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Sugar intake interacts with temperature to influence reproduction and immunity in adult mosquitoes

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Draft

Abstract

Disease transmission by insect vectors will depend on integrated physiological responses to interacting environmental variables. We explored how interactions between temperature and sucrose concentration affected immunity and fecundity, two variables that contribute to vectorial capacity, in the mosquito, *Culex pipiens* (Linnaeus, 1758). We provided female *C. pipiens* with either 2 % or 20 % sucrose and exposed them to low (22 °C), moderate (25 °C) or high (30 °C) temperatures for 8 d. We then measured the strength of the melanisation response in one subpopulation of females, and the number of eggs laid as a measure of fecundity in another subpopulation. Temperature interacted with diet to weaken immunity under 2 % sucrose at 22°C and 25°C. This effect disappeared at 30°C, suggesting that high temperatures allowed mosquitoes to compensate for the effects of decreased sucrose. Conversely, increasing temperature increased egg production on a diet of 20 % sucrose, but heat exposure on a diet of 2 % sucrose decreased fecundity. Overall, we suggest that heat exposure requires investment in thermal protection, which may prompt reconfiguration of the immune system and/or decreased investment in reproduction. Thus, our understanding of the effects of climate change rest on which physiological system we measure, under which combinations of stressors.

Key words: vector, insect, multiple stressors, heat, *Culex pipiens*, mosquitoes, diet

Introduction

The incidence and severity of disease is changing as the climate changes, largely due to alterations in the physiology of both hosts and parasites under new environmental conditions (Harvell et al. 2002; Rohr and Raffel 2010; Parham et al. 2015). These environmental changes

encompass shifts in multiple, interacting stressors that require simultaneous responses by several physiological systems. On a mechanistic level, these physiological responses may compete for resources, or act synergistically through mechanisms such as cross-tolerance or cross-talk (Sinclair et al. 2013; Kaunisto et al. 2016). Therefore, the phenotype of an animal exposed to multiple stressors is often different than the phenotype after exposure to isolated stressors (Kaunisto et al. 2016). These differences in phenotype have important implications for how we understand environment-driven changes in physiology, and our ability to predict population-level effects of phenomena such as disease occurrence and transmission (Rohr and Palmer 2013; Todgham and Stillman 2013). However, despite this importance, our understanding of how multiple stressors interact to affect physiology is limited (Todgham and Stillman 2013), especially in animals such as insects (Kaunisto et al. 2016). Thus, we are inhibited from making critical predictions about climate change, such as variation in the transmission of human and wildlife diseases by vector insects (e.g., mosquitoes), until we further understand the impact of interacting stressors.

Mosquitoes are important vectors of human and wildlife pathogens, and their ability to vector disease is influenced by the effects of environmental stressors and climate change on their physiology (Harvell et al. 2002; Murdock et al. 2012a; Murdock et al. 2013; Parham et al. 2015). Increased temperature can increase the efficacy of the immune response (Suwanchaichinda and Paskewitz 1998), thereby influencing the ability to respond to and carry a pathogen or parasite. Conversely, immune performance can decrease at higher temperatures due to trade-offs with other physiological systems or other constraints that contribute to the shape of the thermal performance curve (Murdock et al. 2012b). Furthermore, environmental stress experienced

during larval stages can carry over into the adult stages (Muturi et al. 2011). For example, decreased nutrient availability as larvae result in smaller adults that are less fecund and less capable vectors (Moller-Jacobs et al. 2014). However, although there is ample evidence that stressors such as temperature and nutrient availability individually influence mosquito physiology, fitness, and vectorial capacities, we know little about the interaction of related stressors, particularly in the adult stage.

Adult female mosquitoes require blood to produce eggs, but also feed on sugar to provide energy for flight and maintenance (Foster 1995); therefore, the availability of sugar can influence physiology and vectorial capacity of these insects (Gary and Foster 2006). Decreased sugar availability may reduce longevity and fecundity (Foster 1995), but also increase the frequency of blood-feeding and thus overall lifetime reproductive success (Gary and Foster 2001). Sugar availability is likely to change with climate change, as elevated temperatures and drought affect plant physiology and nectar availability. For example, temporal mismatches with host plants may occur (Memmott et al. 2007), or decreases in the available volume of nectar in a plant (Carroll et al. 2001). Because drought is often interconnected with increased temperatures, we focus on the interaction between changes in temperature and sucrose concentration as an example of the effects of interacting stressors.

