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Stable isotope analysis reveals detrital resource base sources of the tree hole mosquito, *Aedes triseriatus*

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Abstract. 1. Detritus that forms the basis for mosquito production in tree hole ecosystems can vary in type and timing of input. We investigated the contributions of plant- and animal-derived detritus to the biomass of *Aedes triseriatus* (Say) pupae and adults by using stable isotope (^{15}N and ^{13}C) techniques in laboratory experiments and field collections.

2. Laboratory-reared mosquito isotope values reflected their detrital resource base, providing a clear distinction between mosquitoes reared on plant or animal detritus.

3. Isotope values from field-collected pupae were intermediate between what would be expected if a single (either plant or animal) detrital source dominated the resource base. However, mosquito isotope values clustered most closely with plant-derived values, and a mixed feeding model analysis indicated tree floral parts contributed approximately 80% of mosquito biomass. The mixed model also indicated that animal detritus contributed approximately 30% of mosquito tissue nitrogen.

4. Pupae collected later in the season generally had isotope values that were consistent with an increased contribution from animal detritus, suggesting that this resource became more nutritionally important for mosquitoes as plant inputs declined over the summer.

Key words. *Aedes triseriatus*, ^{13}C, detritus, ^{15}N, stable isotope, tree hole.

Introduction

Determinants of adult mosquito production from larval habitats include abiotic factors such as temperature and rainfall, and biotic factors such as predation, parasitism, and competition (Blaustein & Chase, 2007; Juliano, 2009). Although such broad ecological factors affect mosquito production from small, discrete container habitats, resource inputs are often the primary and fundamental limits to larval growth and subsequent adult emergence (Carpenter, 1983; Hard *et al*., 1989; Lounibos *et al*., 1993; Kitching, 2000, 2001; Kaufman *et al*., 2002; Kneitel, 2007). This resource limitation often manifests itself through severe intra- and interspecific competition that affects numbers of adults, their size, and their vectorial capacity, and ultimately impacts disease transmission dynamics (Hawley, 1985; Alto *et al*., 2005; Bevins, 2007). Barrera *et a*l. (2006), for example, concluded that larvae of *Aedes aegypti*, the primary vector of dengue world-wide, are commonly food limited and compete for resources, leading to reduced body sizes of adult females. This reduced size may in turn affect dispersal and biting rates of adult females (Maciel-de-Freitas *et al*., 2007).

Aedes triseriatus is a common container breeding mosquito in eastern North America and the primary vector of La Crosse encephalitis virus. Larvae develop in water-filled tree holes and discarded vehicle tyres that are normally heterotrophic microbial habitats, driven largely by particulate

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inputs and subsequent microbial processing (Walker *et al*., 1991). Although tree holes are consistent recipients of plant detritus in the form of senescent leaf material (Carpenter, 1983; Lounibos *et al*., 1992; Leonard & Juliano, 1995), other inputs include flower parts, twigs, and terrestrial invertebrate carcasses (Lounibos *et al*., 1992; Yee *et al*., 2007a,b). Recent studies have emphasised the potential importance of animal (invertebrate) detritus inputs as they relate to container-breeding mosquito nutrition and to outcomes of larval competition (Daugherty *et al*., 2000; Yee & Juliano, 2006; Harshaw *et al*., 2007; Yee *et al*., 2007a,b; Murrell & Juliano, 2008). Insect carcasses appear to be roughly 10-fold higher in food value for mosquito larvae compared to senescent leaf material (Yee & Juliano, 2006; Yee *et al*., 2007b), potentially allowing co-existence of competing larval species in tree holes and increased production of *Ae. triseriatus* from these habitats (Harshaw *et al*., 2007; Yee *et al*., 2007b). Tree hole dwelling larvae have even been shown to alter their foraging (browsing) behaviours in response to different types of detritus (Kesavaraju *et al*., 2007).

