fig. 6).

Discussion

Senescent plant material is the most abundant allochthonous input in tree holes, furnishing a nutrient base for macroinvertebrates and heterotrophic microorganisms in these containers (Kitching, 2001). Previous work had indicated that this relatively labile, water-soluble fraction of leaf material is critical to *Ae. triseriatus* larval growth (Walker *et al.*, 1997), and in this experiment, mosquito production fell as much as 38% in microcosms treated with leaves that were leached prior to being added as the primary nutrient source. This study supported the hypothesis that leaf detritus, although necessary, is not sufficient for mosquito production. In contrast, the hypothesis that labile leaf components would result in productivity comparable to that observed in response to unleached senescent leaves was not supported; both labile and refractile leaf components were needed for optimal production. The failure of leachate alone to support larval development (experiment 1) re-emphasizes their reliance on the slower decay of particulate matter, and the surfaces such material provides for microbial production, in these systems. Furthermore, the absence of mosquito production from leachate-only microcosms indicates an ephemeral and insufficient nutrient supply in leachate from the larval mosquito perspective. Additionally, leachate might be expected to have negative effects associated with inhibitory substances (e.g., tannins) found in this fraction; however, increasing amounts of leachate in experiment 2 were generally stimulatory to mosquito production. Palik *et al.* (2006) measured performance of *Ae. aegypti* larvae on sugar maple leaves and leachate, and concluded that poor nutritional quality rather than secondary metabolites in the water soluble fraction explained the relatively poor growth. That leaf surfaces are important to microorganism-mosquito larvae interactions is well established, as they support and maintain microbial biofilms that are grazed heavily by larvae (Kaufman *et al.*, 2001, 2002). The presence of leaf material and associated microbial biofilm appear essential for mosquito production based on these findings; however, it is likely that natural bark-lined tree holes also provide surface area for the development of microbial biofilms and mosquito browsing along the container walls. Although microcosm walls provided a substrate for mosquito browsing, it is possible that the composition of the containers (polyvinyl chloride) was not an adequate substrate or did not provide adequate surface area for promoting microbial growth. Further experiments are needed to determine the role of leaves as a structural element in tree hole mosquito habitats.

We expected that the generally positive effect of labile leaf components (leachate) on mosquito success could be reclaimed by the re-introduction of these components to microcosms containing leached leaves. In our second experiment, mosquito survival (larvae and adults) in microcosms containing leached leaves and 100% of the leachate produced by an equivalent leaf amount was not significantly different from survival in those containing unleached leaves and no additional leachate, suggesting that for this parameter the effect of leachate could be restored. In contrast, although development time and adult mass exhibited a positive response to the reintroduction of leachate, complete recovery of levels obtained in the unleached leaf treatments was not observed. We postulate that this reflects both the overall inadequacy of leached leaves for driving adult production and the artifacts of our

preparing bulk leachate for redistribution. Initially, we presumed the failure of leachate restoration to stimulate equivalent adult production was due to the effect of filtering the leachate. A plausible explanation is that the available nutrients present in the leached leaf material were assimilated by microorganisms prior to filter-sterilization during the leaching period. Filtering the leachate before adding it to microcosms would, therefore, remove two critical components from the microcosms: incorporated nutrients and established populations of microorganisms.

Surprisingly, the results of the third experiment showed that filtration of leachate did not influence mosquito production parameters in the direction anticipated. Greater bacterial productivity was observed following filtration, presumably due to the removal of established and slower growing bacterial populations, damage to cells during filtration and subsequent release of nutrients, and/or removal of protozoan predators from the leachate via filtration. That substantial bacterial populations were present prior to filtration is evidenced in this experiment, wherein DMCs of bacterial cells in leachate were 7.2 log cells/ml. Predatory protozoan populations, particularly small flagellates, can establish quickly in these mosquito habitats (Kaufman *et al.*, 2002), and their additions to microcosms in the unfiltered leachate would likely have a negative effect on bacterial productivity (Jurgens & Matz, 2002).

