TITLE: Linking nutrient stoichiometry to Zika virus transmission in a mosquito

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ABSTRACT

Food quality and quantity serve as the basis for cycling of key chemical elements in trophic interactions, yet the role of nutrient stoichiometry in shaping host-parasite interactions is under appreciated. Most of the emergent mosquito-borne viruses affecting human health are transmitted by mosquitoes that inhabit container systems during their immature stages, where allochthonous input of detritus serves as the basal nutrients. Quantity and type of detritus (animal and plant) were manipulated in microcosms containing newly hatched *Aedes aegypti* mosquito larvae. Adult mosquitoes derived from these microcosms were allowed to ingest Zika virus infected blood and then tested for disseminated infection, transmission, and total nutrients (percent carbon, percent nitrogen, ratio of carbon to nitrogen). Treatments lacking high quality animal (insect) detritus significantly delayed development. Survivorship to adulthood was closely associated with the amount of insect detritus present. Insect detritus was positively correlated with percent nitrogen, which affected Zika virus infection. Disseminated infection and transmission decreased with increasing insect detritus and percent nitrogen. We provide the first definitive evidence linking nutrient stoichiometry to arbovirus infection and transmission in a mosquito using a model system of invasive *Ae. aegypti* and emergent Zika virus.

Key Words: Nutrition, ontogeny, infection, Zika virus

INTRODUCTION

The environment experienced during development often greatly shapes adult phenotypes. For animals with complex life cycles, ecological factors during the juvenile stage can influence the adult stages via carry-over effects, including in fish (Green and McCormick 2005, McCormick and Gagliano 2008), mosquitoes (Alto et al. 2005), and other insects (Kingslover et al. 2011). In insects, and mosquitoes in particular, different temperatures, habitats, and food environments can affect different life history states, and thus adults may result with differential contributions to overall lifetime fitness (Kingslover et al. 2011). For mosquitoes, this means that larvae exposed to different stressors may have different adult life history traits including mass, development time, survivorship to adulthood, and population growth, as well as and resilience to future stress including pathogen challenge (Nasci 1991, Alto et al. 2012, Da-Silva Araújo et al. 2012). These life history traits in addition to vector competence, defined as the susceptibility to pathogen infection and transmission potential, are some of the many factors that influence vectorial capacity. Vectorial capacity is an index of risk of pathogen transmission (Alto et al. 2008, Bara et al. 2014), and if defined as the number of infectious bites a host receives per day.

Nutrition is an early developmental factor known to modulate life history traits. It is an important factor regarding host-pathogen interactions; as host nutrition influences multiple measures of adult performance, immune response, and the resources necessary for pathogen replication (Lee et al. 2006, 2008; Telang et al. 2012, Yee et al. 2015). Larval nutritional stress can reduce the immune response of mosquitoes allowing

for increased pathogen infection (Sindbis virus; Muturi et al. 2011) or reduce resources for pathogen development (malaria; Vantaux et al. 2016ab). Further, nutritionally stressed larvae result in adults with reduced mass. As larger bodied mosquitoes imbibe greater volumes of blood than their smaller counterparts, one may predict that large mosquitoes would ingest higher doses of pathogen in the infected blood and may have higher infection rates, as arboviral infection is often dose dependent (Takken et al. 1998, McCann et al. 2009).

Past studies have explored the role of nutrition in the larval environment primarily in terms of stress induced by varying quantities of laboratory or plant detritus as a substrate for microbial growth; these microbes provide the direct source of larval nutrition. Recent studies utilizing stable isotope analysis have shown that additions of animal detritus increase nitrogen availability. In particular, increases in animal detritus have positive effects on larval growth and adult phenotypes such as decreasing development time, larger mean mass, greater survivorship to adulthood, and higher estimated population growth (Yee and Juliano 2006, Murrell and Juliano 2008, Winters and Yee 2012, Yee et al. 2015). Within natural and artificial aquatic container systems such as treeholes and tires (communities dominated by immature stages of mosquitoes), primary production is nearly absent. Most of the incoming energy originates from allochthonous inputs of detritus, mainly in the form of senescent plant material (primarily leaves) and terrestrial invertebrate carcasses (Carpenter 1983, Kling et al. 2007). Invertebrate carcasses, which make up the bulk of animal detritus, have greater available nitrogen stores and a faster rate of decay than plant material, allowing for more rapid release of nutrients into the system (Yee and Juliano 2006). This

suggests that animal detritus scarcity could have important effects on vector competence as a limiting factor of growth, impacting a variety of adult traits, including immunity.

At the molecular level, immunity has several components including the production of cells that actively destroy pathogens. For example, within this pathway, nitric oxide (NO) plays a critical role in innate immunity in both vertebrates (Wink et al. 2011) and insects like mosquitoes (Hillyer & Estéves-Lao 2010). Free radicals like NO are very unstable and react quickly with other molecules to acquire a stable electron configuration (Clements 2012). As an important component in immunity, it seems possible that nitrogen limitation could affect immunity against pathogens. Specifically, reactive nitrogen is linked to mosquito immunity as nitrate and hydrogen peroxide are used to synthesize NO in the mosquito midgut (Hillyer 2010).

In this study, we aimed to investigate the influence of various ratios of animal:plant detritus on infection and transmission of Zika virus in *Aedes aegypti*. We test the hypothesis that nutrient limitation (specifically Nitrogen limitation) during the larval stages will be associated with higher infection and transmission potential of *Ae. aegypti* for Zika virus. We measure total nutrients in terms of percent carbon (%C), percent nitrogen (%N), and ratio of carbon to nitrogen (C:N) in both the mosquitoes and basal resources (detritus). Inclusion of measurements of both detritus and mosquitoes allows us to provide a link between basal nutrition and the adult phenotype, in terms of nutrient stoichiometry. Although %C and %N are correlated to C:N, the latter value is reflective of the stoichiometry of the animal, in essence showing how they may balance the two elements in their body across detrital environments. Although carbon and

nitrogen are contained within C:N, individually they cannot enlighten us about how they act in tandem. Although we focus on *Ae. aegypti* and the Zika model system, this study has general application in addressing a gap in our understanding of how mosquito larval nutrition relates to adult nutrient stoichiometry and interactions with pathogens ingested from infected vertebrate blood.

MATERIALS AND METHODS

Mosquitoes and Detritus Treatments

Aedes aegypti mosquitoes used in these studies were collected as larvae from containers in Key West, FL. Larvae were reared in approximately 1.0 L of tap water in plastic trays (25 x 30 x 5 cm) with 0.40 g larval food comprised of equal amounts of liver powder and brewer's yeast at hatching and supplemented with the same amount of food 3-4 d later. Pupae were transferred to water-filled cups in 0.3 m^3 screened cages for emergence to adulthood. Adults were provided with 10% sucrose solution from cotton wicks and weekly blood meals from live chickens (IACUC protocol 201507682). Females laid eggs on damp paper towels in cups with water held in the cages. All life stages were maintained with a light:dark photoregime of 12:12 h at 28°C. The F_{17} generation of parental *Ae*. *aegypti* were used in these experiments.

Larval rearing treatments consisted of ten groups that varied in the relative ratio and amount of animal (freeze-dried crickets, *Gryllodes sigillatus*, Fluker Farms, Port Allen, LA, USA) to plant (senescent red maple leaves, *Acer rubrum,* collected at the Lake Thoreau Center, Hattiesburg, MS, USA 31°19'37.63"N, 89°17'25.22"W) detrital sources. Plant and animal detritus were dried for 48 hrs at 45 °C prior to use. Each detritus

155 treatment was performed in triplicate for a total of 30 experimental units (hereafter,
156 containers). Detritus types were expressed in relative terms (1 unit of detritus equals
157 0.15 g) of animal:plant as follows: 1:0, 2:0, 4:0, 0:5, 0:10, 1:5, 1:10, 2:5, 2:10, 4:10.
158 These detritus treatments allow for a range of nitrogen and carbon values in adults and
159 generally were based off past studies examining how different detrital environments
160 affect mosquito performance and stoichiometry (e.g., Winters and Yee 2012, Yee et al.
161 2015). To permit microbial growth for mosquito larvae to feed on, detritus was soaked
162 for 5 d before introduction of larvae in 2.0 L plastic buckets (height, 19.05 cm; top and
163 bottom diameters, 19.30 cm and 16.31 cm, respectively) containing 2000 mL tap water
164 and 1000 µL of tire water inoculum. Tire water inoculum was obtained from several tires
165 occupied by mosquitoes and maintained on the UF-FMEL campus in Vero Beach, FL.
166 The inoculum provided a source of microorganisms, acquired from a semi-natural
167 setting, as food for larvae. Treatment containers were maintained with a light:dark
168 photoregime of 12:12 h at 28°C.