Plant material inputs into larval container habitats in the field are typically 10–100× those of animal detritus, but invertebrate carcass inputs can periodically exceed those of plant-derived material (Daugherty *et al*., 2000; Yee *et al*., 2007b), and invertebrate material can be the primary nutrient inputs in larval mosquito habitats such as pitcher plants (Gray *et al*., 2006; Hoekman *et al*., 2009). Previous experimental investigations of animal vs. plant detritus effects on tree hole dwelling mosquitoes have been carried out in laboratory microcosms, and one such study indicated that a 1:10 ratio of insect carcass to senescent leaf material is near optimal for *Ae. triseriatus* development (Yee *et al*., 2007b). Path analysis indicated that the insect detritus was largely responsible for the production of mosquito biomass in that study (Yee *et al*., 2007b). However, the contribution of animal- vs. plant-derived detritus to mosquito production from natural tree holes or other container systems has not yet been determined. This represents an important unanswered question in our understanding of how organic inputs into tree hole ecosystems are translated into mosquito biomass, and the related vectorial capacity of *Ae. triseriatus* and similar mosquitoes that breed in a wide variety of detritus-dependent habitats.

Stable isotopes, usually ^{13}C and ^{15}N, are now commonly employed to examine food webs in terrestrial and aquatic systems, and to determine the trophic status of components (Post, 2002; Grey, 2006; Hebert *et al*., 2006; Hood-Nowotny & Knols, 2007; Layman *et al*., 2007; Pasquaud *et al*., 2007). $\delta^{13}C$ analyses can identify dietary sources of primary consumers because consumer tissue is typically close to the $\delta^{13}C$ values of the food source (Goedkoop *et al*., 2006; Fry, 2006). In contrast $\delta^{13}N$ values typically increase with trophic level, making them useful for establishing trophic structure. Bi-plots of the isotope values usually help accentuate differences between consumer groups in a mixed food web (Phillips & Koch, 2002; Pasquaud *et al*., 2007). Based on food source and consumer isotope values, and elemental concentrations in source and consumer biomass, mass balance-concentration dependent mixing models allow estimation of dietary contributions to consumers (e.g. Phillips & Koch, 2002; Fry, 2006).

Because animal tissues are typically enriched in both ^{13}C and ^{15}N relative to plant material (Fry, 2006), we sought to make use of this distinction to address the question of the detrital dietary resources for *Ae. triseriatus* in tree holes at our study site in Michigan. Based on the high nutritional content, rapid turnover of, and stimulation of mosquito growth by insect carcasses, we hypothesised that animal detritus would form a substantial portion of the resource base for mosquito biomass in tree holes.

Materials and methods

We used a combination of laboratory studies and field collections to provide material for isotopic analysis. In laboratory studies, mosquitoes (*Ae. triseriatus*) were reared with single sources of detrital material in microcosms (e.g. Kaufman & Walker, 2006). The microcosms included 300 ml of distilled water, a microbial inoculum from natural tree holes (Kaufman *et al*., 2002), and the detrital source. The detrital sources were: senescent oak (*Quercus alba*) leaves, beech (*Fagus grandifolia*) flower parts, laboratory-reared fruit flies (*Drosophila melanogaster* – Diptera) adults, or earthworms (unidentified taxa). Dry mass per microcosm of detritus was: oak leaves, 1 g; beech flowers, 0.6 g; earthworms, 0.4 g; and *Drosophila*, 0.3 g. Oak leaves, beech flower parts and earthworms were collected from the litter layer at our tree hole study field sites near the Michigan State University (MSU) campus (E. Lansing, Michigan). Plant material was added after drying (48 h, 45 °C), and animal material was lyophilised prior to microcosm introduction. Forty neonate mosquito larvae were added to each microcosm and adults were collected as they emerged over a period of several weeks. Adult mosquitoes from previous studies (Kaufman & Walker, 2006; Yee & Juliano, 2006) that had been stored with desiccant were also assayed for isotope content. In one of the previous studies (Yee & Juliano, 2006), cricket tissue was used as detrital source, and we subsequently obtained laboratory-reared *Grylloides sigillatus* from the same source colony (and fed the same diet) used in that study courtesy of Dr Scott Sakaluk (Illinois State University, Normal, Illinois).