The fact that high initial water column bacterial productivity was not associated with better mosquito performance is unsurprising in light of recent studies that document the greater importance of leaf-associated bacteria and fungi to larval *Ae. triseriatus* (Kaufman & Walker, 2006; Kaufman *et al.*, 2001). In the third experiment, the best overall mosquito performance, suggested by the number of adult females produced, occurred when initial bacterial productivity was lowest. As mentioned above, this may suggest that more protozoan or fungal biomass was present. We have observed that eukaryotic DNA quantities in water column samples from similar larval microcosms are inversely related to prokaryotic DNA quantities (Kaufman unpub. data). Additionally, the best mosquito performance was essentially under conditions of the highest overall nutrient input (unleached leaves and unfiltered leachate) – conditions that would be most analogous to N and DOC amendments that have been shown to enhance *Ae. triseriatus* growth in similar microcosms (Kaufman & Walker, 2006; Kaufman *et al.*, 2002). Indeed, the results of canonical correlation analysis here indicate that mosquito survival was greatest and male emergence time fastest when microbial abundance was greatest and nitrogen and phosphorus levels were high.

The success of mosquitoes in the unleached leaf treatments compared with leached leaf treatments (no leachate added) in this study, and in Walker *et al.* (1997), was presumably associated with sustained microbial productivity through larval development. In some treatments, high initial levels of bacterial productivity were not maintained, with differences among treatments becoming negligible by the end of the experiment due to reduced bacterial productivity in response to grazing pressure from larvae and protozoans (Kaufman *et al.*, 2001) without corresponding reductions in overall abundance. It should be noted that the microbial analyses herein do not account for compositional changes in the microbial community. Indeed, microbial communities exposed to protozoan and invertebrate feeding pressure are known to undergo a structural shift, potentially resulting in the dominance of indigestible forms (Jürgens & Matz 2002; Matz & Jürgens, 2003; Pernthaler *et al.*, 1997; Šimek *et al.*, 1997, Sommaruga & Psenner, 1995). Such shifts in the composition of bacterial and fungal communities in response to larval grazing have recently been described for tree holes (Kaufman *et al.*, 2008). Hence, microbial abundance may remain relatively unchanged throughout an experiment despite underlying changes in bacterial susceptibility to foraging mosquito larvae and therefore, quality of the larval environment. That declines in bacterial productivity do not correspond with drops in abundance of the same magnitude also suggest that either community composition or metabolic activity were altered. The

absence of changes in bacterial abundance also suggests sufficient nutritive material is available to maintain microbial communities in the absence of stemflow events (which would bring in pulses of limiting nutrients such as N and P).

In nature, rapid degradation of leaves may occur upon entering tree holes, particularly under high nutrient conditions (Macia & Bradshaw, 2000). Inorganic nutrients (N, P) promote decomposition because, despite available particulate carbon pools, microbial production and decomposition enzyme production is often limited by these nutrients and DOC (Kaufman & Walker, 2006; Kaufman *et al.*, 2002). Once the initial flush of nutrients from the leaf matrix has been exhausted, the growth of heterotrophic microorganisms available for mosquito consumption is limited until stemflow or detritus replenish the system. Although initial fluxes of nutrient-rich leachate seemed critical for developing larvae in previous studies, they may not be the norm in natural tree holes. More likely, the situation many mosquito larvae experience is one driven by the slow decay of low quality leaf material. As *Ae. triseriatus* larvae hatch from mid- to late spring, leaf inputs are primarily in the form of senescent leaves that have been subjected to some degree of leaching throughout the winter. This cohort of larvae probably experiences a “leached leaf” environment and adult production likely depends on additional inputs that stimulate microbial production immediately (e.g., less refractile plant material) and also spur faster decay of the existing detrital pool (e.g., N input that promotes leaf – associated fungi). Indeed, these data suggest that alternate nutrient inputs (e.g. animal detritus, fecal material, or pollen) may play a critical role in driving mosquito production (Kaufman unpub. data). The interplay of particulate detrital input, subsequent solubilization and incorporation by microbial heterotrophs, and harvesting of microbial biomass to allow mosquito production is relatively understudied yet critical in understanding how the many species of mosquitoes that breed in container habitats successfully emerge as disease vectors.

Acknowledgments

We thank Lane Frasier for her help with laboratory analysis and mosquito sampling and two anonymous reviewers for their helpful comments. This project was supported by National Institutes of Health award AI21884.

