Assessing Thermal Tolerance of Infected and Uninfected *Bombus impatiens* to a Variable Thermal Environment

by

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ABSTRACT

In insect species such as the common eastern bumble bee, *Bombus impatiens,* the relationships between body size, temperature, and disease are closely intertwined. Given the significant selection pressure that the latter two of these stressors impose, it is critically important to understand how climate change will impact species who carry out critical ecosystem services as mean temperatures and thermal variability increase. This work assesses the ecophysiology of *B. impatiens* in a simulated heatwave. It compares the thermoregulation, behavior, and metabolic rate of bumble bees when uninfected versus infected with a sublethal intestinal trypanosome, *Crithidia bombi*. Using a thermally controlled chamber, I measured thoracic body temperature, behavior, and respiration rates in infected and uninfected *B. impatiens* workers from 24 to 36 ºC. I found a significant interactive effect of infection status and body size on thermoregulation, with infection masking the side-mediated effects seen in uninfected bees. The likelihood of an individual fanning– a physiological adaptation to heat stress– increased at higher temperatures regardless of infection. Finally, the respiration rate of infected individuals demonstrated a negative relationship with infection intensity. Obtaining this fine-scale understanding of interacting stressors will provide insight on outcomes for important pollinators with respect to global climate change.

INTRODUCTION

Bumble bees (*Bombus* spp. [Hymenoptera: Apidae]) are critically important pollinators of wild plants and agricultural crops across the Northern Hemisphere (Goulson et al., 2008). Bumble bee populations are threatened by a number of anthropogenic stressors such as habitat loss, pesticides, and climate change (Goulson et al., 2008; Tosi et al., 2022; Vasiliev and Greenwood, 2021). Many aspects of climate change contribute to pollinator decline, including changes in surface temperature, increasing magnitude and frequency of extreme weather events, and changes in precipitation patterns (Vasiliev and Greenwood, 2021). In response to the rapidly changing climate, bumble bee species have shifted in latitudinal range and elevation in order to survive (Chen et al., 2011; Kerr et al., 2015). However, bees can only shift their range so much before reaching their critical thermal limits (CT_{min} and CT_{max}); the minimum and maximum environmental temperatures at which an organism can maintain muscle control (Oyen and Dillon, 2018). Thus, it is important to understand the physiological effects of thermal stress on bumble bee species as global temperatures continue to rise and increase in variance.

Bumble bees exhibit a great deal of interspecific trait variation. For example, body size can vary by up to twenty-fold between species (Van Wyk et al., 2021). Furthermore, intraspecific worker size variation is extremely species-specific. In *Bombus impatiens* (Cresson, 1863), body size can vary by up to ten-fold within a colony, thus making this species a useful model system for studying the interactions between body size and other environmental stressors (Alford, 1975; Cumber, 1949). As bumble bees populations across North America continue to decline, understanding how responses to disease and thermal stress are mediated by body size is critically important given the ecological relevance of these species (Cameron et al., 2011).

In addition to anthropogenic stressors, bumble bees also face stress from disease. The pathogen *Crithidia bombi* (Kinetoplastea: Trypanosomatidae; hereafter referred to as *Crithidia*) is an intestinal trypanosome that infects *Bombus* species globally (Salathé and Schmid-Hempel, 2011). Bumble bees infected with *Crithidia* display deficiencies in cognition and foraging efficiency, as well as lowered individual lifespan and reproductive potential (Brown et al., 2000; Gegear et al., 2006; Shykoff and Schmid-Hempel, 1991b). *Crithidia* prevalence in a community– which can reach up to 80%– is influenced by a number of factors such as genetic composition of the host population and seasonality (Gillespie, 2010; Shykoff and Schmid-Hempel, 1991a). Host populations with low genetic diversity experience higher *Crithidia* prevalence than populations with high genetic diversity (Whitehorn et al., 2010). Furthermore, *Crithidia* prevalence peaks in the middle of the foraging season (Popp et al., 2012). Horizontal *Crithidia* transmission occurs when a susceptible worker bee forages from a flower contaminated with the feces from an infected worker from another colony (Durrer and Schmid-Hempel, 1994). Given that workers infected with *Crithidia* spend more time on flowers and defecate more than uninfected workers, pathogen transmission is increased when there is more foraging activity (Figueroa et al., 2019). Furthermore, foraging workers bring pathogens from outside of the colony to their nest mates, facilitating transmission within intracolony contact networks, such as between workers and the brood (Folly et al., 2017; Otterstatter and Thomson, 2007).