Detritus (plant and animal) and *Ae. triseriatus* pupae were collected from tree holes at our E. Lansing study site periodically in late spring and summer of 2005 and 2006. We collected detritus samples primarily from the surface or near the surface of the tree hole water column with the assumption that these reflected recent inputs into the system. In the cases of invertebrate carcasses and flower parts, this assumption was realistic; however leaf detritus was problematic in that senescent leaves could have entered the system during the previous fall or been blown in anytime hence. We sought to obtain representative detrital inputs at the study site, but did not quantify the relative abundance of detritus categories. All samples were frozen (−80 °C), lyophilised, and stored with desiccant before grinding and analysis. Invertebrate samples or mosquitoes collected from individual tree holes were pooled

as separate subsamples. Quantities collected were sometimes of insufficient mass to allow for losses in sample preparation prior to isotope analysis, such as in the cases of two or fewer mosquito pupae, hence not all samples collected could be analysed.

All samples were ground to fine powder using stainless steel ball bearings in microcentrifuge tubes on a multiple-sample, high speed shaker (Retsch MM300, Glen Mills, Clifton, New Jersey). Large bulk samples of plant material (e.g. leaves used in microcosm experiments) were first subsampled by taking small sections from several different leaves or flowers, followed by grinding in a mortar and pestle before being processed with the bead beater. Subsamples of the pulverised material were then weighed into tin cups and stored with desiccant until isotopic analysis.

Because animals partition ^{13}C differently in lipid pathways compared with other tissues (Post *et al*., 2007), we compared isotope values from mosquito pools that were subjected to lipid extraction with untreated mosquito pools from the same study. The source of the mosquitoes was a microcosm experiment in which we had manipulated levels of added nitrate (Kaufman & Walker, 2006). Lipid was extracted using a dichloromethane–methanol biphasic extraction procedure that we've used previously for microbial lipid analysis (Kaufman *et al*., 1999). This method is a modification of the standard Bligh and Dyer (1959) lipid extraction and differs primarily in the replacement of chloroform with dichlomethane as the non-polar lipid extractant (see also Peterson & Klug, 1994 and references therein).

Carbon (^{13}C, ^{12}C) and nitrogen (^{15}N, ^{14}N) isotope content of the samples were determined with an elemental analyser (EA3000, Eurovector, Milan, Italy) coupled to a stable isotope ratio mass spectrometer (Elementar, Mt Laurel, New Jersey) following procedures detailed in Ostrom *et al*. (1997) and Gandhi *et al*. (2004). Total C/N ratios were also determined for each sample. Stable isotope values are expressed in parts per mil (‰) according to the following equation:

$$\delta X = [(R_{\text{sample}}/R_{\text{standard}}) - 1] \times 1000 \qquad (1)$$

where X is ^{13}C or ^{15}N, and R is the corresponding ratio $^{13}C/^{12}C$ or $^{15}N/^{14}N$. R_{standard} was V-PDB or atmospheric nitrogen, for $\delta^{13}C$ and $\delta^{15}N$, respectively. For $\delta^{13}C$ and $\delta^{15}N$, laboratory standards were analysed after every 10 unknown samples, with an accuracy and precision of $\leq 0.2‰$ for both $\delta^{13}C$ and $\delta^{15}N$.

To avoid statistical problems with distribution and transformation of proportionate (ratio) data, we compared isotope values or C/N ratios using the non-parametric Wilcoxon/Wallis rank sum methods. We used JMP® Statistical Discovery Software, V5.1 (www.jmpin.com, SAS Institute, Inc., Cary, North Carolina) for theses analyses and for descriptive statistic calculations.

To estimate dietary contributions of detrital sources to mosquito tissue, we used the mixture model of Phillips and Koch (2002), available at www.epa.gov/wed/pages/models.htm. This model takes into account the total carbon and nitrogen concentrations of the diets (detrital inputs) and consumer (mosquito), the isotope values of the dietary sources and consumer, and the trophic fractionation (shifts in isotope ratio between organism and diet) of each isotope by the consumer. Fractionation of food resource isotope values was estimated from the results of laboratory-reared mosquitoes on single detritus sources. For the mixing model, we used the average isotope values for endmembers adjusted for fractionation factors determined in the laboratory studies, and the mixture was the average isotope values of field-collected mosquitoes (Phillips & Koch, 2002).