The ability to respond to multiple stressors depends on the interaction between physiological systems, through mechanisms such as cross-talk and cross-tolerance, as well as trade-offs between systems (Sinclair et al. 2013). Due to these interactions, not all physiological systems will respond in the same direction to the same stressors. For example, cold acclimation

allows crickets to better respond to cold stress but decreases their ability to respond to pathogens at low temperatures, through a putative trade-off between immunity and the response to cold (Ferguson et al. 2016). Thus, it is pertinent to examine the response of multiple physiological systems when examining the effects of multiple stressors on the ability for an animal to survive under new environmental conditions.

To understand how multiple stressors interact to influence mosquito fitness and ability to transmit disease, we exposed adult *Culex pipiens* (Linnaeus, 1758) mosquitoes to combined temperature and nutrient levels and measured the outcome on immune activity and fecundity. Immune activity, such as encapsulation and melanisation responses, can be used as a proxy for resistance to parasites, and changes in parasite resistance alter a mosquito's vectorial capacity (Murdock et al. 2012b; Murdock et al. 2013). We find that high temperatures alone are beneficial for fecundity, but that when combined with decreased sugar availability, reproduction suffers. In contrast, immunity is stronger under a combination of high temperature exposure and decreased sugar availability. Due to technical constraints, we used two different sources, and thus subpopulations, of *C. pipiens* females. This discrepancy in populations prevents us from interpreting any interaction between fecundity and immunity within an individual. However, we are able to determine how multiple environmental stressors interact to influence a single trait, and provide insight into the complexity of measuring only one trait within one subpopulation.

Methods

Mosquito colony maintenance

We obtained *Culex pipiens* mosquitoes from Carolina Biological Supply (Burlington, North Carolina, USA) from a long-standing colony maintained for many years at room temperature (approx. 22 °C). These mosquitoes were used to test the immune response of *C. pipiens*.

However, during the course of this study, this culture was discontinued and we subsequently sourced this species from the laboratory of Dr. Carl Lowenberger at Simon Fraser University (Burnaby, BC, Canada). Mosquitoes from this second source were used for all fecundity experiments.

We reared larvae in geothermal water on a diet of Nutrafin Max Tropical Fish Flakes and Fleischmann's active dry yeast. Upon emergence, we maintained adult mosquitoes at 25 ± 2 °C, 60 % humidity and 14 L:10 D light cycle in 30 cm × 30 cm × 30 cm Plexiglas cages in the Weston Animal Care Facility at Acadia University. For general colony maintenance, we provided adults with a Kimwipe soaked in 10 % sucrose solution.

Exposure to changes in temperature and sucrose concentration

Four days following emergence, we provided adult, female mosquitoes with a blood meal from the arm of a human volunteer to allow mosquitoes to undergo one gonotrophic cycle before exposure to changes in temperature and sucrose concentration. We allowed this one gonotrophic cycle to pass because females may rely heavily on larval lipid reserves for egg production in the first gonotrophic cycle (Zhou et al. 2004), and we wanted to avoid masking the effects of decreased sugar. Following this first blood meal, we haphazardly divided the mosquitoes into

two cohorts and provided each with either a 2 % or 20 % sucrose solution. We allowed mosquitoes to lay their eggs, and removed these eggs for colony maintenance. At 24 *h* after oviposition was completed, we further divided the mosquitoes in each sucrose cohort into individual cages at one of three temperatures: 22 °C, 25 °C or 30 °C for a total of six treatment groups (exposure to one of three temperatures combined with either a diet of 2 % or 20 % sucrose). We maintained mosquitoes at these temperatures and sucrose concentrations for 8 *d* and then proceeded to measure the strength of the immune response or fecundity in the second gonotrophic cycle.