169 Eggs were hatched at room temperature for 60 min in a deoxygenated water in a
170 250 mL Erlenmeyer flask attached to a vacuum to induce synchronous hatching with
171 0.20 g/L of larval food (Kauffman et al., 2017). Larvae were transferred to 5 L of tap
172 water in 5 L plastic trays with an additional 0.20 g/L food. The following day, the first
173 instar larvae were rinsed with tap water to remove larval food and 160 larvae were
174 placed in each treatment container. The initial larval density (0.08 larvae/mL) is within
175 the range of densities observed in field conditions in Florida among tires occupied by
176 *Ae. aegypti* and competitor *Ae. albopictus* (Alto et al. 2005). Treatment containers were
177 maintained in a walk-in environmental chamber at the UF-FMEL with a light:dark cycle

of 12:12 h set at 28±1°C. Containers were checked every day and rearranged haphazardly within the environmental chamber. When present, pupae were transferred from treatment containers to polystyrene *Drosophila* culture vials with 2 to 5 mL of tap water (up to 5 pupae per tube) according to treatment conditions and sealed with a cotton plug. The date and sex of newly emerged adults from each replicate were recorded. Both males and females were housed together by treatment, replicate, and emergence date in paperboard cages with mesh screening (height by diameter: 10 cm x 10 cm) and rearranged haphazardly each day in the environmental chamber. For logistical reasons, females were added into cages over a period of three days because it would have been impractical to blood feed large numbers of cages. Adults were provided with 10% sucrose solution on cotton pads. Females were 9 to 15 d old at the start of trials in which mosquitoes were allowed to ingest Zika virus infected blood.

Infection Study

Females were fed defibrinated bovine blood (Hemostat Laboratories, Dixon, CA) containing freshly propagated Zika virus. To encourage blood feeding, mosquitoes were deprived of sucrose, but not water, 24 h before blood-feeding trials. Infection experiments were performed in a biosafety level-3 laboratory at the UF-FMEL. Isolates of Asian lineage of Zika (strain PRVABC59, GenBank KU501215.1) from Puerto Rico were prepared in African green monkey (Vero) cells and used in the infection study. Monolayers of Vero cells were inoculated with 500 µL of diluted stock virus (multiplicity of infection, 0.1) and incubated at 1 h at 37 °C and 5% CO_2 atmosphere, after which 24 mL media (M199 medium supplemented with 10% fetal bovine serum,

penicillin/streptomycin and mycostatin) were added to each flask and incubated for 4 d. Freshly harvested media from infected cell cultures were combined with defibrinated bovine blood and adenosine triphosphate (0.005 M) and presented to mosquitoes using an artificial feeding system (Hemotek, Lancashire, UK) with hog casing membranes for 1 hr feeding trials. Carbon dioxide from the sublimation of dry ice was used to stimulate blood feeding three times every 20 min. Samples of infected blood were taken at the time of feedings and stored at -80 °C for later determination of virus titer. Mosquitoes were fed 6.5 - 7.5 $\log_{10}$ plaque forming units (pfu)/mL of Zika.

Following blood feeding trials, fully engorged mosquitoes were sorted using light microscopy and held in cages, maintained at 12:12 hour L:D photoperiod and at 28 °C. Partially fed (average of 2%) and unfed females (average of 9%) were discarded. Mosquitoes were provided with an oviposition substrate and 10% sucrose solution on cotton pads.

Zika virus Disseminated Infection, and Transmission Potential

Mosquito tissues were tested for Zika infection 15 d after ingestion of infected blood. Mosquito legs and saliva were tested for Zika RNA as indicators of Zika disseminated infection (Turell et al. 1984) and transmission potential, respectively (i.e., the presence of viral RNA in saliva is a proxy for transmission). Partitioning mosquito tissues for testing allowed us to determine treatment-induced changes in barriers to infection (e.g., midgut escape barrier and salivary gland barriers). Mosquitoes were cold anesthetized (4 °C), and the legs were removed using light microscopy. Legs were placed in 1 mL of incomplete media (M199) and stored at -80 °C until testing. Using forceps, one wing was damaged to immobilize the mosquito and the proboscis was inserted into a

capillary tube for a 1-h collection of saliva in type B immersion oil using methods described by Alto et al. (2014). Following collection of saliva, mosquito bodies were stored at -80 °C until nutrient analysis testing. Saliva and oil were expelled into 300 µL of media (M199) and stored at -80 °C until testing. Each treatment replicate yielded multiple mosquitoes and so infection measures are reported as percent infection per replicate.

RNA Extraction and Reverse Transcriptase Quantitative Polymerase Chain Reaction (RT-qPCR)

Legs and bodies were homogenized using a TissueLyser (Qiagen, Valencia, CA) in 1000 µL media after which a 140 µL sample of homogenate was clarified by centrifugation and used for RNA isolation with the QIAamp viral RNA mini kit (Qiagen, Valencia, CA) following the manufacturer's protocol. Saliva samples were processed similarly, but with no homogenization. Viral RNA was eluted in 50 µL buffer and quantitative RT-PCR was used to determine the presence and quantity of viral RNA using the Superscript III One-Step qRT-PCR with Platinum® Taq kit (Invitrogen, Carlsbad, CA) with the C1000 Touch Thermal Cycler, CFX96 Real-Time System (Bio-Rad Laboratories, Hercules, CA). The mastermix used 2.2µL molecular grade water, 1µL forward primer, 1µL reverse primer, 10µL 2X reaction mix, 0.4µL ZIKV probe, 0.4µL Taq polymerase, and 5µL of mRNA template. Primers and probe sets synthesized by IDT (Integrated DNA Technologies, Coralville, IA) had the following sequences:

Forward Primer, 5′- CTTCTTATCCACAGCCGTCTC-3′

Reverse Primer, 5′- CCAGGCTTCAACGTCGTTAT-3′

Probe, 5′-/56-FAM/AGAAGGAGACGAGATGCGGTACAGG/3BHQ_1/- 3′

The program for qRT-PCR consisted of a 30-min step at 50°C linked to a 40-cycle PCR (94°C for 12 s and 58°C for 60 s). A standard curve was used to quantify viral load (titer) of Zika in mosquito tissues by comparing cDNA synthesis to a range of serial dilutions of Zika in parallel with plaque assays of the same dilution of virus, expressed as pfu equivalents/mL (Bustin 2000).

Carbon and Nitrogen Analysis

Mosquito species can vary in nutrient content, both based on larval diet and inherent differences among species (e.g., Yee et al. 2015). Carbon is the principle building block of life, and can vary with across diet. In addition, as container systems for developing *Aedes albopictus*, like tree holes, are nitrogen limited (Carpenter 1983) we focused on percent body nitrogen as well. For nutrient analysis, mosquitoes and detritus were prepared by drying in an oven for at least 48 hrs at 50°C. Each weighed sample (mosquito and detritus) was encapsulated in 5 x 9 mm pressed tin capsules (Costech Analytical, Valencia, CA, USA) before analysis. Mosquito body samples and detritus samples were analyzed for total nutrients (%C, %N, C:N) using a ECS 4010 Elemental Combustion System (Costech Analytical Technologies, California). Although %C and %N are correlated to C:N, the latter value is reflective of the stoichiometry of the animal, in essence showing how they may balance the two elements in their body across detrital environments. Although carbon and nitrogen are contained within C:N, individually they cannot enlighten us about how they act in tandem.