Literature Cited

- Bachmann RW, Canfield DE. Use of an alternative method for monitoring total nitrogen concentrations in Florida lakes. *Hydrobiologia*. 1996; 323:1–8.
- Bärlacher F. The role of fungi in the nutrition of stream invertebrates. *Botanical Journal of the Linnaen Society*. 1985; 91:83–84.
- Carpenter SR. Comparisons of equations for decay of leaf litter in tree hole ecosystems. *Oikos*. 1982a; 39:17–22.
- Carpenter SR. Stemflow chemistry effects on population dynamics of detritivorous mosquitos in tree hole ecosystems. *Oecologia*. 1982b; 53:1–6.
- Carpenter SR. Resource limitation of larval treehole mosquitoes subsisting on beech detritus. *Ecology*. 1983; 64:219–223.
- Cornelissen JHC. An experimental comparison of leaf decomposition rates in a wide range of temperate plant species and types. *Journal of Ecology*. 1996; 84:573–582.
- Craig, GB. Biology of *Aedes triseriatus*: some factors affecting control. In: Calisher, CH.; Thompson, WH., editors. *California serogroup viruses*. Alan R. Liss; New York, New York: 1983. p. 329–341.
- Crompton WG, Isenhardt TM, Mitchell PD. Nitrate and organic N analyses with second-derivative spectroscopy. *Limnology and Oceanography*. 1992; 37:907–913.
- Cummins KW, Klug MJ. Feeding ecology of stream invertebrates. *Annual Review of Ecology and Systematics*. 1979; 10:147–172.

- Daugherty MP, Alto BW, Juliano SA. Invertebrate carcasses as a resource for competing *Aedes albopictus* and *Aedes aegypti* (Diptera: Culicidae). *Journal of Medical Entomology*. 2000; 37:364–372. [PubMed: 15535579]
- Fish D, Carpenter SR. Leaf litter and larval mosquito dynamics in tree hole ecosystems. *Ecology*. 1982; 63:283–288.
- Fish, D. Phytotelmata, Flora and Fauna. In: Frank, JH.; Lounibos, LP., editors. *Phytotelmata: Terrestrial plants as hosts for aquatic insect communities*. Plexus; Medford, N.J.: 1983. p. 1-27.
- Gessner MW, Schwoerbel J. Leaching kinetics of fresh leaf-litter with implications for the current concept of leaf-processing in streams. *Archiv fuer Hydrobiologie*. 1989; 115:81–90.
- Grattan RM, Suberkropp K. Effects of nutrient enrichment on yellow poplar leaf decomposition and fungal activity in streams. *Journal of the North American Benthological Society*. 2001; 20:33–43.
- Hard JJ, Bradshaw WE, Malarkey DJ. Resource- and density-dependent development in tree hole mosquitoes. *Oikos*. 1989; 54:137–144.
- Harshaw L, Chrisawn C, Kittinger B, Carlson J, Metz G, Smith L, Paradise CJ. Decaying invertebrate carcasses increase growth of *Aedes triseriatus* (Diptera: Culicidae) when leaf litter resources are limiting. *Journal of Medical Entomology*. 2007; 44:589–596. [PubMed: 17695012]
- Jürgens K, Matz C. Predation as a shaping force for the phenotypic and genotypic composition of planktonic bacteria. *Antonie van Leeuwenhoek*. 2002; 81:413–434.
- Kaufman MG, Walker ED, Smith TW, Merritt RW, Klug MJ. Effects of larval mosquitoes (*Aedes triseriatus*) and stemflow on microbial community dynamics in container habitats? *Applied and Environmental Microbiology*. 1999; 65:2661–2673. [PubMed: 10347058]
- Kaufman MG, Bland SN, Worthen ME, Walker ED, Klug MJ. Bacterial and fungal biomass responses to feeding by larval *Aedes triseriatus* (Diptera: Culicidae). *Journal of Medical Entomology*. 2001; 38:711–719. [PubMed: 11580044]
- Kaufman MG, Goodfriend W, Kohler-Garrigan A, Walker ED, Klug MJ. Soluble nutrient effects on microbial communities and mosquito production in *Ochlerotatus triseriatus* habitats. *Aquatic Microbial Ecology*. 2002; 29:73–88.
- Kaufman MG, Walker ED. Indirect effects of soluble nitrogen on growth of *Ochlerotatus triseriatus* larvae in container habitats. *Journal of Medical Entomology*. 2006; 43:677–688. [PubMed: 16892624]
- Kaufman MG, Chen S, Walker ED. Leaf-associated bacterial and fungal taxa shifts in response to larvae of the tree hole mosquito, *Ochlerotatus triseriatus*. *Microbial Ecology*. 2008; 55:673–684. [PubMed: 17899246]
- Kirchman, DL. Measuring bacterial biomass production and growth rates from leucine incorporation in natural aquatic environments. In: Paul, J., editor. *Methods in Microbiology*. Academic Press; New York, New York: 2001. p. 227-237.
- Kitching RL. An ecological study of water-filled tree-holes and their positions in the woodland ecosystem. *Journal of Animal Ecology*. 1971; 40:281–302.
- Kitching RL. Food webs in phytotelmata, “Bottom-up” and “top-down” explanations for community structure. *Annual Review of Entomology*. 2001; 46:729–760.
- Leonard PM, Juliano SA. Effect of leaf-litter and density on fitness and population performance of the tree hole mosquito *Aedes triseriatus*. *Ecological Entomology*. 1995; 20:125–136.
- Macia A, Brandshaw WE. Seasonal availability of resources and habitat degradation for the western tree hole mosquito, *Aedes sierrensis*. *Oecologia*. 2000; 125:55–65.
- Matz C, Jürgens K. Interaction of nutrient limitation and protozoan grazing determines the phenotypic structure of a bacterial community. *Microbial Ecology*. 2003; 45:384–398. [PubMed: 12704556]
- Menzel DW, Corwin N. The measurement of total phosphorus in seawater based on the liberation of organically bound fractions by persulfate oxidation. *Limnology and Oceanography*. 1965; 10:280–282.
- Mercer DR, Anderson JR. Tannins in treehole habitats and their effects on *Aedes sierrensis* (Diptera: Culicidae) production and parasitism by *Lambornell clarki* (Ciliophora: Tetrahymenidae). *Journal of Medical Entomology*. 1994; 31:159–167. [PubMed: 8158619]
- Merritt RW, Dadd RH, Walker ED. Feeding behavior, natural food, and nutritional relationships of larval mosquitoes. *Annual Review of Entomology*. 1992; 37:349–376.

