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Late-instar Behavior of *Aedes aegypti* (Diptera: Culicidae) Larvae in Different Thermal and Nutritive Environments

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ABSTRACT The effects of temperature on ectotherm growth have been well documented. How temperature affects foraging behavior is less well explored, and has not been studied in larval mosquitoes. We hypothesized that temperature changes foraging behavior in the aquatic larval phase of the mosquito, *Aedes aegypti* L. Based on empirical results in other systems, we predicted that foraging effort would increase at higher temperatures in these insects. We tested this prediction over three temperature conditions at two food levels. We measured behaviors by video recording replicated cohorts of fourth-instar mosquitoes and assessing individual behavior and time budgets using an ethogram. We found both food level and temperature had significant impacts on larval foraging behavior, with more time spent actively foraging at low food levels and at low temperatures, and more occurrences of active foraging at both temperature extremes. These results are contrary to some of our predictions, but fit into theoretical responses to temperature based upon dynamic energy budget models.

KEY WORDS ecology & behavior, climate change, immature insects, mosquito borne diseases, metabolism/life processes

Ectotherms derive their body temperature from the environment and this has important consequences for their life histories. For most ectothermic organisms, this means higher metabolic rates and activity at higher temperatures (Precht et al. 1973, Angilletta 2009), resulting in rapid growth with smaller size at adulthood (Atkinson 1995, Kingsolver and Huey 2008). There is substantial evidence for thermal adaptation, which can alter the shape of a fitness or performance response to temperature for an organism (Huey and Kingsolver 1993). In addition, there are many examples of how ectothermic organisms can moderate the effects of environmental temperature by movement, locomotion, or habitat choice (Angilletta 2009). However, when an organism is forced to experience a certain thermal environment, they may still alter their behavior to maximize performance under those conditions. For example, by adjusting foraging activity, an organism may find an optimum between anabolism and catabolism (Padmanabha et al. 2012). Therefore, an examination of the behavior of an organism under different thermal and other environmental conditions can provide insight into the causes and consequences of temperature–growth rules.

As with other ectotherms, mosquitoes have shown a general adherence to the temperature–growth rules, but also show a plastic response in allometry to different thermal environments (Atkinson 1995). Mosquitoes are a good system for examining behavioral response to temperature because of their bipartite lifecycle, established ethograms, and obligate use of small, constrained, aquatic habitats as larvae (Walker and Merritt 1991). Both theoretical models and empirical data for container mosquitoes suggest a plastic response in growth rate through changes in anabolism and catabolism of resources at different larval temperatures, resulting in changes in allometry and reserve storage strategies in adults (Padmanabha et al. 2012, Reiskind and Zarrabi 2012). This plasticity may be achieved through qualitative and quantitative differences in larval behavior under different thermal conditions, specifically differences in foraging intensity. However, this is has not been previously explored.

All mosquito growth occurs in an aquatic larval phase. There is a tremendous body of literature on how nutrients and temperature affect larval growth and subsequent adult outcomes such as size, fecundity, vector competence, longevity, and population growth rate. However, the effects of temperature and nutrients on larval behavior have been less well studied. There is some evidence that larval mosquitoes behave differently when provided with a different types or levels of nutritive resources (Merritt et al. 1992, Kesavaraju et al. 2007, Phelan and Roitberg 2013, Skiff and Yee 2014). As a general rule, mosquito larvae follow theoretical responses of other ectotherms in responding to poor nutrients with increased foraging effort and may adjust their behavior within a habitat to orient towards higher quality food (Anholt et al. 2000, Kesavaraju et al. 2007). Temperature has received even less

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attention in larval behavior studies. A recent publication suggested that temperature had no effect on diving rate in *Anopheles gambiae* (Giles), although nutrient level did (Phelan and Roitberg 2013). To our knowledge, larval behavior of *A. aegypti* has not been quantified with regards to variation in temperature or nutrient concentration. Older studies have suggested that, given the freedom of movement along a temperature gradient, most *A. aegypti* larvae move to between 23 and 32°C, with younger instars preferring slightly cooler locations (23–27°C) relative to fourth-instar larvae and pupae (28–32°C; Omardeen 1957). A similar experiment with *Aedes taeniorhynchus* (Wiedmann) demonstrated a similar pattern, with older larvae and pupae preferring temperatures >30°C (Linley and Evans 1971). However, how temperature affects larval foraging behavior in mosquitoes remains an open question.

