Indirect Effects of Soluble Nitrogen on Growth of *Ochlerotatus triseriatus* Larvae in Container Habitats

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**ABSTRACT** We conducted experiments in laboratory microcosms to simulate input of stemflow nutrients and flushing of metabolites in the tree hole habitats of larval *Ochlerotatus triseriatus* (Say). In the first experiment, we simultaneously examined the effects of nutrient additions (nitrogen, phosphorus, glucose, or combination) and flushing (removal of one-half of water volume replaced by deionized water) on mosquito production. The combination of nutrients had the greatest positive effects on mosquito production, with nitrogen (N) likely accounting for most of the increase in adult emergence and adult mass. Dilution of the nutrient pool via simulated flushing reduced mosquito growth, suggesting that the primary effect of stemflow input was nutrient addition as opposed to dilution of any latent toxic metabolites. In a second experiment, N additions were crossed with larval presence or absence to examine effects on key microbial processes. N increased leaf decay rates, soluble carbohydrate concentrations, fungal biomass and leaf-associated carbohydrase activity, but it did not stimulate bacterial productivity. Leaf decay was enhanced and bacterial production on leaves and in the water column was depressed in the presence of larvae. We conclude that the inputs of soluble N stimulated fungal growth, which made more fungal biomass available because of both its absolute increase and via the softening of the leaf particulate matter that could allow direct ingestion by larvae.

**KEY WORDS** *Ochlerotatus triseriatus* larvae, tree holes, stemflow, soluble nutrients, bacteria, microbial biomass

*Ochlerotatus triseriatus* (Say) (= *Aedes triseriatus*; see Reinert 2000) is a common container-breeding mosquito in eastern North America. Larvae develop in water-filled tree holes and tires that are found in or near wooded areas. *Oc. triseriatus* is a primary vector of La Crosse encephalitis, and larvae of this species tend to be the predominant macroinvertebrate consumers found in tree holes in Michigan. *Oc. triseriatus* has been the focus of many previous studies of tree holes and similar habitats (Walker et al. 1991, Kitching 2001).

Tree hole systems are thought to be driven largely by particulate inputs and subsequent microbial processing of plant and animal detritus that supports a variety of macroinvertebrates, primarily dipteran larvae (Carpenter 1983, Kitching 2001). An essential component of these systems is the periodic input of water from rainfall events. Most tree holes collect water in the form of stemflow—rainwater flowing down tree trunks. In addition to the fundamental maintenance of the habitat itself, stemflow can add soluble nutrients and potentially serves to flush out toxic or inhibitory metabolites from the system (Carpenter 1982).

Although basic pathways of resource input and use leading to production of *Oc. triseriatus* adults from these habitats are known, many details about what limits that production are not. It is well established that quantity and quality of particulate input influence growth and emergence of adult mosquitoes (Fish and Carpenter 1982, Hard et al. 1989, Leonard and Juliano 1995, Walker et al. 1997), and there is also evidence that soluble inputs via stemflow are important to mosquito production (Carpenter 1982; Walker et al. 1991; Kaufman et al. 1999, 2002) These two types of inputs are linked because large particulates such as leaf litter in the system normally must enter the soluble pool and be incorporated into microbial biomass as a prerequisite for use by mosquito larvae. Therefore, the soluble nutrient pool ultimately has a primary, if indirect, influence on mosquito growth. Indirect effects, including trophic cascades in aquatic ecosystems, are increasingly recognized as key factors in explaining species abundance patterns (Wootton 1994, 2002). However, little is known about indirect nutrient effects in detritus-based food webs, particularly those where mosquito larvae are dominant consumers.

Although leaf material inputs tend to be high in carbon content, much of which is refractory cell wall material, it is relatively low in nitrogen and phospho-
rus (Webster and Benfield 1986, Walker et al. 1997). Stemflow, however, brings in nitrates, phosphates, and other inorganic nutrients, in addition to organic carbon compounds (Carpenter 1982). Leaf litter decay processes are known to be limited by inorganic nutrients, primarily nitrogen and phosphorus, in other aquatic systems (Webster and Benfield 1986), and it is likely that stemflow inputs impact particulate decay in tree hole systems. Carpenter (1982) showed that some inorganic components of stemflow affected leaf decay rates in microcosms, but possible mechanisms were unexplored. He also postulated that any positive effects of stemflow on mosquito growth could be due as much to the flushing out of toxic metabolites as it was to input of nutrients. However, there have been no studies that have examined the effects of nutrient inputs and the dilution or flushing consequences of stemflow inputs into tree hole systems simultaneously.