- Moorhead DL, Sinsabaugh RL. A theoretical model of litter decay and microbial interaction. *Ecological Monographs*. 2006; 76:151–174.
- Murphy J, Riley JP. A modified single solution method for the determination of phosphate in natural waters. *Analytica Chimica Acta*. 1962; 27:31–36.
- Palik B, Batzer DP, Kern C. Upland forest linkages to seasonal wetlands: Litter flux, processing, and food quality. *Ecosystems*. 2006; 9:142–151.
- Pernthaler J, Posch T, Šimek K, Vrba J, Amann R, Psenner R. Contrasting bacterial strategies to coexist with a flagellate predator in an experimental microbial assemblage. *Applied and Environmental Microbiology*. 1997; 63:596–601. [PubMed: 16535516]
- Porter, KG. Integrating the microbial loop and the classic food chain to a realistic planktonic food web. In: Polis, GA.; Winemille, KO., editors. *Food webs, integration of patterns and dynamics*. Chapman and Hall; New York, New York: 1996. p. 51-59.
- Porter KG, Feig YS. The use of DAPI for identifying and counting aquatic microflora. *Limnology and Oceanography*. 1980; 25:943–948.
- Riemann B, Azam F. Measurements of bacterial protein synthesis in aquatic environments by means of leucine incorporation. *Marine Microbial Food Webs*. 1992; 6:91–105.
- Reiskind MH, Greene KL, Lounibos P. Leaf species identity and combination affect performance and oviposition choice of two container mosquito species. *Ecological Entomology*. 2009; 34:447–456. [PubMed: 20161198]
- Rice WR. Analyzing tables of statistical tests. *Evolution*. 1989; 43:223–225.
- SAS Institute Inc.. SAS/STAT User's Guide. version 4th ed.. SAS Institute; Cary, NC: 2000.
- Scheiner, SM. MANOVA: multiple response variables and multispecies interactions. In: Scheiner, M.; Gurevitch, J., editors. *Design and analysis of ecological experiments*. 2nd ed.. Oxford University Press; Oxford, United Kingdom: 2001. p. 99-115.
- Šimek K, Vrba J, Pernthaler J, Posch T, Hartman P, Nedoma J, Psenner R. Morphological and compositional shifts in an experimental bacterial community influenced by protists with contrasting feeding modes. *Applied and Environmental Microbiology*. 1997; 63:587–595. [PubMed: 16535515]
- Smith DC, Azam F. A simple, economical method for measuring bacterial protein synthesis rates in seawater using ³H-leucine. *Marine Microbial Food Webs*. 1992; 6:107–114.
- Sommaruga R, Psenner R. Permanent presence of grazing resistant bacteria in a hypertrophic lake. *Applied and Environmental Microbiology*. 1995; 61:3457–3459. [PubMed: 16535131]
- Tabachnick, BG.; Fidell, LS. Using multivariate statistics. 4th ed.. Allyn & Bacon; Needham Heights, MA: 2001. Profile analysis: The multivariate approach to repeated measures (Chapter 10); p. 423-452.
- Velgi, MI.; Albright, LJ. Improved sample preparation for enumeration of aggregated aquatic substrate bacteria. In: Kemp, PF.; Sherr, BF.; Sherr, EB.; Cole, JJ., editors. *Handbook of methods in aquatic microbiology*. Lewis; Boca Raton, Florida: 1993. p. 139-142.
- Walker ED, Lawson DL, Merritt RW, Morgan WT, Klug MJ. Nutrient dynamics, bacterial populations, and mosquito productivity in tree hole ecosystems and microcosms. *Ecology*. 1991; 72:1529–1546.
- Walker ED, Merritt RW. Behavior of larval *Aedes triseriatus* (Diptera: Culicidae). *Journal of Medical Entomology*. 1991; 28:581–589. [PubMed: 1941923]
- Walker ED, Olds EJ, Merritt RW. Gut content-analysis of mosquito larvae (Diptera, Culicidae) using DAPI stain and epifluorescence microscopy. *Journal of Medical Entomology*. 1988; 25:551–554. [PubMed: 2905011]
- Walker ED, Kaufman MG, Ayres MP, Riedel MH, Merritt RW. Effects of variation in quality of leaf detritus on growth of the eastern tree hole mosquito, *Aedes triseriatus* (Diptera: Culicidae). *Canadian Journal of Zoology-Revue Canadienne De Zoologie*. 1997; 75:706–718.
- Webster JR, Benfield EF. Vascular plant breakdown in freshwater ecosystems. *Annual Review of Ecology and Systematics*. 1986; 17:567–594.
- Yee DA, Juliano SA. Consequences of detritus type in an aquatic microsystem: effects on water quality, micro-organisms and performance of the dominant consumer. *Freshwater Biology*. 2006; 51:448–459. [PubMed: 17476312]