Aedes aegypti L. is the principal vector of dengue fever virus, yellow fever virus, and chikungunya virus. The ability of *A. aegypti* to transmit viruses depends on mosquito abundance, host biting rate, vector competence, extrinsic incubation period, and longevity. Longevity, vector competence, and abundance are sensitive to conditions under which the larvae were raised, including competition, nutrients, and temperature (Alto et al. 2008, Reiskind and Lounibos 2009, Westbrook et al. 2010, Adelman et al. 2013). Understanding the foraging behavior of these mosquitoes under different conditions may provide insight into how larval conditions affect adult characteristics. Furthermore, many larval control approaches require ingestion of insecticides (e.g., *Bacillus thuringiensis* and some fungal pathogens), so understanding how actively and where these vectors are foraging can help design better larvicides (Merritt et al. 1992).

In this study, we hypothesized that *A. aegypti* larvae behave differently when nutrients are rich versus poor, with more active foraging when nutrients are scarce, as seen in other aquatic ectotherms (Anholt et al. 2000). We also hypothesize that larval activity rates are higher at higher temperatures, as seen in many ectotherms (Precht et al. 1973). We predict that larvae will spend more time actively foraging (e.g., browsing surfaces) when nutrients are poor to obtain sufficient nutrients to achieve pupation. We predict that larvae will be more active per unit time (more changes in behavioral state) at high temperatures relative to lower temperatures. To test these hypotheses, we performed an experiment in which we exposed cohorts of mosquito larvae to different temperatures and nutrient levels and examined their behavior through video analysis.

Materials and Methods

Mosquitoes. We used F_9 *A. aegypti* originally collected in Palm Beach County, FL. These mosquitoes were maintained on senesced oak leaf (*Quercus phellos* (L.) 4 g/liter) and yeast:albumin (1:1, 0.3 g/l) infused water and fed on a human volunteer to generate eggs.

Study Design and Location. We performed a 2×3 factorial study with two nutrient levels (high and low) and three temperature levels. Each factor combination was replicated four times, for a total of 24 experimental units consisting of a cohort of 15 neonate *A. aegypti*. Neonate *A. aegypti* were placed, within 24 h of hatching, in square, clear plastic containers (AMAC) and 250 ml of infusion. Nutrient levels were manipulated by providing full-strength infusion (4 g of dried oak leaves [*Q. phellos*] plus 0.3 g of 1:1 yeast:albumin per liter of tap water, aged 3 d), and the same infusion diluted to 0.25× with aged tap water. As with most studies of larval nutrients, this represents a nutrient base that is colonized by microorganisms, and is thus dynamic and subject to environmental factors, including temperature (Merritt et al. 1992). The low nutrient level was chosen to provide a stressful food environment barely sufficient for pupation for some individuals. Distilled water was added to the containers throughout the experiment to maintain initial water levels and account for evaporation, which was higher at higher temperatures.

Temperature was manipulated by using three climate controlled greenhouses at the North Carolina State University Phytotron (http://www.ncsu.edu/phytotron/). The greenhouses experienced diurnally fluctuating temperatures. The coolest greenhouse had a daytime high of 25.9°C and a low of 18.1°C, with a measured average of 21.0°C. The middle greenhouse had a high of 30.4°C and a low of 21.6°C, with a measured average of 24.8°C. The hottest greenhouse had a high of 34.1°C and low of 25.7°C, with an average of 28.5°C. All three greenhouses had a 1-h transition between day (high) and night (low) temperatures. Day and night temperatures which were consist outside of transitional periods, with the high lasting 11.5 h and the low 10.5 h. This range of temperatures is a reasonable approximation of temperatures experienced by *A. aegypti* in the field (Padmanabha et al. 2010). We did not measure water temperature, and recognize that it may lag behind changes in air temperature. The study took place from 9 August to 1 September 2013, and during this time, the larvae were exposed to an average of 13.3 h of sunlight each day.