We have shown previously that soluble organic and inorganic nutrients in concentrations found in stemflow can stimulate microbial productivity and mosquito production in Oc. triseriatus larval habitats (Kaufman et al. 2002). In that study, nutrients were added as a mixture of nitrate, phosphate, and glucose without attempts to mimic the flushing effects of stemflow. Also from that study, glucose was shown to stimulate water column bacterial productivity; however, a nutrient mixture was more consistent in enhancing microbial and mosquito growth, suggesting at least colimitation of organic carbon and inorganic nutrients. Phosphate levels seem to fluctuate more dramatically in natural tree holes, whereas soluble nitrogen moieties were found at consistently high levels in both natural tree holes and microcosms (Walker et al. 1991, Kaufman et al. 1999). This suggested to us that phosphorus would be the key inorganic component of the nutrient mixture.

In this study, we address the inputs of nutrients individually and in combination while simultaneously examining the effects of flushing and dilution typically accompanying rainfall events in tree holes. We hypothesized that flushing with nutrient addition would improve mosquito production and that phosphorus would be an important colimiting nutrient with labile organic carbon. Because the first experiment indicated nitrogen was the key component of a nutrient effect, we examined mosquito and microbial responses to nitrate inputs. We hypothesized that nitrate additions would stimulate leaf-associated fungal and bacterial activity, leading to greater mosquito production.

Materials and Methods

Microcosms. We used laboratory microcosms, each containing 1 g (dry weight) of senesced white oak leaves, Quercus alba L., and 300 ml of deionized water, as experimental units. Microcosms were constructed similarly to those described by Walker et al. (1991) and consisted of an 8-in. length of polyvinyl chloride pipe (3 in. i.d.) sealed at one end and wrapped in foam pipe insulation. The microcosms were covered and secured with nylon window screen that had an access port with removable plug for experimental manipulations and collection of adults. We added a microbial inoculum (3 ml) prepared from water and leaf material homogenates taken from natural tree holes, and allowed the microcosms to incubate (23–25°C) for 4 d before adding 40 first instar larvae of Oc. triseriatus (our own laboratory colony derived from the Walton strain at the University of Notre Dame, Notre Dame, IN) to appropriate treatments. In our experience (Kaufman et al. 2001), this density of larvae with 1 g oak leaves induces a level of competition such that larval development and adult emergence is moderately to severely constrained. Microcosms were kept at room temperature (23–25°C) and shaded from room light with coarse black cloth placed over the shelving unit. Shading was used to eliminate direct light sources and to simulate canopy cover normally associated with natural tree holes. Although primary productivity can be important in some tree holes, most found in temperate forests are thought to be dominated by heterotrophic processes (Kitching 2000). Natural tree holes in our area normally do not contain detectable algal populations (M.G.K., unpublished data).

Experiment 1. In this experiment, we examined effects of nutrient inputs and the dilution of existing nutrient pools and removal of potentially inhibitory metabolites on mosquito production. Treatments (two levels of flush crossed with five nutrient levels at seven replicates per treatment combination) were assigned randomly to microcosms and applied 4, 8, 14, 18, 25, 32, and 44 d after larvae were added for a total of seven additions. Our flushing intervals approximated rainfall event frequencies in our area during the same period of larval development in natural tree holes. Significant rainfall events (>0.6 cm/d, thunderstorms) occurred in our locale (Lansing, MI) at rates of 5 ± 4 (mean ± SD) per month in April and May from 2001 to 2004, but varied greatly. Flushing was simulated by removing 150 ml of water from the microcosms and replacing that with 150 ml of deionized water. Based upon our own observations of flushing by stemflow in natural pan-tree hole, and the dilution of inorganic ions and a conservative tracer (chloride) in previous field and microcosm experiments, a 50% dilution of contents with each rainfall event would be well within natural ranges. No-flush treatments were left stagnant except when nutrients were added. Nutrients were added in concentrated form (3 ml) to give the following concentrations in 300 ml: Nitrogen (N) as KNO₃ at 5 ppm; N; phosphorus (P) as Na₂PO₄ at 0.5 ppm P; glucose at 20 ppm; mixture (mix) is N plus P plus glucose in concentrations listed above; or control, an equivalent quantity of deionized water. Deionized water was added as necessary to replace any evaporative losses during the course of the experiment. We measured levels of total N, total P, soluble carbohydrates, and pH of the water column twice during the experiment, at days 8 and 18 after larval addition. Because we were most interested in overall values of these parameters due to treatments and not concerned about fluxes with time, we used
mean values of the two measurements for statistical analysis. Samples (15 ml) for these water column measurements were collected before any flush or nutrient addition treatment. Adult mosquitoes were collected daily throughout the experiment, frozen immediately, and later lyophilized for dry weight determination. When the experiment was terminated (52 d after larval addition), any remaining larvae and pupae were counted, frozen, and lyophilized for dry weight determination. At this time, leaf material remaining was dried at 50°C for 48 h and weighed.