Results

Laboratory studies

Plant detritus (oak leaves and beech flowers) used as initial sources in microcosm studies had lower $\delta^{13}C$ and $\delta^{15}N$ values relative to the invertebrate detrital sources (Fig. 1). Isotope values of earthworm tissues were roughly intermediate between the plants and insects examined. In general, adult mosquito tissue was enriched, relative to the detritus sources in ^{13}C when reared on plant detritus, and in ^{15}N when reared on animal sources, but mosquitoes reared on cricket carcasses were enriched in both ^{13}C and ^{15}N (Fig. 1) and there was little evidence of any isotope fractionation in mosquito tissue when *Drosophila* was the detrital source. Mosquitoes reared on oak leaves with additions of inorganic nitrogen (KNO_3) to microcosms were enriched in ^{15}N compared with mosquitoes reared on oak leaves without KNO_3 addition (Fig. 1, Table 1).

Lipid extraction did not affect $\delta^{13}C$ or $\delta^{15}N$ values from mosquito tissue; however extraction significantly lowered the C/N ratio (Table 1).

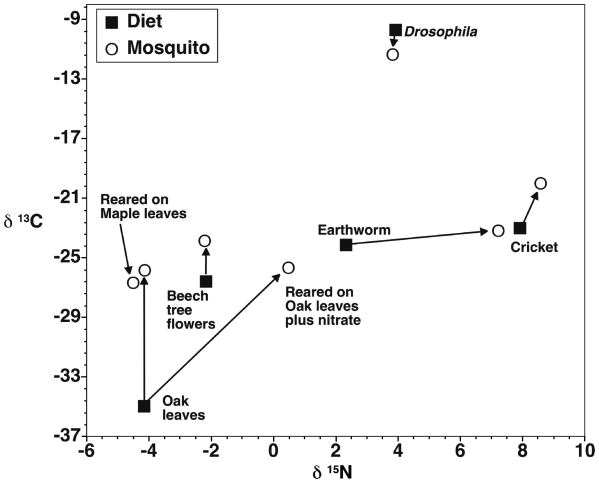

Fig. 1. Stable isotope composition of detrital source and mosquitoes from laboratory microcosm experiments. Values are means from three or four analytical replicates of pooled material. Arrows connect diet source with mosquitoes reared on that source. No maple leaf detrital (diet) material was available for analysis.

Table 1. Nitrate addition during larval growth and lipid extraction effects on isotopic content and carbon/nitrogen (C/N) ratios of *Aedes triseriatus* adult tissue.

Nitrate	Lipid extraction	δ^{15}N	δ^{13}C	C/N
0	0	-2.2 ± 0.2	-26.4 ± 0.4	4.6 ± 0.2
0	+	-2.1 ± 0.1	-25.9 ± 0.2	4.0 ± 0.1
+	0	0.4 ± 0.3	-25.4 ± 0.3	4.7 ± 0.1
+	+	0.6 ± 0.2	-26.3 ± 0.4	4.1 ± 0.1
Non-parametric test			*P*-value	
Nitrate		0.005*	0.298	0.575
Lipid extraction		0.810	0.689	0.005*

*Significant with sequential Bonferroni adjustment.
Values are means $\pm$ 1 SE, $n = 3$.
Non-parametric comparisons are results of Wilcoxon/Kruskal–Wallis rank sums tests.

Field collections

Leaf detritus from tree holes consisted of maple, oak, beech, and unidentified leaves, and flower components were from oak and beech trees. Animal detritus from tree holes consisted mainly of small Diptera, other insects (Coleoptera, Lepidoptera), unidentified arthropods, and earthworms. As was seen in the laboratory studies, the two main categories of detrital material (plants and invertebrates) from field collections were distinct, but differences were not as pronounced as seen in the laboratory studies (Fig. 2). Plant detritus collected directly from tree holes had isotope values very similar to those seen in the laboratory studies (Fig. 1), and animal detritus isotope values were higher than plants. Field-collected mosquitoes had isotope values (Fig. 2) that were intermediate between those that would be expected if either plant or animal material were the sole resource base. Stable isotope values in field mosquitoes were most similar to those seen in mosquitoes reared in the laboratory on leaf material with an external source of nitrogen, or with tree flowers, as the detritus base.