Experiment 1: Strength of the immune response

To measure the strength of the immune response of *C. pipiens*, we followed Suwanchaichinda and Paskewitz (1998). Briefly, we dyed A-25 Sephadex bead beads with a solution of Congo Red (1 g/L) for 10 min to allow visualization of the beads following injection. We washed the beads four times in mosquito saline (1.3 mM NaCl, 0.5mM KCl, 0.2mM CaCl₂) to remove residual Congo Red and maintained the beads in solution to prevent desiccation. We anaesthetized individual mosquitoes using CO₂ and positioned them on a mesh grid to expose the thorax. We used a pulled glass capillary tube with a fine, sharp tip (approximately 50 µM) and injected mosquitoes with one A-25 Sephadex bead each (mean diameter = 50 µM) by gently blowing air, by mouth, into an aspirator attached to the glass capillary tube. Following 24 *h* of implantation of the bead at the respective combinations of sugar and temperature, we anaesthetized mosquitoes using CO₂, removed the wings for later measurement, pulled back the cuticle at the thorax using fine tweezers, and gently removed the bead by prodding it loose from tissue with an insect pin to prevent any disturbance of the melanisation. We photographed each

bead using at 100× magnification using a Nikon camera (Nikon Instruments Inc. Melville, NY, USA) attached to a light microscope. We quantified the strength of the melanisation response following Ferguson et al. (2016). Briefly, we calculated the intensity of the melanin as a grey value using Image J (Rasband 1997).

Experiment 2: Fecundity

At dusk during the light:dark cycle in the insectary, we grouped mosquitoes from each temperature treatment in large, Plexiglas cages and provided the forearm of one of three female human volunteers on which mosquitoes could feed on blood. Once the abdomen of the mosquito was fully distended with blood, we transferred individual mosquitoes to individual plastic cages containing a small dish of geothermal water for oviposition. We returned the mosquitoes to their experimental temperature and sucrose conditions for 24 h, following which we returned mosquitoes to 25 °C and 10 % sucrose until they completed oviposition.

Following oviposition, we anaesthetized mosquitoes ($n = 4-6$ per treatment) using CO₂, and removed the wings for later measurement. We then counted and recorded the number of eggs in each raft. We randomly selected ten eggs from each raft, photographed each egg, and measured the length, width, and cross-sectional area using ImageJ.

Statistical analyses

We compared the darkness of the encapsulation capsule (i.e. grey values) between sucrose cohorts across temperature using ANOVA in R (Angilletta 2006). We log-transformed the grey values to improve normality, and assessed the assumptions of ANOVA by plotting residuals

against fitted values to confirm homogeneity of variance, and standardised residuals against theoretical quantiles to assess normality (Crawley 2007). We used polynomial contrasts (Lenth 2013) to compare means between 2 % and 20 % sucrose at each temperature.

As egg number is positively correlated with body size (Blackmore and Lord 2000), we confirmed this assumption using a Pearson's Rank Correlation to test the strength of this correlation in our study. We then corrected the number of eggs produced by each mosquito by body size, as estimated from wing length (Armbruster and Hutchison 2002). We then compared the number and mean size of eggs (i.e. the mean of ten eggs per raft) between sucrose cohorts across temperature using the same methods we used when comparing melanisation. We used the source of blood (i.e., from two different volunteers) as a covariate, as well as wing size as a proxy for body size as a covariate when comparing egg sizes. We used Pearson's Rank Correlation to test for any relationship between egg size and the number of eggs produced. All analyses were performed in R.

Results

Experiment 1: Strength of the immune response

We did not detect any effect of temperature on immunity (Figure 1; $F_{2,61} = 0.69$, $p = 0.51$) or any interaction between sucrose and temperature ($F_{2,61} = 0.83$, $p = 0.44$); however, sucrose had a significant effect on immunity (Figure 1; $F_{1,61} = 1.63$, $p = 0.01$), where mosquitoes on a diet of 2 % sucrose had a weaker melanisation response at 22 °C ($p = 0.03$) and 25 °C ($p = 0.05$) than mosquitoes on a diet of 20 % sucrose. We did not detect an effect of wing size on the melanisation response ($F_{1,61} = 0.02$, $p = 0.89$).