**Experiment 2.** This experiment was designed to examine the effects of nitrogen addition on leaf decay processes, fungal biomass and activity, and bacterial food resources while simultaneously monitoring larval feeding effects on those parameters. Microcosms were set up and maintained as described above except that only two nutrient treatments (N and control) were used, and one-half of the microcosms did not receive larvae. Nitrogen was added as KNO₃ (5 ppm N) weekly for six consecutive weeks, with the first addition being 1 d before introduction of 40 first instars to the larvae present treatment. The resultant 2 by 2 factorial was replicated with 18 microcosms of each treatment combination. This allowed the destructive sampling of six replicates of each treatment combination on 2 d (day 6 and day 20) during the experiment while leaving six replicates for mosquito production estimates. On the two sampling days, we randomly selected six microcosms from each treatment and separated water, leaf material, and any remaining larvae or pupae. Larvae and pupae were collected for lyophilization as described above. Water samples (1–10 ml, depending on particular analysis) were collected for soluble carbohydrate analysis, bacterial productivity, and total N determination (see below). Leaf material was sampled with a cork borer (18 or 11 mm in diameter, depending on particular analysis). Two leaf discs each were collected for ergosterol measurements, enzyme activity assays, dry weight determination, and bacterial productivity (see below). At the end of the experiment (42 d), any remaining larvae and pupae were collected, frozen, and lyophilized for dry weights, and remaining leaf material was dried and weighed as described above.

**Chemical Analyses.** Total P was determined using persulfate oxidation techniques on unfiltered samples. In this procedure, all phosphorus moieties present are converted to phosphate (Menzel and Corwin 1965) followed by subsequent colorimetric assay of total phosphate (Murphy and Riley 1962). Total N was determined on unfiltered water samples by using persulfate oxidation of all nitrogen moieties to nitrate followed by subsequent analysis using second derivative spectroscopy (Crumpton et al. 1992, Bachmann and Canfield 1996). Water pH was determined with a pH electrode. Soluble carbohydrate was measured on filtered (0.2 µM) water samples with a phenol-sulfuric acid procedure (Dubois et al. 1965) modified for water containing interfering substances (Gerchakov and Hatcher 1972).

**Bacterial Productivity.** Bacterial productivity was estimated with the [³H]leucine method (Kirchen 2001). This technique measures the incorporation of amino acids into protein and is thus a direct measure of microbial biomass accumulation. With short incubation times and nanomolar concentrations of added amino acids, the method is very specific for bacteria, and we have used it successfully in mosquito habitat and microcosm studies previously (Kaufman et al. 2001, 2002). We used the microcentrifuge tube version of this technique (Smith and Azam 1992, Kirchen 2001). Water samples (1 ml) were incubated in 2-ml microcentrifuge tubes. Two leaf discs (18 mm in diameter) from each microcosm were incubated in glass scintillation vials with filter-sterilized water from their corresponding microcosm. All samples were incubated for 30 min at room temperature in the dark. Reactions were stopped with concentrated trichloroacetic acid (TCA, final concentration 10% [vol:vol]), which also precipitates protein. Water samples were processed directly in their incubation tubes, whereas leaf discs were first placed in a sonicator bath with ice for 12 min (Kaufman et al. 2001). The leaf sonicates and subsequent rinses of the leaf discs with 10% TCA were then transferred to microcentrifuge tubes for quantitation of labeled protein in the same manner as water column samples. The TCA-protein precipitates were rinsed twice with 10% TCA, once with cold (5°C) 80% (vol:vol) ethanol, and concentrated by centrifugation. Radioactivity in the samples was then determined with standard liquid scintillation counting techniques.

**Fungal Biomass.** Fungal biomass was estimated from the ergosterol content of leaf subsamples (Newell and Barlocher 1993, Suberkropp and Weyers 1996). Two leaf discs (18 mm in diameter) from each microcosm were preserved in 100% high-performance liquid chromatography (HPLC)-grade methanol and refrigerated in the dark. Ergosterol was extracted and quantified using HPLC and UV detection as described previously (Kaufman et al. 2001).