Estimated Finite Rate of Increase

In many cases, life history traits correlate with per capita rate of change. An estimate of the per capita rate of change is feasible in experiments where populations are established as cohorts and indirect measures of survivorship, fecundity and generation time are available (Livdahl and Sugihara 1984, Juliano 1998). An estimate of the finite rate of increase (λ') was calculated for each replicate container by initially calculating the estimated instantaneous rate of increase (r', Livdahl and Sugihara 1984):

$$\lambda' = \exp(r') = \exp \frac{\ln [(1/N_o) \sum_x A_x f(w_x)]}{D + [\sum_x x A_x f(w_x) / \sum_x A_x f(w_x)]}$$

where N_0 is the initial number of females in the cohort (assumed to be 50%); A_x is the number of females emerging to adulthood on day x; D is the time from emergence to reproduction taken as 12 d for *Ae. aegypti* (Grill and Juliano 1996); $f(w_x)$ is a function based on the relationship between size and fecundity in female mosquitoes. For *Ae. aegypti* $f(w_x) = 2.5\, w_x - 8.616$ (Briegel 1990).

Statistical Analysis

Analysis of variance (ANOVA) was used to test for larval treatment effects on male development time, survivorship to adulthood, and the estimate of the finite rate of increase (λ'). Multivariate analysis of variance (MANOVA) was used to test for treatment effects on adult female development time and mass. Standardized canonical coefficients were used to describe the relative contribution and relationship of the response variables to the multivariate treatment effect. Differences in response variables among treatment groups were identified using the Tukey-Kramer HSD post-

hoc test for multiple comparisons. Stepwise multiple regression analysis was used to relate infection measures (disseminated infection, saliva infection) to detrital conditions (%N, amount of animal detritus) and %C, %N, C:N signatures in adult females (pooled across treatments). All statistical analyses were performed using SAS software (2004). A randomization ANOVA was used to analyze treatment effects on λ' due to no transformations allowing us to meet assumptions of normality and heteroscedasticity.

RESULTS

Mosquito Life History

Multivariate analysis of variance showed significant effects of treatment on female development time and mass (Pillai's trace $_{18,34}$ = 1.72). Standardized canonical coefficients showed that development time contributed almost twice as much as mass to the significant treatment effect (SCCs, development time = -2.50, mass = 1.42). Females with the longest development times were associated with reduced mass (Fig. 1).

Treatment levels lacking animal detritus displayed significantly delayed development time for both female and male mosquitoes ($F_{8,25}$ = 45.42, $P < 0.001$). Plant only treatments showed delayed development compared to treatment levels with animal detritus (with or without plant) (Figs. 1 and 2). No significant differences were found when treatment levels included at least one unit of animal detritus. No male survivors were observed in the treatment 0:5.

There was a significant effect of treatment on mosquito weights. Mosquitoes were the heaviest in treatment levels with at least two units of animal detritus, regardless of

how much plant detritus was present (Fig. 1). Mosquitoes were intermediate in weight with one unit of animal detritus, regardless of the amount of leaf detritus was preset (Fig. 1). The lightest mosquitoes were produced in habitats with only leaf detritus present (Fig. 1).

Survivorship to adulthood was closely associated with the amount of animal detritus present ($F_{9,27} = 10.53$, $P<0.001$). Increases in basal resources, especially inclusion of animal detritus, yielded higher survivorship compared to plant detritus only situations (Fig. 3). The highest survivorship was observed in treatments containing 2:5 and 2:10 units of animal:plant detritus. An intermediate to high survivorship was seen in treatments containing 1:10, 2:0, 4:0, and 4:10 units of animal:plant detritus, and intermediate to low survivorship in treatments containing 1:0 and 1:5 units of animal:plant detritus. The lowest survivorship was seen in treatments lacking animal detritus (Fig. 3).

A randomization ANOVA showed marginally non-significant treatment effects on λ' ($F_{9,19} = 2.28$, $P = 0.062$, Fig. 4). In general, λ' values were significant higher in combinations of animal and leaf detritus compared to leaf-only treatment levels. In most cases populations were estimated to be growing ($\lambda' > 1$).

Zika Virus Disseminated Infection and Transmission

Disseminated infection (stepwise regression: animal, $F_{1,20} = 65.44$, $P < 0.001$, $R^2=0.75$; animal+leaf, $F_{2,20} = 4.54$, $P = 0.046$, $R^2= 0.05$; %N, $F_{1,24} = 9.23$, $P = 0.006$) and transmission potential (stepwise regression, $F_{1,22} = 20.30$, $P < 0.001$) decreased with increasing animal detritus and %N (Figs. 5, 6, and 7). Disseminated infection was highest with treatment levels containing only one unit of animal detritus, intermediate in

338 treatment levels containing two units of animal detritus, and low in treatment levels
339 containing four units of animal detritus. Overall, adult females showed an average of
340 4.66 ± 0.09% nitrogen, 54.39 ± 0.54% carbon, and a 12.20 ± 0.26% C:N ratio across all
341 detritus ratios.
342
343 **DISCUSSION**
344 We found support for our hypothesis that variation in animal and leaf detritus would
345 alter Zika virus infection and transmission by *Ae. aegypti*. The infection component of
346 our study revealed that quantity and quality of nutrition, and the associated changes in
347 nutrient stoichiometry, altered disseminated infection and transmission potential of Zika
348 virus. Particularly, animal detritus was positively correlated with %N, which affected Zika
349 virus infection. Disseminated infection and transmission decreased with increasing
350 animal detritus and %N. Thus, we provide the first definitive evidence linking nutrient
351 stoichiometry to arbovirus infection and transmission in a mosquito, using a model
352 system of *Ae. aegypti* and Zika virus. Future studies should consider using lower
353 generation of mosquitoes (e.g., F1 generation from field-collected parents) which are
354 likely to be more representative of populations in the wild. The observed resource
355 quality mechanism mediating interactions between *Ae. aegypti* and Zika may apply to
356 other arboviruses and mosquito species. For instance, stoichiometric composition was
357 similar for both *Ae. aegypti Ae. albopictus* across different diet environments (Yee et al.
358 2015). These two species are often implicated in the same transmission cycles (e.g.,
359 chikungunya, Zika, dengue; Gubler 1998, Coffey et al. 2014, Boyer et al. 2018), so
360 further work will be needed to determine if our findings of the relationships between

361 stoichiometry and viral infection are applicable to *Ae. albopictus*. Although the
362 mechanism for the observed results in the current study is unclear, it may relate to
363 increased immune activity and reduced pathogen propagation, as observed in other
364 systems (Cotter et al. 2011, Cornet et al. 2014, Brunner et al. 2014, Howick and
365 Lazzaro 2014).

366 We were interested in producing females who would exhibit a range of nitrogen and
367 carbon values and thus we used different combinations of high-quality animal and low-
368 quality leaf detritus. Although we did produce a gradient (4.27 – 5.29 %N across diets),
369 our diets yielded nitrogen values at the lower end of those produced elsewhere. For
370 instance, in a laboratory experiment Yee et al. (2015) produced female *Ae. aegypti* with
371 a range of 7.60 - 10.19 %N across diets using the same types but higher amounts of
372 detritus per individual. Carbon levels were more similar, with our study producing adults
373 with 51.38 – 58.78 %C whereas Yee et al. (2015) had a range of 45.75 - 55.45 %C.
374 Thus, our diets produced females that were likely more stressed for limited nitrogen,
375 although at present there are no published data from wild mosquitoes to know if the
376 lower values produced in this study fall within the typical range for field collected adults.
377 However, as our animals seemed more stressed for nitrogen and females in higher
378 nitrogen containers had lower average disseminated infection, this does suggest that
379 nitrogen does play a role in affecting vector-pathogen interactions; a mechanism which
380 has not been explored elsewhere.