Experiment 2: Fecundity

Wing size was positively correlated with number of eggs produced (Pearson's Rank Correlation: $t_{26} = 2.24$, $p = 0.03$; $r = 0.42$) and had a significant effect on the number of eggs produced (ANOVA; $F_{1,22} = 5.05$, $p = 0.03$). Therefore, to help discern the effect of adult diet on eggs without body size confounding these effects, we corrected the number of eggs produced by an individual by its wing size. Temperature had a significant effect on the number of eggs produced by mosquitoes (Figure 2A; $F_{2,22} = 7.06$, $p < 0.01$) and interacted with sucrose to affect fecundity ($F_{2,22} = 3.90$, $p = 0.03$), such that mosquitoes at high temperatures (30 °C) produced fewer eggs when provided with only 2 % sucrose compared to 20 % sucrose (Figure 2A; $p = 0.05$). Egg sizes (length, width, area) were all unaffected by either temperature or sucrose concentration (Table 1; Figure 2B,C,D) and neither wing size nor blood meal source affected egg size (Table 1). Egg number was not correlated egg length ($t_{26} = 0.59$, $p = 0.56$, $r = 0.11$), width ($t_{26} = -1.16$, $p = 0.26$, $r = -0.22$) or total area ($t_{26} = -0.35$, $p = 0.73$, $r = -0.07$).

Discussion

The future of disease transmission, and human and wildlife health, hinges on how temperature will interact with other environmental variables to affect mosquito populations and their vectorial capacities. Temperature may interact with nutrient availability (e.g., sugar) outside of blood meals for mosquitoes, thereby affecting mosquito physiology. We demonstrate that the encapsulation ability of adult *Culex pipiens* was weakened when mosquitoes were restricted to a lower sucrose concentration between blood meals; however, this effect of sucrose concentration was only detected at temperatures below 30°C. Sucrose concentration also interacted with

temperature to affect fecundity: *C. pipiens* laid fewer eggs on 2 % sucrose, but only at 30 °C.

Together, these data suggest that it was the interaction between temperature and nutrient availability that determined both fecundity and immunity, which will have cascading effects on the vectorial capacity of mosquitoes.

Increasing temperature increased egg production in *C. pipiens*, except when food was limited under the hottest conditions (30 °C). Similarly, *Gryllus texensis* (Cade and Otte 2000) increase egg production at higher temperatures when food is abundant, but decrease fecundity when food limited under the same thermal environment (Adamo et al. 2012). This suggests that physiological consequence of a single stressor, such as the energy expenditure required for thermal protection (Feder and Hoffmann 1999), is only apparent when an additional stressor is present (e.g. nutrient limitation). Thus, our interpretation of the impact of a stressful event is limited without understanding it in the context of multiple stressors. Understanding mechanisms underlying responses to single stressors may help us to better predict the outcomes of combined stressors on insect physiology (Kaunisto et al. 2016), and any population-level changes in mosquito abundance arising from stressor-induced changes in fecundity.

In contrast to what we might expect from reproduction, immunity improved in *C. pipiens* at 30 °C under food-limited conditions. It is not unusual for insect immunity to improve at higher temperatures (Adamo and Lovett 2011; Wojda 2017); however, paradoxically, we only observed this increase under nutrient limitation. The insect immune system has the capacity to compensate for decreased resource availability during a stress response (Adamo et al. 2016; Adamo 2017), which may account for this shift in immune function under combined heat and nutrient stress.

For example, food-limited *Manduca sexta* enhance constitutive immune defences while decreasing investment in inducible immune defences (Adamo et al. 2016). Further, this type of reconfiguration can also occur in response to thermal stress (Salehipour-Shirazi et al. 2016). Here we suggest that a combination of stressors (e.g. heat and nutrient limitation) may trigger a pattern of reconfiguration of immunity that is absent or unobserved under only one stressor in isolation.

The effects of combined nutrient and temperature stress have seemingly juxtaposed effects on immunity and fecundity. Principally, combined stress was detrimental to fecundity, but beneficial to immunity. Thus, although combined stressors (e.g., temperature, nutrition) may be synergistic for one physiological response (e.g. immunity), they may be antagonistic for another (e.g. reproduction), which indicates that we must be cautious in extrapolating stress responses across physiological systems. Further, we must also be cautious in how we extrapolate stress responses across populations of a species. In this study, we used (out of necessity) two different populations to measure separate physiological responses. These two populations likely produce genotype $\times$ environment interactions (e.g. Lazzaro et al. 2008) that limit how we interpret any interaction between immunity and fecundity. However, we do note that these combined stressors had a significant impact on fitness-related traits in both populations, which indicates that heat and nutrient availability are likely to affect vectorial capacity when combined, regardless of which system they impact in a population. Overall, our interpretation of what environmental conditions are stressful appears plastic, and depends on how environmental conditions interact with each other, and likely the genotype of the animal.