**Enzyme Assays.** We examined leaf-associated carbohydrase activity using methylumbelliferyl (MUF) substrate analogs for plant polymers. These substrate analogs contain the same linkage found in the plant polymer and when the bond is enzymatically broken, MUF is released. Free MUF fluoresces at 360 nm and can be readily measured with fluorometers designed for DNA quantification. The technique is very sensitive and has been used to measure extracellular enzyme activity in aquatic systems (Hendel and Marxen 1997, Schulte et al. 2003). We used three MUF substrates: 4-methylumbelliferyl-β-D-celllobioside for cellobiohydrolase activity (releases cellubiose from cellulose polymer); 4-methylumbelliferyl-β-D-xyloside for xyllosidase activity (releases xylose from xylooligosaccharides and hemicellulose polymers); and 4-methylumbelliferyl-α-L-arabinofuranoside for arabinosidase activity (releases arabinose from hemicellulose polymers). All analogs plus free MUF (methylumbelliferyl-sodium salt) for standards were obtained from Sigma-Aldrich (St. Louis, MO).
For enzyme activity assays, we homogenized two leaf discs (11 mm in diameter) from each microcosm in 3 ml of phosphate buffer, pH 7.2, with a Virtis Tissuemizer (Tekmar Corp., Cincinnati, OH). The homogenate was placed in a sonicating bath (model 50T, Aquasonic, West Chester, PA) with ice for 5 min. Particles were removed via centrifugation, and 0.5-ml aliquots were incubated with 50 nmol of MUF substrate in microcentrifuge tubes. Remaining leaf extract was used for a composite heat-denatured control to which the same amount of MUF substrates was added. Solutions were incubated in a 25°C water bath for 1–1.5 h. Aliquots from each reaction tube were removed with 50-µl capillary tubes and read on a Hoefer DyNA Quest 200 fluorometer (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). The time between substrate addition and reading was recorded to calculate activity rates. Fluorometer readings were converted to pico moles per hour with a standard curve of free MUF. Because enzyme activity levels for the three substrates were found to be similar in magnitude among treatments, we summed the activities of all three enzymes for presentation and statistical analysis purposes.

Statistics. We used standard multivariate analysis of variance (MANOVA) techniques for groups of related variables (Scheiner 2001). Variables were grouped as mosquito production, water chemistry parameters, and leaf mass loss, or microbial response parameters. Mosquito production variables used in the MANOVAs were survival (number of individuals collected as adults plus any living larvae and pupae), total number of adults per microcosm, total adult weight per microcosm, total numbers of female adults per microcosm, average adult female weight per microcosm, and average female development time per microcosm. Because production of adult females was absent in some flush–nutrient treatment combinations in experiment 1 (see Results), we included only survival, number of adults, and total adult weight in the full factorial MANOVA analysis. We analyzed female production variables in a separate MANOVA by using only the nonflushed treatments and nutrient as the sole main effect. Additionally, because leaf mass loss in experiment 2 was measured only at the end of the experiment, it could not be included as a variable in the factorial MANOVA analysis of microbial parameters. Leaf mass loss was therefore analyzed as a one-way analysis of variance (ANOVA). Posthoc contrasts were performed for individual nutrient treatments versus the control in the MANOVA analysis of mosquito production parameters in experiment 1. Significance levels for posthoc contrasts were adjusted from an α level of 0.05 using the sequential Bonferroni technique to reduce type I error (Rice 1989). Standardized canonical coefficients (SCC) were calculated for each MANOVA and used to assess the contribution of each variable to the main effects (Scheiner 2001). When necessary, data were transformed [log (x) or log (x + 1) or arcsine-square root (√x)] before analysis, and distributions of the data were analyzed with normal quantile plots to verify they met assumptions of the analyses. We used JMP Statistical Discovery software, version 5.1 (www.jmpin.com; SAS Institute, Cary, NC) for all analyses and descriptive statistic calculations.

Results

Experiment 1. Both simulated flushing and nutrient additions had significant impact on Oc. triseriatus survival and adult emergence in this experiment, and these two main effects also showed a significant interaction (Fig. 1; Table 1). In the flush treatments receiving P or deionized water, few or no females were produced, and the subsequent analysis of the nonflushed treatments only showed strong nutrient effects on adult female production. Standardized canonical coefficients (Tables 1 and 2) indicated that significant effects of nutrients were primarily manifested in biomass of adults produced, whereas flushing effects were most pronounced in numbers of adults. The relatively high values of SCC for adult numbers and biomass in the flush × nutrient interaction term showed that negative effects of the flush treatment dampened nutrient effects (Fig. 1; Table 1). The combination of nutrients generally provided the greatest stimulus of mosquito production, enhancing total adult numbers and biomass, and average individual weights for females relative to the control (Fig. 1; contrasts in Tables 1 and 2). However, each nutrient separately had significant effects on some aspects of mosquito production (contrasts in Table 1). After the mix treatment, N additions had the strongest stimulatory effect on overall adult production (Fig. 1; Table 1). Although contrasts of N versus control for female production parameters were not significant with Bonferroni P value adjustment (P value level of significance for N group was 0.0167), the results suggest it was also the primary factor in the effects of the mix treatment on female production (Table 2). N also seemed to reduce overall survival rates. Glucose additions also tended to enhance overall mosquito production, but effects compared with control treatments seemed to be most pronounced in overall survival (Fig. 1), and glucose had no effect on adult female production. Additions of P generally had negative or no effects on adult production (Fig. 1, Tables 1 and 2). As noted, P was the only nutrient class that failed to produce females in any replicates of the flushed treatments.