Fig. 1. Mosquito production variables from microcosms provisioned with leachate only, leached leaves with water, and leached leaves with leachate intact. (A) Survival (40 initial larvae). (B) Average female development time per microcosm. (C) Average male development time per microcosm. (D) Total female mass per microcosm. (E) Total male mass per microcosm. Values are means $\pm$ SE ($n = 6$ for all variables in A, D, and E; $n = 0-6$ for variables in B and C). For each significant mosquito parameter determined by ANOVA, treatment means with the same letter are not significantly different, ($P > 0.05$, Fisher's protected LSD test).

Fig. 2. Mosquito production variables from microcosms provisioned with 0, 10, 50, or 100% leachate and leached or unleached leaves. (A) Survival (20 initial larvae). (B) Average female development time per microcosm. (C) Average male development time per microcosm. (D) Total female mass per microcosm. (E) Total male mass per microcosm. Values are means $\pm$ SE ($n = 6$ for all variables in A, C, D, and E; $n = 0-6$ for variables in B). For each significant mosquito parameter determined by ANOVA, significant differences between leaf treatments are indicated by an asterisk; leachate treatment means with the same letter are not significantly different ($P > 0.05$, Fisher's protected LSD test).

Fig. 3. Mosquito production variables from microcosms provisioned with filtered, unfiltered leachate or water and leached or unleached leaves. (A) Survival (40 initial larvae). (B) Average female development time per microcosm. (C) Average male development time per microcosm. (D) Total female mass per microcosm. (E) Total male mass per microcosm. Values are means $\pm$ SE (n = 6). For each significant mosquito parameter determined by ANOVA, significant differences between leaf treatments are indicated by an asterisk; leachate treatment means with the same letter are not significantly different ($P > 0.05$, Fisher's protected LSD test).