381 We observed that variation in the amount and relative ratio of animal to plant
382 detritus altered individual life history trait responses of mosquitoes including
383 development time to adulthood, mass (net growth), and survivorship to adulthood.

384 Greater amounts of animal detritus and %N consistently shortened development time
385 and resulted in heavier adults with higher survivorship. Thus, we were able to
386 demonstrate that %N reflected, in presence of animal detritus, affected a variety of life
387 history traits and rate of female infection. These observations are consistent with a
388 study that demonstrated reduced development time and increased adult mass for *Ae.*
389 *aegypti* and *Ae. albopictus* but not *Culex quinquefasciatus* in animal versus leaf only
390 environments (Yee et al. 2015). Nutrient analyses showed that *Aedes* tissues varied in
391 their C:N ratio dependent on animal and leaf detritus ratios, whereas *Cx.*
392 *quinquefasciatus* showed a less plastic response in C:N ratio (Yee et al. 2015). This
393 suggests that nutrient content, and not type of detritus, influenced life history traits.

394 In the current study, an estimate of the finite rate of increase (λ') showed trends for
395 different responses to the quantity and quality of nutrition. Specifically, the presence of
396 animal detritus, either alone or in combination with leaf detritus, increased population
397 growth relative to treatments with only leaf detritus present. However, this effect was
398 only marginally non-significant. We hypothesized that increased variance attributable to
399 treatments that had no survivors ($\lambda' = 0$) was, in part, responsible for lack of significance
400 as the low nutrient treatments showed drastically delayed development time and
401 reduced survivorship. To test this hypothesis, we re-ran the analysis omitting replicates
402 with no survivors (i.e., where $\lambda' = 0$). Results showed a significant treatment effect ($F_{9,17}$
403 $= 8.77$, $P < 0.001$) in the anticipated direction, despite reductions between treatment
404 means among treatments, thus providing support for the hypothesis that increased
405 variance was a contributing factor to the marginally non-significant result. This trend
406 should not be taken lightly, as nutrient pulses within microcosms such as tree holes or

tires are common. Further, spatio-temporal pulses of nitrogen in the form of animal detritus may account for rapid flux in mosquito populations with varying competence and longevity affecting disease transmission dynamics (Kling et al. 2007, Yee 2008, Yee and Juliano 2012).

Frost et al. (2008) observed rich nutrition in *Daphnia magna* water fleas enhanced growth and reproduction of a bacterial parasite (*Pasteuria ramosa*) and Vantuax et al. (2016b) reported a lesser likelihood of infection in females exposed to a reduced quantity of laboratory diet in the larval stages. Discrepancies in these observations may be, in part, attributable to the notion that elemental nutrition may alter parasite and pathogen infection dynamics at different stages of the infection cycle (Alto et al. 2015, Borer et al. 2016). Further, living pathogens, such as malaria parasites or *P. ramosa*, must acquire nutrients from the host environment to grow and reproduce whereas viruses must hijack host replication machinery to replicate. Calorie restriction has been shown to either decrease or increase resistance to parasitism (reviewed in Cotter et al. 2011). The mechanism(s) may be, in part, related to the observation that different immune traits (e.g., phenoloxidase activity and lysozyme-like antibacterial activity) respond differently to nutrient uptake, as demonstrated in the Egyptian cotton leafworm (Cotter et al. 2011). At present, the mechanism for why *Ae. aegypti* females would be less susceptible to infection by Zika when nitrogen levels are greater is unclear. However, given that container systems that produce adults are often limited by nitrogen (Carpenter 1983), this area of research could prove fruitful for linking fine-scale patterns of resource environments to human outbreaks of arbovirus induced disease. Although the present study was limited by the amount of tissue required to perform elemental

analysis for C, N, and P, future investigations should include of the role of phosphorus and other essential elements to understand the role of limiting nutrients in infection.

Our study showed that infection traits map onto different regions of nutrient space as observed by other studies (Cotter et al. 2011). The effects of dietary intake on nutrient stoichiometry and subsequently on immunity in insects has only been investigated in a handful of disparate taxa (Lee et al. 2008). However, it is known that activation or maintenance of immunity often involves use of protein reserves (Lee et al. 2006), which is likely consistent with nitrogen availability. Thus, our results provide a starting point to investigate the wider role of nutrients, including nitrogen, in affecting mosquito-pathogen interactions of important human diseases.

Adult mosquitoes derived from nutrient rich environments containing insect detritus had greater mass and body size than adults from treatments with less detritus, especially those with little or no animal detritus. Although larger mosquitoes consume greater volumes of blood, therefore ingesting higher doses of Zika, we considered the possibility that they might have higher rates of infection. However, the pattern that we observed was the opposite of this prediction. Specifically, larger adults from nutrient rich larval environments had lower disseminated infection and saliva infection rates than smaller conspecifics. This observation suggests that differences in infection rates are not attributable to differences in volume of infected blood ingested. Rather, we speculate that the overall health of the mosquito determined by larval nutrient environments, may influence infection and progression of infection (advanced states of infection). Another possibility is that larger blood meals may provide a greater influx of nitrogen. Consequently, some blood meal resources for reproduction may trade off with

453 energy reserves to fight an infection in order to live long enough to reproduce
454 successfully which would likely be adaptive and favored by selection. Other studies
455 investigating mosquito larval nutrition (amount of plant detritus or quantity of laboratory
456 diet) and competition have observed similar impacts on adult life history traits or
457 competence (LaCrosse virus, Grimstad and Haramis 1984; Sindbis virus, Alto et al.
458 2005; dengue virus, Alto et al. 2008ab). However, we are the first to quantify nitrogen
459 limitation and demonstrate it's role in arboviral infection and transmission potential.
460 Invasive *Ae. albopictus* and native *Ae. triseriatus* container mosquitoes derived from
461 nutrient rich larval environments were less likely to exhibit disseminated infection and/or
462 to transmit dengue-2 virus (Zhang et al. 1993) and LaCrosse encephalitis virus
463 (Grimstad and Haramis 1984, Grimstad and Walker 1991, Paulson and Hawley, 1991),
464 respectively, than conspecifics from nutrient-deprived larvae. Additionally, these nutrient
465 effects carried-over to the next generation as demonstrated with maternal effects on
466 offspring infection with LaCrosse virus. This may have important epidemiological
467 consequences given that vertical transmission is a mechanism for persistence of
468 LaCrosse in the environment (Patrician and DeFoliart 1985). Thus, we propose that
469 larval diet, with specific reference to the nutrients it contains, is a mechanism that
470 affects nutrient composition and allocation patterns in *Ae. aegypti*; it may be an
471 important but overlooked component to understanding transmission potential of
472 arboviruses across different resource environments.

473 Larval nutrition alters several phenotypic traits related to mosquito fitness that are
474 relevant to their ability to transmit pathogens (Beldomenico and Begon 2010) such as
475 longevity (Steinwascher 1982, Haramis 1985), host-seeking behavior (Nasci 1986,

476 Klowden et al. 1988), biting persistence (Nasci 1991), blood-feeding success (Nasci
477 1986), and fecundity (Steinwascher 1982, Vantaux et al. 2016a). It is likely that
478 enhanced infection associated with nutrient deprivation may also have consequences
479 for these other life history traits, so mathematical models are needed to evaluate the net
480 effect on risk of arbovirus transmission (Bara et al. 2014).

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488 analysis of mosquitoes.