Water column chemistry and leaf mass loss were significantly affected by flushing and nutrients, and these main effects showed significant interaction (Fig. 2; Table 3). The flush main effect was influenced primarily by changes in N and soluble carbohydrate, whereas nutrient main effects were explained mainly by changes in N and pH (Table 3, SCC). Leaf mass loss was affected most by nutrient treatment and tended to be negatively correlated with other variables in the analysis (Table 3, SCC). The interaction effect was attributable mainly to different responses of pH to flush and nutrient inputs. Frequent experimental nutrient additions obviously contributed to patterns seen
for total N, P, and soluble carbohydrate levels in their respective treatments, but changes in pH and soluble carbohydrate levels were also associated with additions of N (Fig. 2).

**Experiment 2.** Weekly nitrate additions had effects similar to those seen in experiment 1 on mosquito production in that the effect was manifested primarily in the numbers and mass of adults (Fig. 3; Table 4).

Microbial activity and leaf processing were significantly affected by nitrate addition, larval presence, and sampling day (Fig. 4; Tables 5 and 6). Sampling day and nitrate effects showed a significant interaction and the P value for the larvae × day interaction was 0.0517. Examination of Fig. 4 and Table 6, and the SCC values in Table 5, show that microbial responses were different for nitrate and larval effects. Nitrate addition

![Fig. 1. Mosquito production variables from experiment 1. (A) Survival (40 initial larvae). (B) Total adult weight. (C) Number of adults. (D) Average female weight. (E) Number of females. (F) Average female developmental time. Values are means ± SE (n = 7 for all variables in A, B, C, and E. n = 3-7 for variables in D and F).](image)

**Table 1. MANOVA results, posthoc contrasts, and SCC for mosquito production variables in experiment 1**

<table>
<thead>
<tr>
<th>Source</th>
<th>MANOVA</th>
<th>SCC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Roy’s max. root</td>
<td>P</td>
</tr>
<tr>
<td>Whole model</td>
<td>2.70</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Flush</td>
<td>1.31</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Nutrient</td>
<td>2.43</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Flush × nutrient</td>
<td>0.21</td>
<td>0.0211</td>
</tr>
<tr>
<td>Nutrient contrasts (vs. control)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphate</td>
<td>0.18</td>
<td>0.0241</td>
</tr>
<tr>
<td>Nitrate</td>
<td>0.44</td>
<td>0.0001</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.32</td>
<td>0.0013</td>
</tr>
<tr>
<td>Mix</td>
<td>1.21</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

All P values of nutrient contrasts are significant with sequential Bonferroni adjustment.
effects were explained mainly by changes in enzyme activity levels and soluble carbohydrate, and these were positively correlated with fungal biomass (ergosterol) in the analysis (Table 5, SCC; Fig. 4). In addition, nitrate had strong effects on leaf decay (Table 6). The larval effect resulted from changes in bacterial productivity, primarily on leaf surfaces (Table 5, SCC; Fig. 4). Larval presence significantly enhanced leaf decay, but not to the same degree as nitrate addition (Fig. 4; Table 6). Enzyme activity, fungal biomass, and soluble carbohydrates all tended to increase between day 6 and 20 of the experiment, whereas measures of bacterial growth declined (Fig. 4). The significant effect of sampling day was mainly explained by increases in enzyme activity and decreases in leaf surface bacterial productivity between the 2 d (Table 5, SCC). Although the interaction between larvae and day was not significant, SCC values and Fig. 4 suggest leaf surface bacterial productivity response to larvae was increasing on the second sampling day.

**Discussion**

Results from experiment 1 suggest that diluting tree holes with stemflow inputs could be detrimental to
mosquito production and contrasts with earlier investigations where mosquito emergence and biomass in treatments receiving natural stemflow exceeded controls by factors of 2–5 (Walker et al. 1991, Kaufman et al. 1999). However, there were substantial differences in the technique used in this study. In previous studies, stemflow was added by dripping field-collected stemflow slowly into the microcosms. Our approach was designed to dilute and remove metabolites immediately, and we probably removed more fine particulates than occurs with a gradual replacement of the surface water layers. The flushing of natural tree holes during a rain event has not been thoroughly studied, however, we have observed rapid flushing of some tree holes during a storm event such that even large particulate matter is removed. Additionally, in experiment 1, flushing was done two to three times more frequently over comparable time periods than in previous work. Our frequency of treatment more closely matched rainfall event frequencies typically occurring in spring and early summer, after larval hatch and during early larval development. Furthermore, although our simulated stemflow contained ions or organic compounds, even the mix treatment did not contain the magnitude and diversity of soluble compounds (and particulates) present in natural stemflow. The results in experiment 1 would, however, argue against the flushing out and dilution of contents being a necessary mechanism for reduction of toxic metabolites, at least early in the season or in newly formed container habitats. Flushing is conceivably more important as a positive production factor in more mature habitats and later in the larval development sequence where buildup of potentially inhibitory metabolites is most likely.