Fig. 4. Bacterial response to leachate, filtration, and leaf condition at the onset of experiment 2 (day 0). Bacterial abundance (direct microscopic counts) is shown for (A) water column and (B) leaf surface. Bacterial productivity (leucine incorporation rate) is shown for the water column (C). Open bars represent leached leaves and shaded bars represent unleached leaves. Values are means $\pm$ SE ($n = 6$). Significant differences between leaf treatments are indicated by an asterisk; leachate treatment means with the same letter are not significantly different ($P > 0.05$, Fisher's protected LSD test).

Fig. 5.

Water column chemistry response to leachate, filtration, and leaf condition at the onset of experiment 3 (day 0). Shown are total nitrogen (A) and total phosphorous (B). Open bars represent leached leaves and shaded bars represent unleached leaves. Values are means $\pm$ SE ($n = 6$). For each significant parameter determined by ANOVA, significant differences between leaf treatments are indicated by an asterisk; leachate treatment means with the same letter are not significantly different ($P > 0.05$, Fisher's protected LSD test).

Fig. 6.
Bivariate plot depicting the relationship between nitrogen concentration and mosquito survival in response to leaf condition and leachate type in experiment 3.

Multivariate (MANOVA) results for the effect of leachate only, leached leaves with water, and leached leaves with leachate on mosquito production variables and standardized canonical coefficients (SCCs) of the first canonical variate[‡].

Table 1

Source	MANOVA			SCC		
	Pillai's trace	F	df	P	Total Female Mass	Total Male Mass
Treatment	0.770	2.92	6,28	0.02*	-0.968	0.829
						1.582

An asterisk indicates significance. $P \leq 0.05$ for MANOVA.

[‡] Only the first canonical was significant.

Table 2

Multivariate analysis of variance (MANOVA) for the effect of 0,10, 50, or 100% leachate and leached or unleached leaves on mosquito performance variables and the standardized canonical coefficients (SCC) of the first canonical variate[†].

Source	MANOVA				SCC				
	Pillai's trace	F	df	P	Female mass	Male mass	Female emergence time	Male emergence time	Survival
Leaf	0.865	24.44	5,19	<0.0001*	0.415	-0.263	0.035	-2.009	0.942
Leachate	1.296	3.19	15,63	0.0006*	-0.099	0.082	0.220	-2.175	0.686
Leaf × Leachate	1.044	4.37	10,40	0.0004*	-0.238	0.745	-0.047	2.318	-0.681

An asterisk indicates significance. $P \leq 0.05$ for MANOVA.

[†] Only the first canonical was significant.

Table 3

Multivariate analysis of variance (MANOVA) for the effect of leachate (filtered or unfiltered) or water and leached or unleached leaves on mosquito performance variables and the standardized canonical coefficients (SCC) of the first canonical variate[†].

Source	MANOVA				SCC				
	Pillai's trace	F	df	P	Female mass	Male mass	Femal emergence time	Male emergence time	Survival
Leaf	0.560	5.86	5,23	0.0012*	-0.065	0.662	0.397	-1.579	1.659
Leachate	0.832	3.42	10,48	0.0019*	0.616	0.276	0.208	-0.216	0.772
Leaf × Leachate	0.334	0.96	10,48	0.4479	-0.109	-0.896	-0.692	1.777	-0.728

An asterisk indicates significance. $P \leq 0.05$ for MANOVA.

[†] Only the first canonical was significant.

Table 4

Canonical correlation analysis of mosquito and microbial variables denoting standardized canonical coefficients (SCC) of the first canonical and correlation of original variables with the first canonical*.

	First canonical variate	
	Standardized canonical coefficient	Correlation
Mosquito performance variables		
Female mass	0.016	0.582
Male mass	0.225	0.181
Female emergence	0.048	0.247
Male emergence	-0.617	-0.129
Survival	0.966	0.889
Microcosm variables		
Nitrogen	1.384	0.942
Phosphorus	-0.333	0.566
Water column microbial productivity	0.022	0.507
Water column microbial abundance	-0.298	0.390
Leaf surface microbial abundance	-0.018	0.550

* Only the first canonical was significant.