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491 **LITERATURE CITED**

492 Aalto SL, Decaestecker E, Pulkkinen K (2015) A three-way perspective of stoichiometric
493 changes on host-parasite interactions. Trends Parasitol 31:333–340.
494 Alto BW, Lounibos LP, Higgs S, Juliano SA (2005) Larval competition differentially
495 affects arbovirus infection in *Aedes* mosquitoes. Ecology 86:3279–3288.
496 Alto BW, Reiskind MH, Lounibos LP (2008) Size Alters Susceptibility of Vectors to
497 Dengue Virus Infection and Dissemination. Am J Trop Med Hyg 79:688–695.
498 Alto BW, Muturi EJ, Lampman RL (2012) Effects of nutrition and density in *Culex*
499 *pipiens*. Med Vet Entomol 26:396–406.
500 Alto BW, Connelly CR, O'Meara GF, Hickman D, Karr N (2014) Reproductive biology
501 and susceptibility of Florida *Culex coronator* to infection with West Nile virus. Vector
502 Borne and Zoonotic Dis 14(8):606-614.
503 Bara J, Rapti Z, Cáceres CE, Muturi EJ (2014) Effect of larval competition on extrinsic
504 incubation period and vectorial capacity of *Aedes albopictus* for dengue virus. PLoS
505 ONE 10(5):e0126703.doi:10.1371/journal.pone.0126703.
506 Beldomenico PM, Begon M (2010) Disease spread, susceptibility and infection intensity:
507 vicious circles? Trends Ecol Evol 25 (1): 21-27.
508 Borer ET, Laine AL, Seabloom EW (2016) A multiscale approach to plant disease using
509 the metacommunity concept. Annu Rev Phytopathol 54: 397-418.
510 Boyer S, Calvez E, Chouin-Carneiro T, Diallo D, Failloux A-B (2018). An overview of
511 mosquito vectors of Zika virus. Microbes Infect 11-12: 646-660.
512 Briegel H (1990) Metabolic relationship between female body size, reserves, and
513 fecundity of *Aedes aegypti*. J Insect Physiol 36:165-172.

514 Brunner FS, Schmid-Hempel P, Barribeau SM (2014) Protein-poor diet reduces host-
515 specific immune gene expression in *Bombus terrestris*. Proc R Soc B 281:
516 20140128.
517 Buckner EA, Alto BW, Lounibos LP (2016) Larval temperature-food effects on adult
518 mosquito infection and vertical transmission of dengue-1 virus. J Med Entomol
519 53:91–98.
520 Bustin SA (2000) Absolute quantification of mRNA using real-time reverse transcription
521 polymerase chain reaction assays. J Mol Endocrinol 25:169–193.
522 Carpenter SR (1983) Resource limitation of larval treehole mosquitoes subsisting on
523 beech detritus. Ecology 64:219–223.
524 Clements AN (2012) The Biology of Mosquitoes. Vol. 3. Transmission of viruses and
525 interactions with bacteria. CABI, Oxford.
526 Cornet S, Bichet C, Larcombe S, Faivre B, Sorci G (2014) Impact of host nutritional
527 status on infection dynamics and parasite virulence in a bird-malaria system. J Anim
528 Ecol *83*: 256-265.
529 Coffey LL, Failloux A-B, Weaver SC (2014) Chikungunya virus-vector interactions.
530 Viruses 6: 4628-4663.
531 Cotter SC, Simpson SJ, Raubenheimer D, Wilson K (2011) Macronutrient balance
532 mediates trade-offs between immune function and life history traits. Funct Ecol
533 25:186-198.
534 Da-Silva Araújo M, Gil LHS, de-Almeida e-Silva A (2012) Larval food quantity affects
535 development time, survival and adult biological traits that influences the vectorial
536 capacity of *Anopheles darlingi* under laboratory conditions. Malaria J 11:261.

537 Green BS, McCormick MI (2005) Maternal and paternal influences determine size,
538 growth and performance in tropical reef fish larvae. Mar Ecol Prog Ser. 289:263–72.
539 Grill CP, Juliano SA (1996) Predicting species interactions based on behaviour:
540 predation and competition in container-dwelling mosquitoes. J Anim Ecol 65: 63-76.
541 Grimstad PR, Haramis LD (1984) *Aedes triseriatus* (Diptera: Culicidae) and La Crosse
542 virus III. Enhanced oral transmission by nutrition-deprived mosquitoes. J Med
543 Entomol 21: 249-256.
544 Grimstad PR, Walker ED (1991). *Aedes triseriatus* (Diptera: Culicidae) and La Crosse
545 virus. IV. Nutritional deprivation of larvae affects the adult barriers to infection and
546 transmission. J Med Entomol 28: 378-386.
547 Gubler DJ (1998) Dengue and dengue hemorrhagic fever. Clin Microbiol Rev 11:480-
548 496.
549 Haramis L (1985) Larval nutrition and adult ecology. Mosquito Ecology: Proceedings of
550 a Symposium (ed. by L.P. Lounibos & J. Rey), pp. 431-439. University of Florida
551 Press, Gainesville, FL.
552 Hillyer JF (2010) Mosquito immunity. Adv Exp Med Biol 708: 218-238.
553 Hillyer JF, Estéves-Lao TY (2010) Nitric oxide is an essential component of the
554 hemocyte-mediated mosquito immune response against bacteria. Dev Comp
555 Immunol 34:141-149.
556 Juliano SA (1998) Species introduction and replacement among mosquitoes:
557 Interspecific resource competition or apparent competition? Ecology 79: 255-268.
558 Kauffman E, Payne A, Franke MA, Schmid MA, Harris E, Kramer LD (2017) Rearing of
559 *Culex* spp. and *Aedes* spp. Mosquitoes. Bio Protoc 7:e2542

560 Kingsolver JG, Woods HA, Buckley LB, Potter KA, MacLean HJ, Higgins JK (2011)
561 Complex life cycles and the responses of insects to climate change. Int
562 Comp Biol. 51:719–32.
563 Kling LJ, Juliano SA, Yee DA (2007) Larval mosquito communities in discarded vehicle
564 tires in a forested and unforested site: detritus type, amount, and water nutrient
565 differences. J Vector Ecol 32:207–17.
566 Klowden MJ (1986) Effects of sugar deprivation on the host-seeking behaviour of gravid
567 *Aedes aegypti* mosquitoes. J Insect Physiol 32: 479-483.
568 Lee KP, Cory JS, Wilson K, Raubenheimer D, Simpson SJ (2006) Flexible diet choice
569 offsets protein costs of pathogen resistance in a caterpillar. Proc R Soc B 273:823–
570 829.
571 Lee KP, Simpson SJ, Wilson K (2008) Dietary protein quality influences melanization
572 and immune function in an insect. Funct Ecol 22:1052-1061.
573 Livdahl, TP, Sugihara G (1984) Non-linear interactions of populations and the
574 importance of estimating per capita rates of change. J Anim Ecol 53: 573-580.
575 McCann S, Day JF, Allan S, Lord CC (2009) Age modifies the effect of body size on
576 fecundity in *Culex quinquefasciatus* Say (Diptera: Culicidae). J Vector Ecol 34:174-
577 181
578 McCormick MI, Gagliano M (2008) Carry-over affects-the importance of a good start. In:
579 Proc 11th Int Coral Reef Sym Ses. 10: 305–10
580 Murrell EG, Juliano SA (2008) The role of detritus type in interspecific competition and
581 population distributions of *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae).
582 J Med Entmol 45:375–383.

583 Muturi EJ, Kim CH, Alto BW, Berenbaum MR, Schuler MA (2011) Larval environmental
584 stress alters *Aedes aegypti* competence for Sindbis virus. Trop Med Int Health
585 16:955–964.
586 Nasci RS (1986) The size of emerging and host-seeking Aedes aegypti and the relation
587 of size to blood-feeding success in the field. J Am Mosq Control Assoc 2: 61-62.
588 Nasci RS (1991) Influence of larval and adult nutrition on biting persistence in *Aedes*
589 *aegypti* (Diptera: Culicidae). J Med Entomol 28:522-526.
590 Patrican LA, DeFoliart GR (1985) Lack of adverse effect of
591 transovarially acquired La Crosse virus infection on the reproductive capacity of
592 *Aedes triseriatus* (Diptera: Culicidae). J Med Entomol 22: 604–611.
593
594 Steinwascher K (1982) Relationship between pupal mass and adult survivorship and
595 fecundity for *Aedes aegypti*. Environ. Entomol. 11: 150-153.
596 Takken W, Klowden MJ, Chambers GM (1998) Effect of body size on host seeking and
597 blood meal utilization in *Anopheles gambiae sensu stricto* (Diptera: Culicidae): the
598 disadvantage of being small. J Med Entomol 35: 639-645.
599 Telang A, Qayum AA, Parker A, Sacchetta BR, Byrnes GR (2012) Larval nutritional
600 stress affects vector immune traits in adult yellow fever mosquito *Aedes aegypti*
601 (Stegomyia aegypti). Med Vet Entomol 26: 271-81.
602 Turell MJ, Gargan TP, Bailey C (1984) Replication and dissemination of Rift Valley fever
603 virus in *Culex pipiens*. Am J Trop Med Hyg 33: 176-181.
604 Vantaux A, Lefèvre T, Cohuet A, Dabiré KR, Roche B, Roux O (2016a) Larval nutritional
605 stress affects vector life history traits and human malaria transmission. Sci Rep 6:1–
606 10.