A potential confounding factor in experiment 1 is that our flushed microcosms also had their contents temporarily mixed by the subsequent addition of solutions. Macia and Bradshaw (2000) showed that benthic particulates in tree hole habitats of *Ochlerotatus sierrensis* (= *Aedes sierrensis*; see Reinert 2000) were a poor-quality resource for larvae, and it is possible that resuspension of this material in our flushed microcosms partially explained the negative effects. However, any mixing or resuspension would have been short lived, and the negative effects in the

| Table 3. MANOVA results and SCC for water chemistry parameters and leaf mass loss in experiment 1 |
|---------------------------------|----------------|----------------|
| Source | MANOVA | SCC |
| Roy’s max. root | df | P | Phosphate | Nitrate | Soluble carbohydrate | pH | Leaf wt |
| Whole model | 16.12 | 9.59 | <0.0001 | 0.735 | 1.085 | 1.720 | −0.494 | 0.278 |
| Flush | 5.47 | 5.54 | <0.0001 | 0.826 | 2.388 | 0.260 | 1.221 | −0.645 |
| Nutrient | 15.23 | 5.57 | <0.0001 | −0.551 | −0.755 | 0.771 | 2.188 | 0.001 |
| Flush × nutrient | 1.75 | 5.57 | <0.0001 | | | | | |

Fig. 3. Mosquito production variables from experiment 2. (A) Survival (40 initial larvae). (B) Total adult weight. (C) Number of adults. (D) Average female weight. (E) Number of females. (F) Average female development time. Values are means ± SE (n = 8).
flushed treatments are more consistent with dilution of nutrient pools and removal of food items in the water column. It also seems unlikely that other consequences of mixing (e.g., increased aeration) would have detrimental effects on larval growth.

Nutrient enhancement of mosquito production in this experiment is more consistent with the idea that stemflow contributes limiting nutrients or provides nutrients in a more readily available form, to the microbial food base for larvae. Somewhat surprisingly, nitrogen seemed to be more of a limiting nutrient than phosphorus. Carpenter (1982) also found nitrate enhanced *Oc. triseriatus* production by using similar microcosm methods but found positive effects from phosphate and sulfate additions as well. Both dissolved nitrogen moieties and phosphate levels in natural tree holes are highly variable and linked to stemflow inputs, but N levels are higher and more stable overall due partly to the continued presence of ammonia from larval excretion and microbial metabolism (Walker et al. 1991). Nitrogen seems to be the more common limiting nutrient for decay of leaf litter in freshwater systems (Webster and Benfield 1986, Grattan and Suberkropp 2001), although P limitations are not unusual (Xie et al. 2004). The ratios of the two elements related to carbon may be more important as primary

### Table 4. MANOVA results and SCC for mosquito production variables in experiment 2

<table>
<thead>
<tr>
<th>Source</th>
<th>Roy’s max. root</th>
<th>df</th>
<th>P</th>
<th>Survival</th>
<th>No. of adults</th>
<th>Total adult wt</th>
<th>Female developmental time</th>
<th>No. of females</th>
<th>Avg female wt</th>
</tr>
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<tr>
<td>Nitrate</td>
<td>5.62</td>
<td>6.9</td>
<td>0.0028</td>
<td>0.910</td>
<td>-1.926</td>
<td>2.674</td>
<td>0.696</td>
<td>0.573</td>
<td>0.683</td>
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</table>

![Fig. 4. Microbial activity variables, soluble carbohydrates, and leaf mass loss from experiment 2.](https://academic.oup.com/jme/article-abstract/43/4/677/902325)
Limiting factors in general for all food webs, but this has not been extensively examined in detritus-based systems (Sterner and Elser 2002).