In addition to varying greatly within and between species, body size also shapes how temperature impacts host-pathogen dynamics and how bees handle thermal stress. Intraspecific comparisons of bumble bee species have shown that larger bees can tolerate more extreme temperatures than smaller bees (Oyen et al., 2016). This difference could be the result of a thermal inertia driven lag between core body temperature and air temperature (Oyen et al.,

2016). Furthermore, there is a paradoxical relationship between body size and disease transmission dynamics. In specific regard to *Crithidia*, smaller bees experience higher infection intensity; however, larger bees experience significantly higher transmission rates (Van Wyk et al., 2021). This relationship may be explained by differences in physiology and behavior amongst different size classes: larger bees are more likely to forage and have larger fecal volumes (Van Wyk et al., 2021). Additionally, there are metabolic differences based on body size, where insects with a larger body mass exhibit higher metabolic rates (Ehnes, 2011). Thus, it is imperative that body size is considered in evaluations of environmental stressors on metabolic rate.

In bumble bee species, the division of labor amongst workers is critical for proper colony functioning (Michener, 1974). One crucial aspect of colony functioning is maintaining the brood at optimal temperatures and respiratory gas levels for larval development (Weidenmüller et al., 2002). When temperatures or carbon dioxide levels in the nest exceed optimal conditions, workers engage in fanning behavior for a cooling effect (Weidenmüller et al., 2002). Conversely, workers will engage in non-flight thermogenesis – the shivering of flight muscles to produce heat from their thorax– to incubate the brood if environmental temperatures are lower than what is optimal for brood development (Esch et al., 1991; Schultze-Motel, 1991). Brood maintenance and thermoregulation pose an energetic tradeoff to bumble bees. When ambient temperatures stray from the ideal range of 28 ºC to 32 ºC, brood maintenance decreases as more workers are diverted to thermoregulation tasks (Vogt, 1986). Thermoregulation of the brood is critical for colony success as suboptimal brood temperatures result in smaller-bodied offspring, which have decreased fitness as a result of reduced foraging ranges and poorer foraging performance compared to larger bees (Guirard et al., 2021).

Temperature and disease both exert significant selection pressure on insect species (Johnston and Bennett, 1996). Thus, it is imperative to explore how climate change impacts bumble bee ecophysiology and host-pathogen dynamics. The goal of my research is to assess the thermal tolerance of infected *Bombus impatiens* workers to a simulated heatwave. Specifically, I want to assess the body temperature, behavior, and respiration rates of *B. impatiens* across a temperature ramping regime while infected with *Crithidia*. Given that pathogen stress may take away from energetic demands such as thermoregulation, I hypothesize that bees infected with *Crithidia* will have body temperatures closer to ambient than healthy bees. Given that larger bees tolerate extreme temperatures better than smaller bees, I additionally expect to find an interaction between body size and infection status such that small, infected bees will exhibit the poorest thermoregulation. Furthermore, I hypothesize that thermoregulatory behavior will be impacted by ambient temperature such that the likelihood of moving will decrease with rising ambient temperatures and the likelihood of fanning will increase. Additionally, I hypothesize that pathogen stress will inhibit thermoregulatory behavior such that infected bees fan at lower rates than healthy bees. Finally, I hypothesize that infection poses a steeper metabolic cost on individuals, and thus respiration rates will be higher for sick bees. Obtaining this fine-scale understanding of interacting stressors using a model system will inform us of the outcomes for bumble bee species with respect to rising mean temperatures and increases in the incidence of heatwaves associated with global climate change.