Video Recording. Cohorts of mosquitoes were monitored for molting to synchronize the stage of video recording at the early fourth instar. The containers of larvae were video recorded, with a CMOS digital camera (Mightex, Inc.,Model BCE-B050-U, Pleasanton, CA), on the first day that the first larvae, in a container, molted to the fourth instar. Larval behavior was recorded for 35 min by taking a side and overhead shot of each container. To minimize differences between treatments, all recording was conducted between 10 am and 3 pm each day to provide more consistent light conditions from day to day. To further minimize day-to-day differences in light, a translucent umbrella was always placed above the camera set-up. The three greenhouses are side by side and receive identical light conditions, although there was some variation for any given recording session due to cloud cover.

Larval Outcomes. The total number of larvae at time of behavior capture, total number surviving to adulthood, sex, time to emergence, wing-length (mm),

and weight (mg) were measured for each individual. From these data, an average value for females and males was generated for each replicate. Because of very poor survival at low nutrient levels (Fig. 1), time to emergence, wing-length, and weight are only compared between temperature treatments at high nutrient levels.

Behavior Analysis. Behavior of three to five individual larvae for each replicate was scored using a modified ethogram based on Walker and Merritt's ethogram of *Ochlerotatus triseriatus* (Say) behavior (Table 1) (Walker and Merritt 1991). Observations were made for 7 min30 s from 15 min 00 s to 22 min 30 s of each video. At exactly 15 min 00 s, a snapshot of the container was taken. A grid of 60 numbered squares was laid on this image. A random number generator was used to select squares, which was done until a square was chosen that had a larva in it. If more than one larva was present in the cell, then the left-most larva was chosen first. If a square was chosen that contained a larva already measured, the process was repeated until another larva was chosen. If a cell was chosen with more than one larvae and the left most larva was already measured, the next left-most larva in the cell was chosen. Behavior was scored using the free software JWatcher (Blumstein and Daniel 2007). Number of occurrences of each behavior and total time in each behavior for each individual was recorded. Many behaviors were quite rare and were not analyzed (autogrooming, allogrooming, startle response, and asleep). Mean time or occurrences of the three to five individuals' behavior was calculated for each replicate, and compared statistically. One replicate of the moderate temperature/low nutrient treatment was impossible to analyze from 15 min 00 s to 22 min 30 s because of larval clumping. This phenomenon was observed throughout the period video-taped, although data were collected from 7 min 30 s to 15 min 00 s, and this replicate is included in the analysis.

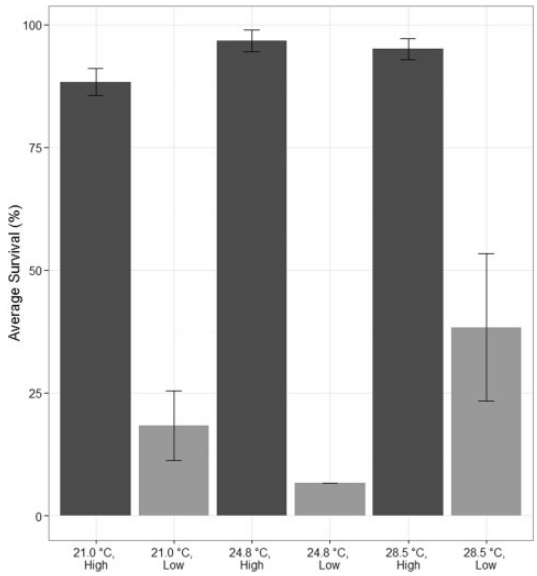

Fig. 1. Average larval survival across temperatures (x-axis) and nutrient conditions (dark grey = high nutrients; light grey = low nutrients). The top of each bar represents the average survival for the four replicates of each condition (error bars show ± 1 S.E.M).

Aggregation of Larvae. We were also interested in whether nutrition or temperature affected aggregation. To examine aggregation, we took images from each replicate every 5 min (at 5, 10, 15, 20, and 25 min) for a total of five images for each replicate. For each image, we measured the location of every larva's head and used these measurements to calculate the nearest neighbor distances (NNDs) between larvae. Random distributions of simulated larvae were created with a random number generator set to produce the same number of three-dimensional coordinates as in the measured data. These coordinates had the same boundaries as the physical dimensions of the water volume in the containers.