607 Vantaux A, Ouattarra I, Lefèvre T, Dabiré KR (2016b) Effects of larvicidal and larval
608 nutritional stresses on *Anopheles gambiae* development, survival and competence
609 for *Plasmodium falciparum*. Parasit Vectors 9:1–11.
610 Wink DA, Hines HB, Cheng RYS, Switzer CH, Flores-Santana W, Vitek MP, Ridnour
611 LA, Colton CA (2011) Nitric oxide and redox mechanisms in the immune response. J
612 Leukoc Biol 89:873-791.
613 Winters AE, Yee DA (2012) Variation in performance of two co-occurring mosquito
614 species across diverse resource environments: Insights from nutrient and stable
615 isotope analyses. Ecol Entomol 37:56–64.
616 Yee DA (2008) Tires as habitats for mosquitoes: a review of studies within the eastern
617 United States. J Med Entomol 45:581–593.
618 Yee DA, Juliano SA (2012) Concurrent effects of resource pulse amount, type, and
619 frequency on community and population properties of consumers in detritus-based
620 systems. Oecologia 169:511-522.
621 Yee DA, Kaufman MG, Ezeakacha NF (2015) How diverse detrital environments
622 influence nutrient stoichiometry between males and females of the co-occurring
623 container mosquitoes *Aedes albopictus*, *Ae. aegypti*, and *Culex quinquefasciatus*.
624 PLoS ONE 10(8):1–19. e0133734.
625 Yee DA, Juliano SA (2006) Consequences of detritus type in an aquatic microsystem:
626 Effects on water quality, micro-organisms and performance of the dominant
627 consumer. Freshwater Biol 51:448–459.

628 Young GB, Golladay S, Covich A, Blackmoore M (2014) Stable isotope analysis of
629 larval mosquito diets in agricultural wetlands in the coastal plain of Georgia, U.S.A. J
630 Vector Ecol 39:288–297.
631 Zhang S, He G, Xu L, Lin Q, Zhang S (1993) Effects of larval nutrition on susceptibility
632 of *Aedes albopictus* to dengue 2 virus. Arbovirus Research in Australia 6: 44-48.

633 **Figure 1.** MANOVA of bivariate least square means of female mass and development
634 time across different nutrient environments as represented by different ratios of animal
635 (crickets) and leaf (red maple) detritus. Means that do not share same letters are
636 significantly different, and bars indicate standard error of the mean. Lowercase letters
637 are for mass and uppercase letters are for development time.