Carbon and nitrogen isotope values in mosquitoes collected from natural tree holes tended to increase during the course of a season in 2006 (Fig. 3). In 2006, the monthly trend was significant for both carbon and nitrogen isotope values (rank sum test, $P = 0.012$ for δ^{13}C and $P = 0.002$ for δ^{15}N). Mosquitoes were collected only in June and July in 2005, and there was no difference between months for either isotope (rank sum test, $P = 0.647$ for δ^{13}C and $P = 0.160$ for δ^{15}N).

Because leaf material, tree floral parts, and invertebrates were ubiquitous inputs into tree holes at our study sites, we used isotope values and carbon and nitrogen concentrations for these three resource types in the mixing model. We also used the overall mean of isotope values from all field-collected mosquitoes from both years for the mixture (i.e. consumer) parameters. Estimates of diet contributions to mosquito biomass (Table 2) indicated that 82% of pupal biomass could be attributed to beech flower detritus, but that 31% of the nitrogen in pupal tissue was derived from

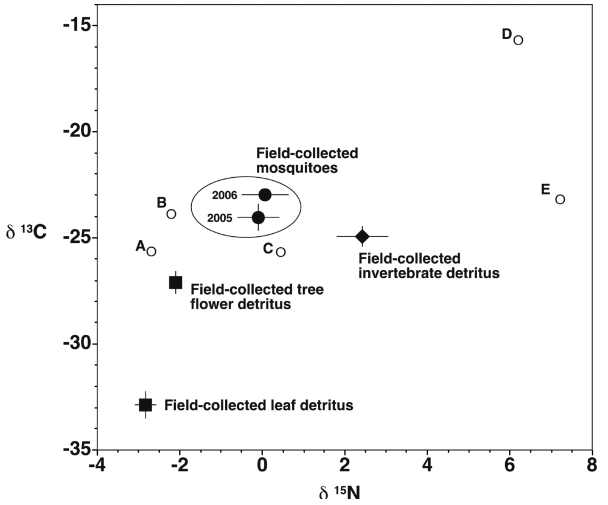

Fig. 2. Stable isotope composition of detrital material and mosquitoes collected from field sites. Values are mean $\pm$ 1 SE, $n = 16$–40. Mean values of laboratory-reared mosquitoes are illustrated for reference with small open circles: A, reared with oak leaves; B, reared with beech flowers; C, reared with oak leaves plus nitrate; D, reared with insect (crickets, *Drosophila*) carcasses; E, reared with earthworm carcasses.

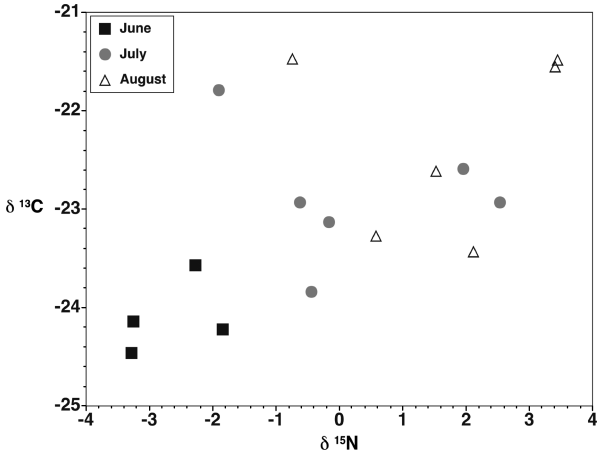

Fig. 3. Stable isotope composition of mosquitoes collected from individual tree holes in 2006. Values are single analytical replicates from pooled pupae collected from an individual tree hole.

Table 2. Concentration-dependent mixing model estimates of dietary contributions to field-collected mosquito biomass and components.

	Mosquito tissue (%)			Detritus values			
	Biomass	Carbon	Nitrogen	δ^{13}C	δ^{15}N	(C)	(N)
Leaf	8.6	8.8	3.4	-24.3	-2.8	46	1
Beech flower	81.8	82.5	65.6	-23.4	-2.1	45	2
Invertebrate	9.6	8.7	31.0	-24.1	4.7	40	8

Isotope values are based on means for each diet type and adjusted for fractionation by mosquitoes determined in laboratory studies. Concentrations of carbon and nitrogen are mean % C and %N for each diet.

invertebrate detritus. Surprisingly, the model indicated that leaf material contributed relatively little to mosquito growth. We should point out that the source parameters in Table 2 did not adequately circumscribe (i.e. enclose the mixture values in a triangle formed by the three sources – Phillips & Koch, 2002) all subsets of the field mosquitoes, and therefore these source estimates do not model the late season (July and August) 2006 mosquitoes. A single isotope (^{15}N), dual source (plant material and invertebrates) version of the model indicated that invertebrate material would have contributed almost nothing to mosquito biomass in June 2006, but approximately 10% in July and 20% in August.