A *z*-score was calculated using the observed NNDs relative to the random NNDs, for each of the five sampled time points of every replicate. This calculation required the subtraction of the arithmetic mean of the NNDs of the simulation from the geometric mean of the NNDs of the sample. This value was then divided by the ratio of the standard deviation of the NNDs of the simulation over the square root of the number of NNDs. These five *z*-scores were averaged across all

Table 1. List of behaviors used for *A. aegypti* larval behavior analysis with their corresponding behavior number in *Oc. triseriatus* as developed by Walker and Merritt (1991)

Letter	Name	Walker and Merritt (1991) no.	Description
a	Asleep	12	Motionless underwater
d	Dive	4	Diving (wriggling or gliding)
f	Friendly	11	Allogrooming or larva directs mouthparts to other larva
g	Glide	9	Mouth swim or underwater, using mouth, not body
h	Hanging	1	Hanging from surface, includes moving with siphon on surface
j	Jump	5	Diving due to startle
o	O-shape	3	Autogroom
r	Rise	13	Rising to surface (wriggling or gliding)
t	Triangle	14	Feeding on wall while hanging from surface
u	U-shape	2	Feeding on water surface while hanging
v	Vacuum	7	Brushing the bottom or side of container
w	Wriggle	8	Flexing movements for lateral (not ascending or descending) movement
z	Mistake or unclear	n/a	If behavior becomes unclear or an error is made in coding (then switched to correct code).

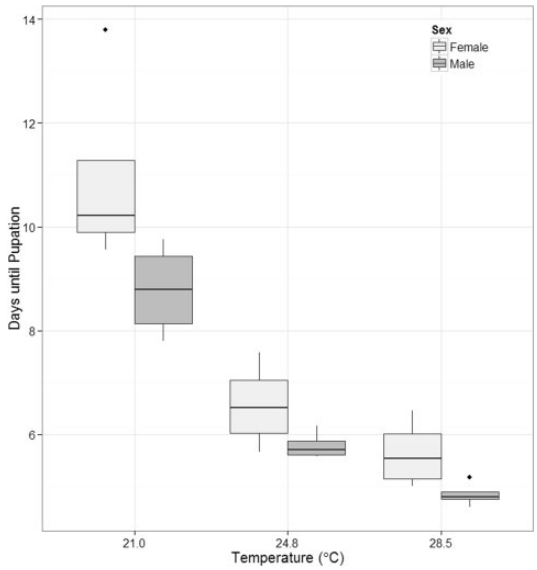

Fig. 2. Box plot of development time, separated by sex. Females are dark grey boxes, males are white boxes. Data from only the high nutrient condition are used. Outliers are represented as solid dots.

time points for each replicate (Malkiel et al. 2006). The distributions of NNDs for each replicate were log-normal (Fig. 5A) and the geometric mean is the appropriate average for this type of distribution (Limpert et al. 2001). For a z-score to be statistically significant (having a probability of <5% of happening by chance), it must have a value >2 or <−2. Z-scores >2 indicate larvae were more widely dispersed when compared to a random distribution, whereas z scores <−2 indicate larvae were clustered (Malkiel et al. 2006).

Statistical Analysis. Percent survival was not normally distributed and was compared using a randomization ANOVA (Manly 1997). Wing length, weight, and time to emergence were normally distributed and homoscedastic. These outcomes were compared between high nutrient treatments for males and females separately using ANOVA. Behavioral data were summarized in JWatcher and exported to Excel, then read into R (R-base package, R-development team, Geneva, Switzerland). As the behavioral time budgets were normally distributed, not heteroscedastic, and each observational unit was exactly 7.5 min, time spent in a given behavior was compared by ANOVA using R. Mean number of occurrences of each behavior was also compared by ANOVA in R. To assess larval aggregation, we compared z-scores for each replicate with ANOVA in R.

Results

Larval Outcomes. There were no significant differences in the number of larvae at time of video capture by nutrients or temperature, although the number of larvae varied between 12 and 15 across all replicates. Larval survival to adulthood was significantly higher at high nutrients than at low, with no significant effect of temperature or the interaction of temperature and nutrients (Fig. 1; randomization ANOVA: temperature: $P = 0.3156$; Nutrients: $P < 0.0001$; T × N: $P = 0.3019$). At high nutrients, there was no significant difference in wing lengths or weights of males or females between temperatures. There was a significant effect of temperature on development time for males and females, with much shorter development times at the highest temperatures (Fig. 2: females: GLM: $F_{2,9} = 27.94$, $P < 0.0001$; males: $F_{2,9} = 119.42$, $P < 0.0001$), as expected from temperature–growth rules.