MATERIALS AND METHODS

STUDY DESIGN

We sourced all bees in our experiment from three Natupol Excel hives purchased from Koppert Biological Systems (Howell MI, USA). We removed 20 workers from each parent colony and individually housed them in vials for a total of 60 replicates. To generate infected replicates, we counted live *Crithidia* cells from the hindgut of bees from infected source colonies maintained in the Adler lab at University of Massachusetts, Amherst. These source colonies were originally infected from wild-caught local *B. impatiens* foragers in 2014 (Hadley, Massachusetts, USA: 42.3639 N, -72.5677). To create the inoculum, we diluted the gut homogenate of source bees with aqueous Ringer's solution to a concentration of 1200 *Crithidia* cells/μL. Then, we added equal parts of 50% sucrose solution for a final inoculum of 600 cells/μL and 25% sucrose. After starving the bees for approximately one hour, we randomly selected 10 bees from each parent colony for a total of 30 bees to receive a 15 μL dose of inoculum, which we administered via pipette. To ensure that the inoculation process did not confound results, we sham inoculated the 30 uninfected replicates with 15 μL of equal parts Ringer's solution and sucrose for a final solution of 25% sucrose. Bees entered the experiment if they consumed the entire dose of inoculum. Following inoculation, we housed the bees in microcolonies according to parent colony and infection status for a total of 6 microcolonies. Each box contained a ball of pollen and a feeder with 30% sucrose solution. We kept the microcolonies in a temperature and humidity controlled thermal chamber at 23 ºC (lwh: 2.4 m x 1.5 m x 2 m; Kolpak EnvironAir, Springfield MA, USA). We confirmed the success of our inoculations by inoculating an additional ten bees and housing them in a separate thermal chamber for the duration of the experiment. These replicates remained at 23 ºC without experiencing any thermal stress.

To ensure that variable hair density would not influence body temperature readings during the experiment, we shaved the bees prior to beginning data collection. To do so, we transported the microcolonies to a 4.9 ºC refrigerator for 1.5 hours to anesthetize the bees. If the bees appeared to be fully awake despite their time in the refrigerator, we placed the microcolony in a –15 ºC freezer for two minutes. We randomly selected one individual at a time and used a scalpel to remove all hair from the intertegular region of the bee. We used small tweezers to hold the bee over an ice pack during this process, as the cold temperature helped to minimize movement and distress for the bee.

Once shaved, we individually housed each bee in preparation for the experiment. Each housing unit consisted of a deli cup with a mesh bottom and a microcentrifuge tube cap filled with pollen. The cup sat on top of two small petri dish feeders with wicks for 30% sucrose and deionized water, respectively. The deli cup lid had a circular hole cut in the center and a layer of polyethylene food wrap over top of it. This was important for taking body temperature readings, as the polyethylene food wrap has a high emissivity of approximately 0.93 $J/s/m^2$, meaning that the thermal camera could obtain more accurate body temperature readings through the wrap compared to through the plastic lid. We also placed a larger petri dish with holes drilled into the bottom on top of the lid to prevent potential escapes. After all 60 bees were successfully shaved and housed, we transported them back to the thermal chamber, where they remained at 23 ºC. In order to maintain humane conditions, we changed the overnight ambient temperature from 23 ºC to 27 ºC for the remainder of the experiment.

THERMOREGULATION AND BEHAVIOR

To assess how a variable thermal environment impacts thermoregulation in *B. impatiens*, we measured thoracic body temperature throughout a simulated heat wave. Beginning at 08:00, we set the temperature of the Kolpak thermal chamber to 24 ºC and then increased by 2 ºC every hour until the chamber reached 36 ºC, at which point we ramped it back down to 27 ºC, the overnight holding temperature, as soon as we finished data collection. To allow the bees to acclimate to the room temperature, we waited 10 minutes to begin measurements once the chamber hit the target temperature. To obtain the maximum body temperature of each replicate, we used a FLIROne Pro camera (Teledyne FLIR, Wilsonville OR, USA). We ensured that the red target on the camera, which indicates the warmest point on screen, was aimed at the shaved intertegular region of the bee. As the body temperature of the bees could be challenging to discern from their surroundings at higher temperatures, we held a damp paper towel underneath the housing units to improve the accuracy of the readings. To prevent bias based on the order in which we took measurements, we cycled through a pattern based on the replicate numbers (1-60) of the bees by beginning at 1 and increasing the start number by increments of 10 each hour. Similarly, we shuffled the placement of the replicates in the thermal chamber over the course of the experiment to avoid any random effects that could arise from room location.