Nutrient effects, however, seemed to be much more complex than what would be expected with any single limiting factor hypotheses. As in a previous study (Kaufman et al. 2002), the combination of labile carbon and inorganic nutrients elicited the strongest response. The combination effect presumably indicated colimitation of nutrients or the simultaneous stimulation of different microbial groups. We have previously shown that glucose stimulates bacterial productivity in these systems either alone or in combination with inorganic nutrients (Kaufman et al. 2002). Although glucose had a positive effect on overall mosquito production, primarily survival, addition of nitrate seemed to be the most important component of the mix treatment. However, positive impacts of N additions were manifested largely through increased total and individual adult mass and this may have resulted in part from reduced survival in that treatment. In effect, nitrate may have increased larval mortality resulting in reduced competition and thus fewer, larger individuals subsequently emerged as adults. Because nitrate was added in the same concentration in the mix treatment with no evidence of increased mortality, any direct toxic effect is unlikely and more probably resulted from changes in N moieties or particular microbial groups. Although nitrate may have had a minor negative effect on survival, its overall effect was positive in terms of more and larger adult mosquitoes being produced.

That N additions increased the transformation of leaf particulate matter into soluble carbohydrate initially suggested that N additions may have indirectly stimulated bacteria that led to enhanced mosquito development. This prompted experiment 2 and our hypothesis that N probably stimulated leaf decay fungi that either provide a direct nutritional source to larvae or provided more soluble organic carbon for bacterial growth. Results from experiment 2 clearly demonstrated that nitrate additions increased leaf-associated enzyme activity and fungal biomass, leading to increased solubilization of leaf carbon polymers and leaf mass loss. Although enzymes active against cellulose and hemicelluloses are produced by bacteria, biomass of fungi associated with leaves in these systems is higher by an order of magnitude (Kaufman et al. 2001) and the measured carbohydrate activity was probably primarily from fungi. Consistent with this are SCC values from the MANOVA analysis (Table 4). Signs of the SCC values show that enzyme activity, carbohydrate content, and ergosterol content were positively correlated for nutrient and day main effects, whereas leaf bacterial activity was negatively correlated.

Fungal biomass estimates from this study, based on a conversion rate of 5.5 μg ergosterol/mg fungal biomass (Gessner and Chauvet 1993) ranged from 4 to 10% of leaf dry weight. Thus, fungal biomass actually exceeded total mosquito mass in these microcosms as it probably does in most natural systems. As in previous studies (Kaufman et al. 2001, 2002), however, there was no clear effect of larval feeding on fungal biomass. We have postulated (Kaufman et al. 2001) that fungal biomass generally remains unavailable to larvae because it is associated with the internal leaf matrix, and larvae feed mainly by grazing on leaf surfaces. Indeed, ergosterol concentrations were generally higher in the N addition treatments when larvae were present (Fig. 4), suggesting possible stimulation of fungi. Although this supports the idea that N stimulates larvae through fungi indirectly, it should be noted that we did not quantify fungi in the water column or elsewhere (sides of containers) in the microcosms, nor did we measure fungal biomass turnover rates. It is possible that fungi not associated with the leaf material (e.g., spores released from the substrate) were available for larval consumption or that fungal biomass production rates in the nitrate treatments were high enough to compensate for any harvest by larvae. Additionally, because leaf mass loss decreased in the presence of larvae (Table 6), it is

Table 5. MANOVA results and SCC for soluble carbohydrate and microbial parameters in experiment 2

<table>
<thead>
<tr>
<th>Source</th>
<th>Roy’s max. root</th>
<th>df</th>
<th>$P$</th>
<th>Soluble carbohydrate</th>
<th>Ergosterol</th>
<th>Leaf enzyme activity</th>
<th>Leaf surface BP</th>
<th>Water column BP</th>
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<td>Whole model</td>
<td>7.48</td>
<td>7.40</td>
<td>&lt;0.0001</td>
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<tr>
<td>Nitrate</td>
<td>4.36</td>
<td>5.36</td>
<td>&lt;0.0001</td>
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<td>Larvae</td>
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<td>5.36</td>
<td>0.0004</td>
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<td>Day</td>
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<td>5.36</td>
<td>0.0001</td>
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<td>Nitrate × larvae</td>
<td>0.28</td>
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<td>Nitrate × day</td>
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<td>0.0004</td>
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<td>Larvae × day</td>
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<td>Nitrate × larvae × day</td>
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<td>0.6171</td>
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BP, bacterial productivity.
possible that larvae were harvesting more fungal material via direct ingestion of “softer” (i.e., more decayed) leaf material in the N addition treatment. In contrast to fungal leaf decay processes, bacterial productivity showed little response to N addition but was reduced by larval feeding. We have consistently observed that larval feeding reduces leaf surface bacteria but have not always seen a clear reduction of water column bacteria (Walker et al. 1991; Kaufman et al. 2001, 2002). In this study, it seems that larvae fed consistently on water column bacteria and then began grazing leaf-associated bacteria more heavily as they matured (Fig. 4). The lack of bacterial growth rate response to N inputs was unexpected because of the increase in soluble carbohydrate pools released by fungal activity. However, these carbohydrate pools are not necessarily readily available to bacteria. The pool of compounds released from leaf material via fungal decay also includes substances such as tannins and other phenolic compounds that would inhibit growth of many microbial groups (Webster and Benfield 1986, Mercer and Anderson 1994). Additionally, there is evidence to suggest that most labile carbon released from leaf decay processes is used by fungi and that fungi outcompete leaf-associated bacteria for these resources (Gulis and Suberkropp 2003).