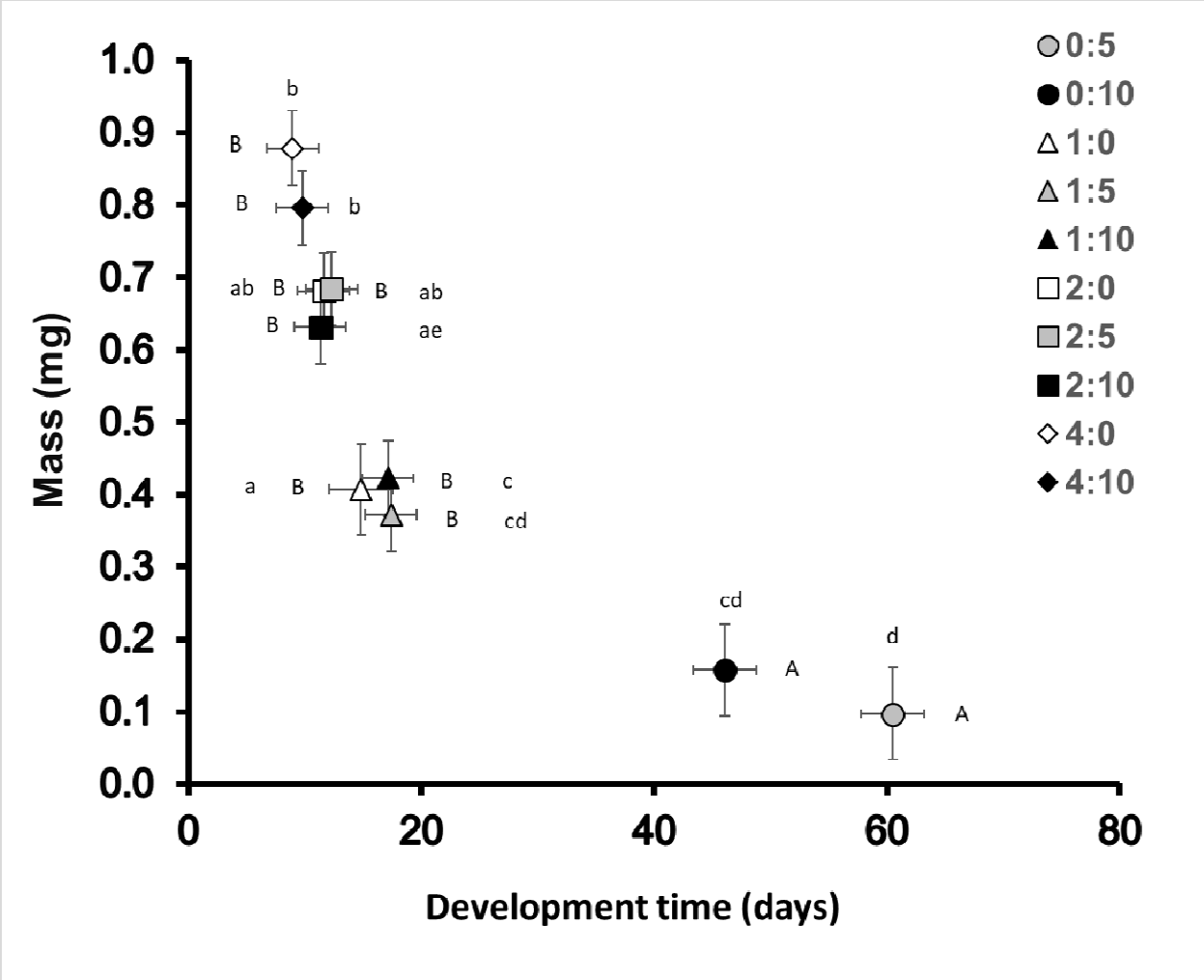

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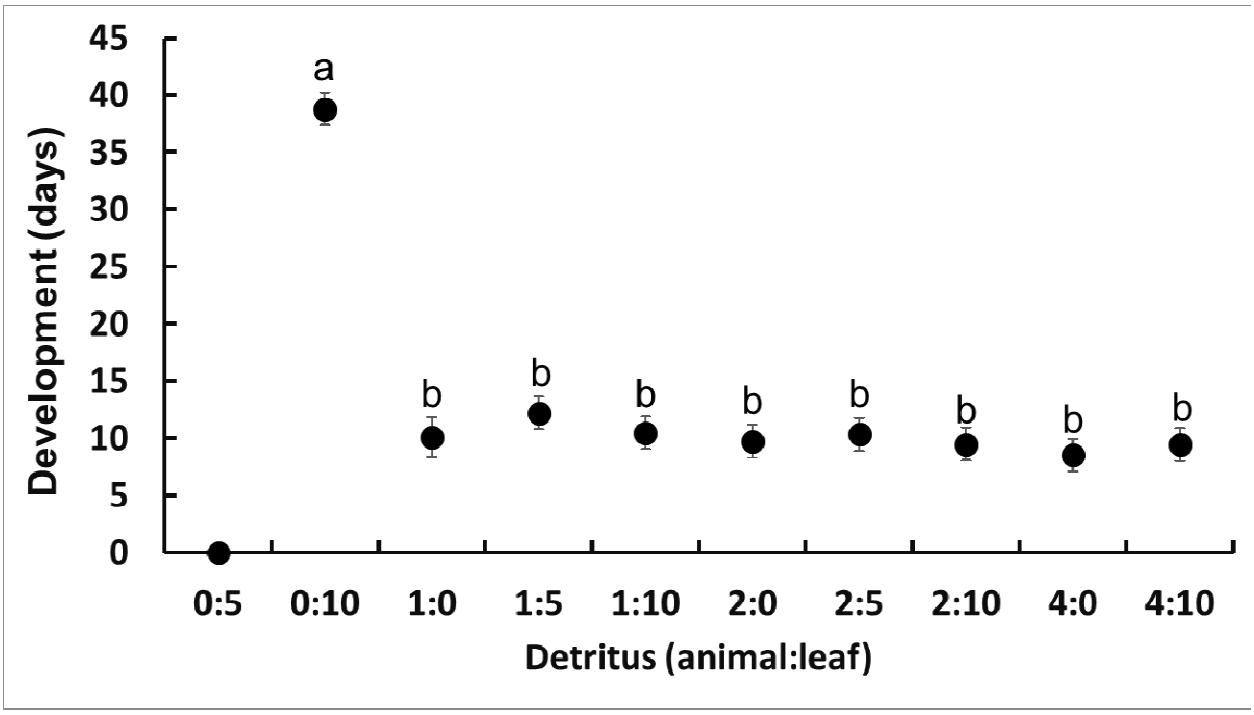
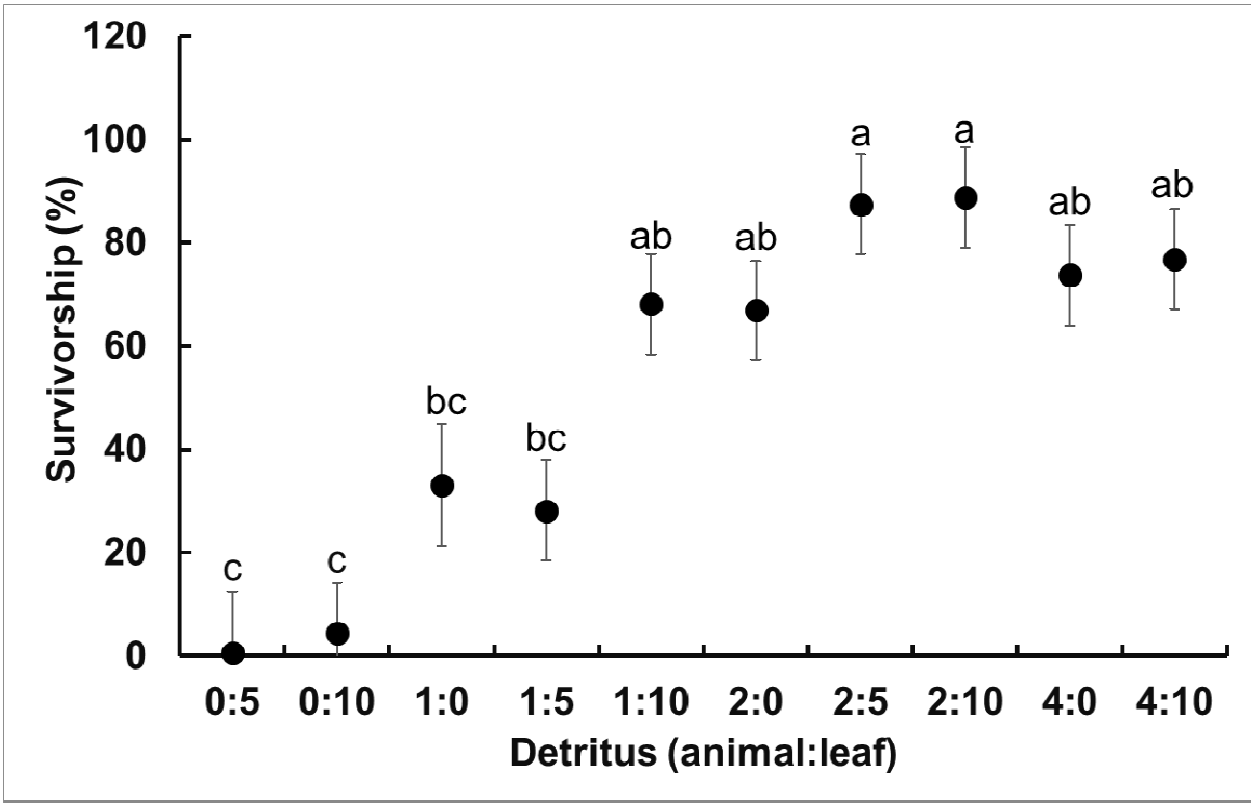
Figure 2. ANOVA of least square means of male development time across different environments as represented by different ratios of animal (crickets) and leaf (red maple) detritus. Means that do not share same letters are significantly different, and bars indicate standard error of the mean.



653 **Figure 3.** ANOVA of least square means of percent survivorship to adulthood
654 (male+female, expressed as percent total of initial cohort of larvae added to containers)
655 across different nutrient environments as represented by different ratios of animal
656 (crickets) and leaf (red maple) detritus. Means that do not share same letters are
657 significantly different, and bars indicate standard error of the mean.



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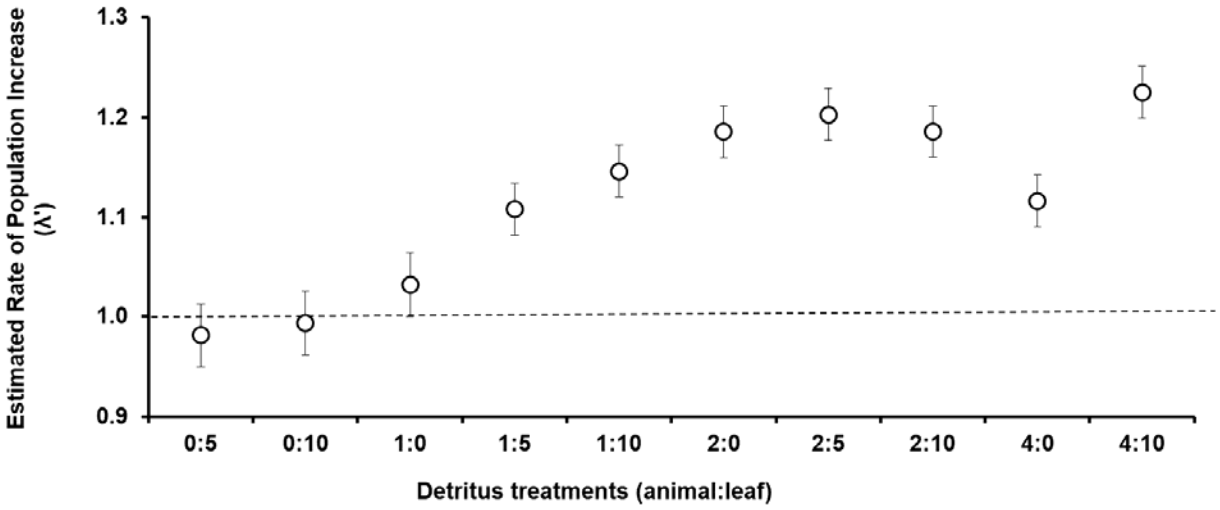
663 **Figure 4.** Values of the estimate of the finite rate of increase (λ′) for *Aedes aegypti*
664 females across animal and leaf environments. Nutrient environments are represented
665 by different ratios of animal (crickets) and leaf (red maple) detritus. Values that share a
666 letter are not significantly different at P > 0.05. The dashed line at λ′ represents
667 populations for which growth is estimated to be zero.