Immunity is thermally sensitive in mosquitoes (Suwanchaichinda and Paskewitz 1998; Murdock et al. 2012b); however, we were unable to detect an effect of temperature on the melanisation response in *C. pipiens*. It is possible that the immune system acclimated to each temperature treatment (Ferguson et al. 2016), which optimised immune activity in each group and masked any effect of temperature on the melanisation response. Conversely, the thermal performance of immunity in *C. pipiens* may be fairly broad, regardless of the different temperature treatments. We must also bear in mind that pathogen physiology will also be influenced by the environment, and that this will impact the outcome of the host-pathogen interaction (Paull et al. 2015; Altman et al. 2016). For example, dissemination of West Nile Virus is higher at 30 °C in *C. pipiens* (Dohm et al. 2002) which suggests that the strength of immune response does not always correlate with survival or transmission of infection. Therefore, it will be essential to move towards including live pathogens in studies of multiple stressors.

Conclusions

Under low nutrient conditions, high temperatures may lead to fewer *C. pipiens* in at least some populations, but individuals with stronger immune responses in others, which may contribute to decreased vectoring of disease in either case. If sugar availability is high, then increased temperatures with climate change may have little effect on populations of *C. pipiens*. Overall, we suggest that multiple stressors are an essential element of understanding changes in vectorial capacity, and that our interpretations of what an animal experiences as “stress” must be couched in the context of a complex environment.

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Figure Captions

Figure 1. The strength of the melanisation response in female *Culex pipiens* exposed to high (20 %) or low (2 %) sucrose and one of three temperatures (22°C, 25°C, 30°C). Melanisation is expressed as the log of the reverse grey value, such that higher values indicate a stronger immune response against a Sephadex bead injected into the thorax of the mosquito (sample sizes for treatment group of 2 % sucrose: $n = 15$ at 22 °C, $n = 16$ at 25 °C, $n = 8$ at 30 °C. For treatment group of 20 % sucrose: $n = 15$ at 22 °C, $n = 14$ at 25 °C; $n = 9$ at 30 °C). Error bars indicate SEM and asterisks indicate significant differences between groups fed on different sucrose concentrations at each temperatures. Differences between groups were analysed with ANOVA followed by polynomial contrasts.

Figure 2. Total number and size of eggs produced by female *Culex pipiens* exposed to high (20%) or low (2%) sucrose and one of three temperatures (22°C, 25°C, 30°C). A. Total number of eggs. Total number of eggs are corrected for wing size, as a proxy for body size. B. Egg length. C. Egg width. D. Total area of eggs calculated from length and width. Sample sizes for treatment group of 2 % sucrose: $n = 5$ at 22 °C, $n = 4$ at 25 °C, $n = 5$ at 30 °C. For treatment group of 20 % sucrose: $n = 4$ at 22 °C, $n = 6$ at 25 °C; $n = 4$ at 30 °C). Error bars indicate SEM and asterisks indicate significant differences between groups fed on different sucrose concentrations at each temperatures. Differences between groups were analysed with ANOVA.

Table 1. Statistical results from ANOVA comparing the effects of temperature and sugar on the sizes of eggs produced by *Culex pipiens* (Linnaeus, 1758) females following a blood meal.

Variable	Egg area	Egg length	Egg width
Temperature	$F_{1,22} = 0.003, p = 0.95$	$F_{1,22} = 0.74, p = 0.74$	$F_{1,22} = 0.25, p = 0.62$
Sugar	$F_{1,22} = 3.92, p = 0.06$	$F_{1,22} = 2.24, p = 0.15$	$F_{1,22} = 3.62, p = 0.07$
Temperature $\times$ Sugar	$F_{1,22} = 0.12, p = 0.91$	$F_{1,22} = 3.15, p = 0.65$	$F_{1,22} = 0.16, p = 0.69$
Wing size	$F_{1,22} = 1.44, p = 0.24$	$F_{1,22} = 1.20, p = 0.28$	$F_{1,22} = 0.54, p = 0.47$
Blood meal source	$F_{1,22} = 3.32, p = 0.08$	$F_{1,22} = 3.15, p = 0.09$	$F_{1,22} = 1.38, p = 0.25$

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2 %
20 %

Log (grey value)

7.0
6.5
6.0
5.5
0

22

25

30

Temperature °C