Discussion

To our knowledge, this study is the first to use stable isotopes for examining the natural food resource base for mosquito larvae. *Aedes triseriatus* reared on single sources of detritus had tissue δ^{13}C and δ^{15}N values that reflected the type of detritus, allowing back-calculated estimation of detrital resource bases for mosquitoes emerging from natural tree holes. Field-collected mosquitoes apparently grew mainly on compounds derived from plant detritus with animal detritus supplements. This is consistent with current conceptions about tree hole ecosystems (Kitching, 2001) and helps to validate microcosm studies that utilise senescent plant material as the resource base (Carpenter, 1983).

The form of plant material resource base, however, might need re-evaluation. Tree floral parts, mainly beech tree flowers, appeared to drive tree hole mosquito production in this study. In 2006 in particular, some tree holes in our study area in May seemed filled to capacity with flower parts. This material did not persist through the summer and was clearly much less refractory than senescent leaf material entering the system. Lounibos *et al*. (1992) also found tree flower inputs into tree holes in Florida to be substantial, but strongly seasonal and ephemeral. Nitrogen content of beech flowers was double that of typical leaf material (Table 2), indicating a much higher quality microbial substrate. In preliminary growth studies (M. G. Kaufman and K. S. Pelz-Stelinski, unpublished), we found that mosquito production from microcosms with beech tree flowers was comparable to that found when *Drosophila* carcasses are used as the detrital source. The latter source is a very high quality growth substrate for mosquito larvae (Yee & Juliano, 2006). Lounibos *et al*. (1993) also showed that *Ae. triseriatus* reared on flowers from live oak trees developed significantly faster than larvae reared on similar amounts of live oak leaves, indicating that this form of plant detritus is nutritionally superior for tree hole mosquitoes. Pulse inputs of these higher quality but inconsistent resources may be critical for *Ae. triseriatus* emergence in the face of intra- and interspecific larval competition (Kaufman & Walker, 2006; Yee *et al*., 2007b).

Field mosquito isotope values were also similar to those of mosquitoes laboratory-reared on leaf material with an external nitrogen addition. The potassium nitrate used in that experiment was enriched ($\delta^{15}N = 3.65$, M. G. Kaufman, unpublished) compared to the oak leaf material ($\delta^{15}N = -4.15$) and was probably incorporated into microbial biomass harvested by larvae. Nitrogen entering the system via stemflow (Kaufman *et al*., 1999; Verdonschot *et al*., 2008) is likely to have very different isotope values than those found in plant detritus, and it has been shown that microbial and larval transformations of nitrogen compounds in tree holes are also dynamic (Walker *et al*., 1991; Kaufman *et al*., 1999; Kaufman & Walker, 2006; Verdonschot *et al*., 2008). Therefore, the external sources of nitrogen incorporated by the leaf microbial community and subsequently assimilated by larvae could greatly alter the δ^{15}N values in field mosquito tissues compared with original leaf δ^{15}N values.

Another source of nitrogen entering the system would be invertebrate carcasses. Our results indicate that invertebrate detritus may contribute proportionately more to nitrogen-containing compounds in pupal biomass than plant material, consistent with the observations that mosquito isotope values changed during the season and that invertebrate carcass influence on growth of *Ae. triseriatus* in tree holes is most pronounced when plant material resources are limiting (Harshaw *et al*., 2007). That carbon may come primarily from one diet source while nitrogen comes from another is not surprising (Stenroth *et al*., 2006), but the ecological consequences of this have not often been addressed. For *Ae. triseriatus* larvae in tree holes, this may mean that emergence is delayed while waiting for nutritional input provided by a particular detritus category. In the case of nitrogen limitations, this might even be in the form of conspecific larval mortality.