Larval Behaviors: Time. Time spent by larvae in a given behavior was affected by both temperature and nutrients. Larvae spent more time hanging from the surface at moderate temperatures relative to low temperatures (Fig. 3A; $F_{2,18} = 5.55$; $P = 0.01329$); however, larvae did not spend more time actively foraging at cool temperatures (Fig. 4; $F_{2,18} = 3.01$; $P = 0.0743$). Larvae spent more time actively foraging (Fig. 3B; $F_{1,18} = 8.83$; $P = 0.0082$) and less time feeding from the surface ($F_{1,18} = 14.61$; $P = 0.0012$) in low nutrient treatments relative to high nutrient treatments. No other behavioral categories were significantly different between nutrient levels or temperature.

Larval Behaviors: Occurrences. Overall, there were no differences in the total number of all behavioral occurrences across nutrient and temperature treatments, such that there were not simply more changes in behavior at a given set of conditions. However, the number of times a larva engaged in a certain behavior was affected by nutrients and temperature. The number of times a larva engaged in either hanging from the surface or feeding on the surface was significantly higher in high nutrient conditions relative to low nutrient conditions (Fig. 4A; hanging: $F_{1,18} = 4.93$, $P = 0.0395$; surface feeding: $F_{1,18} = 13.73$, $P = 0.0016$). Temperature affected the number of times a larva engaged in active feeding, with both high and low temperatures associated with more occurrences of vacuuming behavior (Fig. 4B; $F_{2,18} = 3.84$; $P = 0.041$). The number of occurrences of other behaviors was not significantly affected by nutrient or temperature variation.

Larval Aggregation. Comparing the NND of all larvae to a random distribution showed significant deviation from random, with larvae having a lower than expected distance, suggesting aggregation (Fig. 5A). In 10 individual replicates, larvae were significantly closer to each other than expected by a random distribution (with z scores <−2; Fig. 5B). There was variation in the degree of aggregation across all replicates which differed from a random distribution, but no discernible pattern with regards to temperatures or nutrient levels (Fig. 5B).

Discussion

We found support for our hypothesis that both the nutritive environment and thermal environment affect larval behavior. The behavioral response to nutrients was consistent with previously described effects on mosquito larvae, with low- or poor-quality nutrients