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675 **Figure 5.** Stepwise multiple regression (%C, %N, C:N) on the proportion of positive
676 mosquitoes in each treatment. Each point represents a replicate for each treatment
677 (%N, $F_{1,24}$ = 9.23, P = 0.006).

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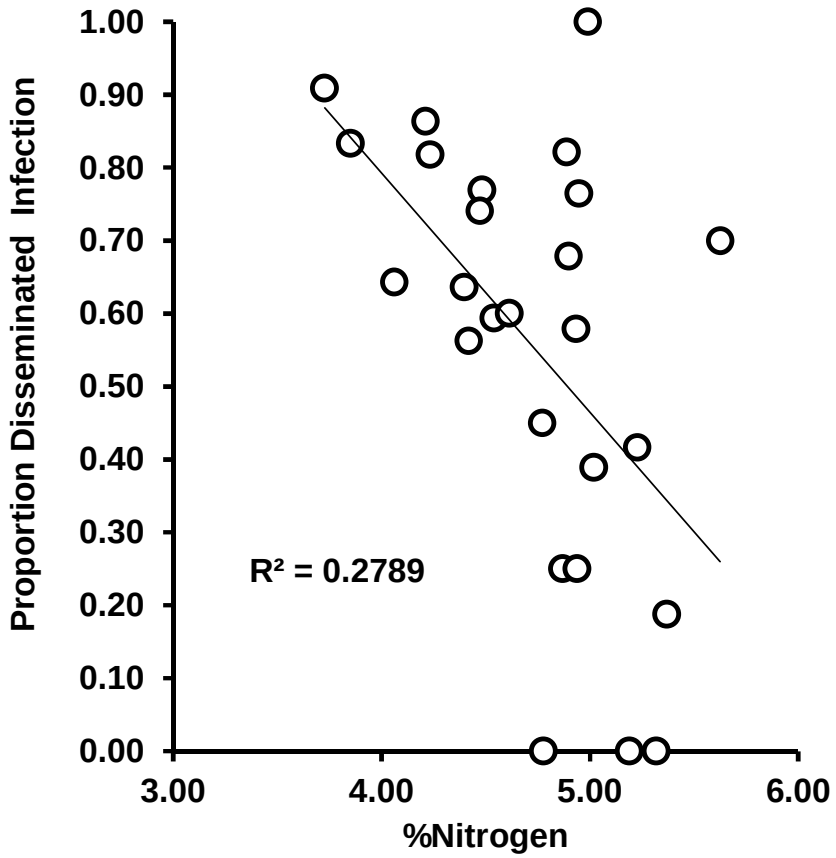

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691 **Figure 6.** Stepwise multiple regression (%C, %N, C:N) on the proportion of positive
692 mosquitoes in each treatment. Each point represents a replicate for each treatment
693 (%N, $F_{1,24}$ = 9.23, P = 0.006).

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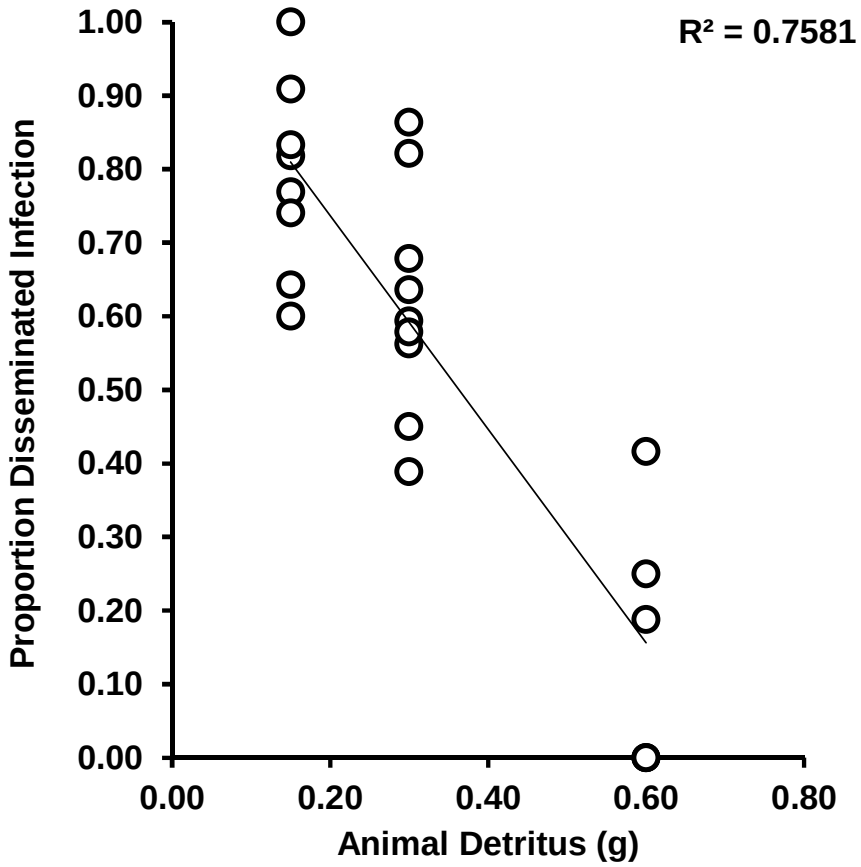

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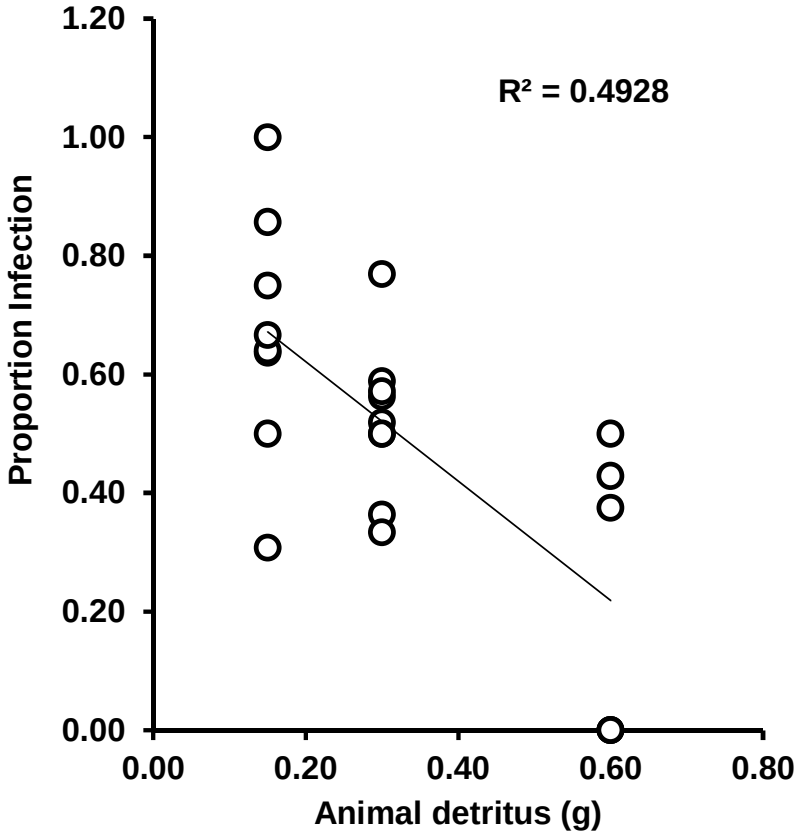
707 **Figure 7.** Stepwise multiple regression (animal detritus and leaf detritus (g)) on the
708 proportion of mosquitoes with positive saliva infection in each treatment. Each point
709 represents a replicate for each treatment (Animal, $F_{1,22} = 20.30$, $P < 0.001$, $R^2 = 0.4617$;
710 Leaf, not significant).



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