Isotopic evidence here (Fig. 1) would indicate direct incorporation of *Drosophila* detritus because of the lack of fractionation between diet and mosquito. However, decomposer microorganisms associated with detritus have isotope values that are usually indistinguishable from the substrate (see discussion below). Yee *et al*. (2007a) suggest that both direct incorporation of animal detritus and harvesting of associated decay microorganisms is important for larval growth. Interestingly, mosquitoes grown on earthworm and cricket carcasses did show isotopic fractionation, possibly because particle size and decay rates differed enough from *Drosophila* to prevent direct ingestion of tissue. Additionally, gut contents were not removed from any invertebrates tested in this study, and this would influence not only carcass decay rates and associated microbial communities, but also digestion and assimilation processes in larval mosquitoes and resultant isotope values (Fry, 2006).

Although our results point to plant-derived material as being the dominant resource base for mosquitoes at the study site, animal detritus sources might be expected to increase in importance in other habitats. Invertebrate carcass inputs into tyres, a habitat commonly exploited by *Ae. triseriatus*, occur at rates than can support mosquito production independent of other inputs (Daugherty *et al*., 2000), and animal detritus supports the mosquito, *Wyeomyia smithii*, in the pitcher plant ecosystem (Gray *et al*., 2006; Hoekman *et al*., 2009). It would be expected that many larval habitats would vary greatly in placement and proximity to sources of plant detritus. Additionally, as our results indicate (Fig. 3), the relative importance of animal detritus inputs varies seasonally and year

to year, as documented for detrital inputs into tyre habitats (Kling *et al*., 2007; Yee *et al*., 2010). Tyres located in forested areas showed decreasing inputs of plant material during a season, while animal inputs remained constant (Kling *et al*., 2007), indicating an increased relative importance of animal inputs over time.

Plant detritus samples in this study appear to be atypical from two perspectives. First, the values seen for leaf detritus are relatively low in ^{13}C compared with what has been reported for most C3 plants and are more similar to what might be expected from some algal groups (Post, 2002). We found δ^{13}C levels of −28 to −37 for leaf material, which are notably lower than ranges of −29.5 to −26 in leaf litter from similar tree taxa reported by Balesdent *et al*. (1993). However, Collier *et al*. (2002) measured a δ^{13}C range of −32 to −30.3 in riparian vegetation at a New Zealand site and the range of values found in C3 plants extends to −34 and lighter (O'Leary, 1988). Location and growth conditions can further influence isotope composition of plants (O'Leary, 1988; Fry, 2006). Second, the ^{13}C fractionation of the plant material by mosquito larvae was much higher (more ^{13}C) than expected, even with the assumption that larvae are consuming microbially transformed material. The isotopic composition of microbial heterotrophs on decaying material is thought to mirror the plant material substrate (Balesdent *et al*., 1993; Fry, 2006) and mosquito larvae harvest this microbial biomass directly (Kaufman *et al*., 2001). Given the accepted range of fractionation of ^{13}C into the next higher trophic levels (0.5–1%), we would need to account for at least three trophic levels between tree flower parts and mosquito consumption (difference of +3.6 δ^{13}C between flowers and mosquito), and nine trophic levels between leaf and mosquito larvae (difference of +8.6 δ^{13}C between leaves and mosquito). Although it is clear that the larvae of many mosquito species feed primarily on microorganisms and not directly on leaf material (Kaufman *et al*., 2001, 2002), and that they also feed upon intermediate microbial grazers such as protozoans and rotifers (Kaufman *et al*., 2002; Kneitel, 2007), it is difficult to conceptualise a food web with that many links in the tree hole system or that mosquitoes harvest only the higher trophic levels. The apparent lack of ^{15}N fractionation between leaves and flowers and the mosquito was also unexpected, but within the range of insects developing in plant-based systems (Spence & Rosenheim, 2005).