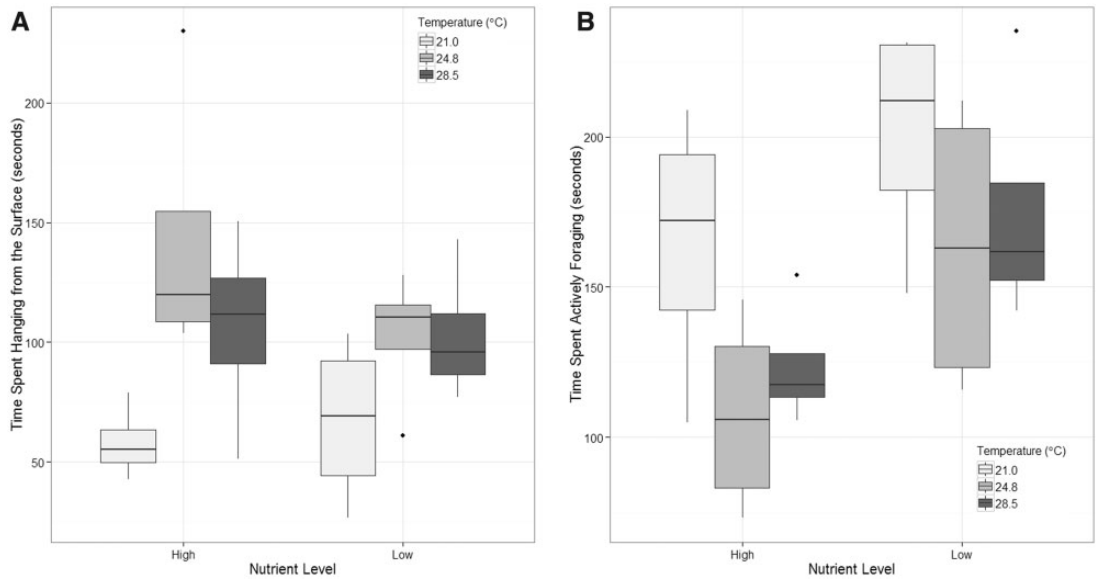

Fig. 3 (A) Box plot of the cumulative time spent hanging from the surface by nutrient level (x-axis) and temperature (gray scale of boxes). Y-axis is in seconds. (B) Box plot of the cumulative time spent actively foraging (vacuuming) by nutrient level (x-axis) and temperature (gray scale of boxes). Y-axis is in seconds. Outliers are represented as solid dots.

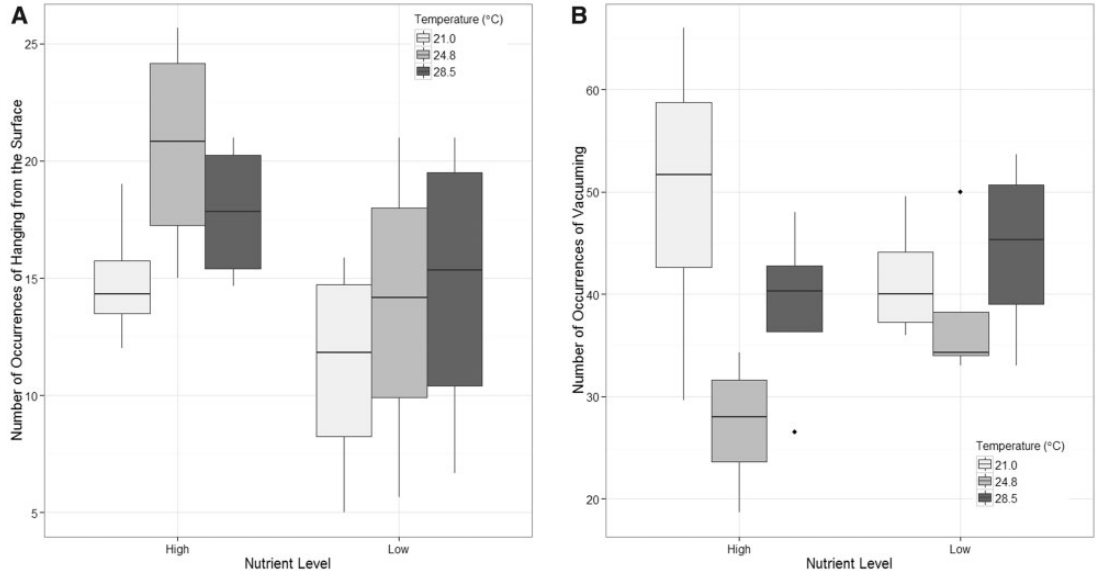

Fig. 4 (A) Box plot of number of occurrences of hanging from the surface and (B) box plot of number of occurrences of vacuuming. Outliers are represented as solid dots.

associated with more active foraging (Workman and Walton 2003, de Valdez 2006, Phelan and Roitberg 2013, Skiff and Yee 2014). This fits into a wider, theoretical response that animals will respond to poor nutritive environments by increasing their foraging effort (Anholt et al. 2000). As we only assessed two levels of nutrition, and both were derived from infusion without any solid substrate, we can only assess whether foraging effort changes between a high and low nutrient environments, and do not know how different nutrients or substrates may affect behavior (Kesavaraju et al. 2007, Skiff and Yee 2014). However, our nutritive conditions were quite different from one another, as evidenced by the dramatic differences in survival to adulthood.