In this study, increased adult mosquito production seemed to be a function of fungal activity and unrelated to bacterial productivity. However, survival of larvae seemed to be enhanced by glucose addition—a factor that we know stimulates bacterial growth in tree holes (Kaufman et al. 2002). This is consistent with the idea that bacteria provide a maintenance type of food resource, whereas fungi and other microeukaryotes provide essential nutrients for growth. We have previously estimated that bacterial productivity in similar microcosms is insufficient to account for larval growth demands and maintain that bacteria are fundamentally inadequate as a complete nutritional base for larvae (Kaufman et al. 2001). This study suggests that fungal biomass might be filling the nutritional gap. That larval presence increased leaf mass loss (Fig. 4) suggests that larvae were harvesting some fungal biomass along with leaf material. For leaf material in the N treatments, larvae would be consuming leaf fragments with a higher concentration of fungal biomass. Larvae are capable of ingesting small, loose fragments of leaf material (Merritt et al. 1992), and this would be more available through the increased activity of carbohydrate enzymes in the N-treated microcosms.

It is also possible that increased leaf decay in the nitrate treatments enhanced protozoan abundance, either directly or indirectly, and larvae were able to harvest that microbial resource. Although we did not measure protozoan abundance here, we were unable to detect nutrient addition effects on flagellate and ciliate populations in a previous study where the equivalent of our mix treatment was used as a stemflow mimic input (Kaufman et al. 2002). Additionally, protozoan abundance is usually linked to bacterial prey, and we saw no effect of nitrate on bacterial growth rates in this study.

A confounding factor in this study was how the form of nitrogen input (KNO₃) influenced pH in the microcosms. N treatments seemed to buffer pH drops otherwise associated with controls and glucose and P treatments, where unflushed microcosms’ pH was ~5 (Fig. 2). The lower pH in those treatments compared with flushed versions probably resulted from higher CO₂ concentrations and organic acids from decomposition processes. Additions of potassium ions would neutralize some the acidity and raise the pH. It is also possible that increased release of leaf components in the nitrate treatments served as additional buffers. In two previous studies (Carpenter 1982, Paradise 2000), performance of Oc. triseriatus was reduced in low pH microcosms. Leaf decay processes also were retarded at lower pH in simulated tree hole habitats (Paradise 2000). Additionally, leaf enzymatic activity would probably be influenced by pH, and we did not address the pH optimality of leaf carbohydrases in this study. This raises the question in our study of whether the nitrate addition effect (alone and in mix treatment) resulted from nutrient stimulus or pH buffering. Ultimately, this may have to be answered with further experimentation, but we suggest that the nutrient stimulus is more likely because pH alone cannot explain mosquito and fungal growth responses in this study. If so, the flushed treatments, which were always near pH 6, would likely have had more positive effects. Additionally, nutrient addition effects on fungal activity and leaf decay processes have been demonstrated independently of pH (Grattan and Suberkropp 2001).

Inputs of nutrients into tree holes and other container systems are diverse and can vary considerably with location and season. How these inputs influence mosquito production is critical in understanding the population dynamics and disease capacities of the species breeding in these habitats. This study suggests one mechanism that may release larvae from competition and lead to a higher abundance of adults or larger individuals. Inorganic nutrient inputs interact with particulate matter already existing in the system and increase its rate of conversion to microbial biomass, ultimately having the effect of increasing its food value to browsing larvae. These inputs, such as a readily available form of nitrogen or some other nutrient, stimulate fungal activity and biomass production, and indirectly enhance mosquito fitness. However, the medium of the input—stemflow—may at times serve to flush out previously developed food resources. Depending on the frequency and magnitude of the events, this may negate or even overwhelm any benefits of nutrient input. The continued pulse inputs of nutrients from sources other than stemflow events (e.g., wind blown particulates that contain labile nitrogen or carbon forms) may ultimately provide the key to successful larval development in habitats such as tree holes.
Acknowledgments

We gratefully acknowledge the assistance of Joel Stouten, Amy Rogerson, Danielle Makowski, Scott Neinhuis, Bill Morgan, and Blair Bullard. This project was funded by National Institutes of Health award AI21884.

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Received 12 September 2005; accepted 27 February 2006.