It seems more likely that the difference in δ^{13}C values between leaf and mosquito reflects an unrecognised fractionation by the microorganisms. It's been shown, for example, that the relatively low δ^{13}C values from methanotrophic bacteria are detectable in midge larvae and other aquatic organisms that consume benthic detritus (Doi *et al*., 2006; Deines *et al*., 2007), but this would not help explain ^{13}C enrichment in the mosquito larvae food web. Nadon and Himmelman (2006) have noted higher than expected enrichment of ^{13}C in primary consumers of marine benthic detritus (+4 δ^{13}C). They suggested selective feeding by the macroinvertebrate consumers, but this was not verified. We examined eubacterial and fungal community structures associated with leaf detritus in tree holes and noted differences in relative abundances of microbial groups when larval feeding ceased (Kaufman *et al*., 2008), but have not yet targeted the Archaea – a group that would be highly active in carbon isotope fractionation (Fry, 2006). Because mosquito larvae feed on many microbial groups (bacteria, fungi, protists) associated with detritus, future studies will need to determine how these components alter detritus δ^{13}C values before they reach mosquito tissue. While previous studies indicate that δ^{13}C values of consumers are marginally higher than their diet, such isotope shifts are not necessarily typical of arthropod consumers. For example, trophic fractionation of δ^{13}C by insects ranges from −2.7 to 5.5% (Ostrom *et al*., 1997; McCutchan *et al*., 2003; Scheu & Folger, 2004) and amphipods feeding on live or decaying seagrass had δ^{13}C values that differed from the source by 9–10% (Crawley *et al*., 2007). While the reasons for this variation are uncertain, it is clear that additional estimates of trophic fractionation for arthropods are needed.

Our results indicate that removal of lipids did not affect δ^{13}C values and adjustment of δ^{13}C values for lipid content of aquatic invertebrates seems to be problematic in general (Kiljunen *et al*., 2006). Removal of lipids is more of a concern in larger animal samples, where fat tissue can be a considerable portion of biomass (Post *et al*., 2007). In addition to the impracticality of removing lipids from small samples, extraction of lipids in mosquitoes adds additional steps to sample processing while reducing the mass of the minimal material available for analysis. Because storage lipids in mosquitoes are usually less than 20% of dry weight (Timmermann & Briegel, 1996), lipid extraction may be inadvisable for δ^{13}C measurements in these insects (Post *et al*., 2007). Using C : N normalisation models in lieu of lipid extraction for aquatic invertebrates is also tenuous (Kiljunen *et al*., 2006; Logan *et al*., 2008). However, additional studies should be conducted to determine if this step is generally unwarranted for mosquitoes.

This study illustrates the utility of stable isotopes in studies of larval mosquito feeding ecology, but also points out some of the limitations. Mixing models for determining diet sources have many caveats (Fry, 2006) and the one employed here may not have adequately addressed the particulars of the tree hole system. Specifically, the isotope values of the three observed primary inputs into tree holes (leaves, flower parts, and invertebrate carcasses) did not adequately circumscribe the targeted consumer (mosquito) values in all cases, with late season 2006 samples falling out of the mixing triangle primarily due to δ^{13}C values. This may have been due, in part, to our estimates of fractionation of ^{13}C from plant material (see above), but may also reflect differences in laboratory vs. field conditions. δ^{15}N values proved more useful in this study in estimating dietary source contributions from a mixture, dovetailing with our findings that nitrogen dynamics are an important driving force for mosquito production in these habitats (Kaufman & Walker, 2006). Additionally, since most of our detrital inputs representatives were collected in the late spring (when larvae had hatched and were starting development), we did not account for possible changes in the isotope values of leaves, flowers, or invertebrates over the course of an entire mosquito season. It's unlikely that the plant material isotope δ values would change substantially, even if

exposed to long periods of decay (e.g. Osono *et al*., 2008; Lau *et al*., 2009), but the invertebrate category in our study already showed high variability in spring collection isotope values and later season values are unknown. Finally, this study did not account for all potential inputs into the system as we only collected the larger particulate fractions. Fine particulate inputs into tree holes and similar habitats may influence larval abundance and species composition in container habitats (Kling *et al*., 2007; Yee *et al*., 2010), and may have different isotope values from their large particulate counterparts. Inclusion of fine particulates in mixing models might help to explain more of the variation in isotope values we saw in field-collected mosquitoes over the season.

Our observations that ephemeral resources added in pulse inputs could be driving *Ae. triseriatus* production in these habitats also point out the need to re-consider established perceptions. In the case of mosquitoes and tree holes, the fact that leaf material is most often observed as the most abundant detrital source could be misleading because the important sources fuelling mosquito development have disappeared from view due to more rapid decay rates and incorporation into microbial and mosquito biomass.

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