Our temperature conditions mimicked those observed for *A. aegypti* in the field (Padmanabha et al. 2010), and represent a large range of mean temperatures (21.9–28.5°C) and an even larger range of temperatures experienced (due to diurnal fluctuations). Despite this temperature variation, we did not see

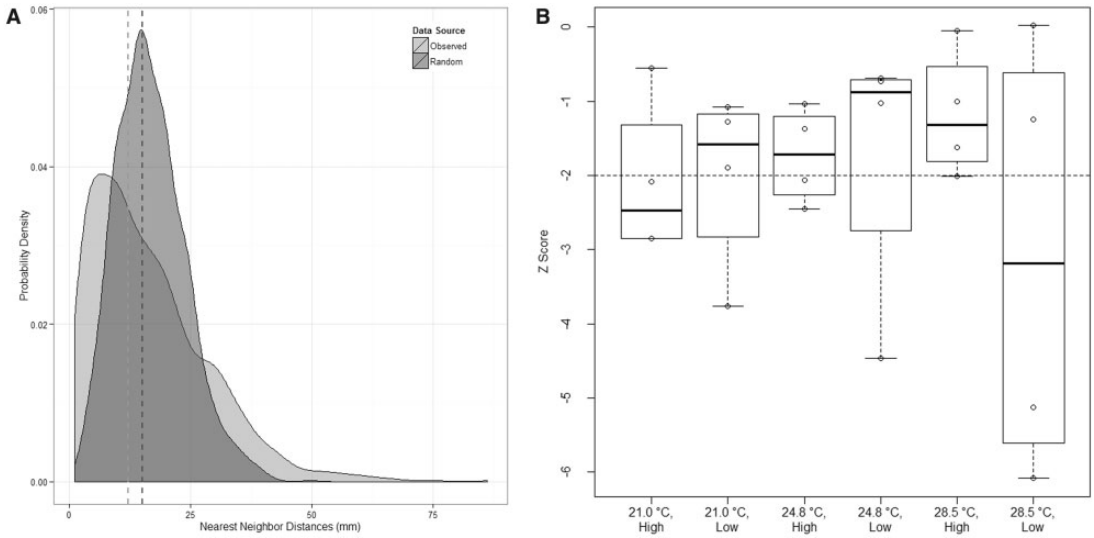

Fig. 5. Aggregation of larvae relative to random: (A) Individuals (the dashed lines mark the means for each distribution) and (B) *z*-scores, by container/replicate (±2 is considered significantly different from random in this plot).

significant differences in wing lengths between temperatures. Nevertheless, many other studies have shown a clear temperature-size effect in *Aedes* mosquitoes across the temperature range we examined (Rueda et al. 1990, Briegel and Timmermann 2001, Padmanabha et al. 2012, Reiskind and Zarrabi 2012, Alto and Bettinardi 2013), and our lack of detection of adult size differences by temperature is likely because of small sample sizes, as we only compared size for the high nutrient levels. Variation in available nutrients may also obscure temperature size relationships in other insects, and the high level of nutrients may have allowed the mosquitoes to achieve maximal size even in the warmest temperature (Diamond and Kingsolver 2010). Furthermore, fluctuating temperatures may dampen the effects of temperature on life-history characters (Carrington et al. 2013). As expected, we did see a very strong effect of temperature on speed of development, with significant differences in developmental rate between temperatures.

The thermal environment did have a significant impact on behavior. We found patterns of behavior consistent with increased vacuuming at low temperatures, but did not find significant support for increased time in active foraging at high temperatures. However, we did see an increase in the number of times a larva vacuumed at both high and low temperatures. Taken as a whole, there is evidence for increased foraging effort at both cool and hot conditions, contrary to our prediction that we would only see increased activity at high temperatures. Indeed, there was stronger support for increased activity at relatively low temperatures than at high temperatures. Although not well-studied in the mosquito literature, the only other study to examine temperature on foraging behavior in a culicid did not detect a temperature effect on diving in *Anopheles gambiae* (Phelan and Roitberg 2014).

The temperature size rule generally predicts larger adult body size at low temperatures for ectotherms. However, dynamic energy models suggest that large bodies require a greater input of nutrients for both growth and maintenance (Padmanabha et al. 2012). This may be somewhat balanced by slower metabolic rates, but a larger body demands increased caloric intake. Likewise, warmer temperatures generally result in smaller adult insects, but may also incur a need for a high caloric intake because of rapid metabolism. Although we did not observe significant differences in measurements of body size, our behavioral observations fit implicit predictions from dynamic energy models and suggest that behavioral choices are indicative of how temperature affects the demands on the organism. Higher foraging activity at both low nutrients and extreme temperatures demonstrate the potential costs to larvae, either in an inability to accumulate sufficient nutrients to pupate, lessened reserves at emergence, or increased risk of depredation during the larval phase.

Although there is strong evidence for size being an important determinant of fecundity and that cooler temperatures may result in more fit individuals (Atkinson 1995, Briegel and Timmerman 2001, Kingsolver and Huey 2008), there is some evidence that temperature induced large size may have costs for adults (Zamudio et al. 1995). The fact that larvae choose a moderate to warm developmental temperature when a gradient exists suggests the large adult size derived from slower growth at cool temperatures is not a favored strategy (Omardeen 1957, Linley and Evans 1971). The increased foraging effort we describe may point to a source of costs of development at cool temperatures. The larger sizes these mosquitoes usually attain when reared in cool larval environments may require more inputs, forcing larvae to spend more time and energy foraging (Padmanabha et al. 2011). This

may result in lower reserves at pupation (Padmanabha et al. 2012). Although not observed when nutrient conditions are good (e.g., Briegel and Timmerman 2001), other studies have noted lower body weights relative to wing size in *Aedes* mosquitoes (Reiskind and Zarrabi 2012), providing empirical support for this hypothesis. Further experiments to assess the connection between foraging behavior, nutrients, and temperature, with a focus on adult outcomes will help strengthen our observations.

We were not able to separate temperature effects on the microorganisms within the habitats from the direct effects of temperature on larval foraging. All natural larval habitats require a living microbial community to support larva, and only recently has there been any success in rearing mosquito larvae axenically (Coon et al. 2014). Therefore, it is possible that foraging activity is not driven solely by temperature but also by the microbial dynamics underlying different thermal conditions. We did make our observations based upon a physiological clock (when the first larvae in a replicate cohort attained fourth instar), which should somewhat control for dynamic levels of available nutrients prior to that life history stage.

Increased activity can also expose larval mosquitoes to higher predation risk (Juliano and Gravel 2002, Kesavaraju and Juliano 2004), although mortality due to predation was later at low temperatures for *Ochlerotatus triseriatus* depredated by *Toxorhynchites rutilus* (Coquillett) (Juliano 1996). If an *A. aegypti* larva encountered a predator at extreme temperatures, the increased foraging could increase the likelihood of depredation. Alternatively, if *Ae. aegypti* curb their foraging behavior in the presence of predators, temperature extremes may have a more pronounced effect on adult fitness by decreasing their ability to acquire sufficient nutrients for growth. Temperature can affect predators as well, making predictions about risk of predation at a given temperature difficult.

Aggregation of mosquito larvae is well-known in open habitats (e.g., ponds, large pools, etc.; Nielson and Nielson 1953, Service 1985, Wallace and Merritt 2004). In more constrained settings, *Culex erythrothorax* (Dyar) has been shown to aggregate more than other *Culex* species (Workman and Walton 2003). Our results, demonstrating a more aggregated distribution than random, suggest *A. aegypti* may aggregate, even in small containers. Aggregation did not vary with nutrient level or temperature. Some previous studies have shown *Aedes* mosquitoes may aggregate at this scale by feeding preferentially on a certain substrate (Kesavaraju et al. 2007), but we find this unlikely in our system as we provide no substrate besides the uniform container walls and floor. It is possible that biofilms develop on these surfaces in a patchy manner, and aggregation represents preferential browsing in these areas. Furthermore, aggregation may increase foraging efficiency, by increasing the availability of particles in a local area (Merritt et al. 1992).

Aedes aegypti larval foraging behavior and growth are important components in mathematical models of disease transmission (Focks et al. 1995, Magori et al. 2009). However, our understanding of how behavior influences growth in response to environmental variation remains weak. Our results empirically demonstrate how larval mosquitoes may adjust foraging tactics in different nutritive and thermal environments, and provide support for a flexible, dynamic set of strategies responsive to variation encountered in nature. Further investigations should directly tie foraging strategies under these variable conditions to measureable adult outcomes and important epidemiological considerations, like vectorial capacity (Murdock et al. 2013, Moller-Jacobs et al. 2014).

Acknowledgments

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