To evaluate behavioral responses to thermal stress and pathogen infection, we observed the behavior of individuals during the body temperature data collection period. At each instance of body temperature observation, we also assessed if the bee was moving, stationary, incubating on the pollen cap, drinking sucrose, or drinking water. We separately evaluated whether or not each bee was fanning, as this is an important thermoregulatory behavior.

We performed this temperature ramping protocol for two days. Additionally, we collected control measurements at the beginning of the experiment. Here, the replicates remained at 23 ºC for the entire day, and we took body temperature measurements every two hours from 08:00 to 18:00.

RESPIRATION

To assess an additional physiological response of *B. impatiens* to a variable thermal environment, we measured the respiration of infected and uninfected bees across a temperature ramping regime using the PreSens Fibox 4 fiber optic oxygen sensor (Product Number: SP-PSt3-NAU-D5-YOP; PreSens Precision Sensing GmbH, Regensburg, Germany). For this experiment, we placed nine bees in air tight jars associated with the PreSens sensor, leaving a tenth empty as a control. We began with the thermal chamber set to 23 ºC. We manually input the temperature and pressure into the sensor. For the former, we entered the temperature of the thermal chamber. For the latter, we used the atmospheric pressure reading for the day of data collection according to the National Weather Service. These readings ranged from 1,006 to 1,028 mb throughout the experiment. The PreSens sensor enabled us to obtain the % O_2 in each sealed jar every ten minutes for one hour, at which point we ramped the thermal chamber to 30 ºC and 37 ºC. In between data collection periods as the chamber rose to the next target temperature, we replaced the lids of the jars with a mesh screen and secured them with a rubber band to return O_2 concentrations to atmospheric levels. Then, we used a pipette to feed each bee 20 µL of 30% sucrose solution to avoid distress or starvation. Upon completion of this data collection, we recorded the mass of the jars (g) with the bees in them, as well as the volume of the empty jars. Additionally, we used a 10 mL graduated cylinder to obtain the volume of the bees via water

displacement. To obtain higher replication, we performed the entire protocol for respiration data for ten additional rounds at a stable ambient temperature (27 ºC) in a temperature and humidity controlled chamber at the Adler lab at the University of Massachusetts, Amherst.

INFECTION INTENSITY

To quantify *Crithidia* prevalence in infected replicates, we anesthetized each bee using CO_2 and dissected the hindgut, excluding the honeycrop and rectum We then macerated and vortexed the hindgut in 300 µL of Ringer's solution. After waiting 4 hours for the supernatant to settle, we counted live *Crithidia* in a 0.02 µL subsample of a 10 µL aliquot on a hemocytometer at 400x magnification. After dissection, we collected the right forewing of each individual bee. If the right forewing was missing or damaged, we collected the left one instead. We taped each wing to white paper and produced a digital scan with a ruler for scale. Given that marginal cell length scales allometrically with body size, we then used these digital scans to measure the marginal cell length (mm) in ImageJ to use as a proxy for body size in statistical analyses (Nooten and Rehan, 2020).

POLLEN CONSUMPTION

Previous research has found that host-pathogen dynamics are impacted by host nutrition status, specifically that pollen-starved bees have lower pathogen loads (Logan et al., 2005). Thus, to understand resource consumption during periods of thermal stress and pathogen infection, we measured pollen consumption over the course of the experiment. We measured the pollen-filled microcentrifuge tube caps (g) at the beginning and end of the temperature ramping experiment. If the pollen had to be replaced during the experiment or if the bee died before the end of the

experiment, the associated pollen consumption data was not included for analysis. Due to the high proportion of replicates whose pollen consumption data had to be excluded (n = 25/60), pollen consumption was not included in any analyses.

STATISTICAL METHODS

All statistical analyses were performed in R ver. 2023.03.1+446 (R Core Team, 2023). Statistical models were built using linear and linear mixed effects models (lme4::lmer; Bates et al., 2015) and the fixed effects were evaluated using Type II Wald χ^2 tests (car::Anova; Fox and Weisburg, 2019). We assessed variance inflation factors (VIFs) between all predictors with a cutoff value of five (car::vif; Fox and Weisburg, 2019). VIF values indicated low collinearity (VIF $\leq$ 1.09 for all variables). The best fit model was chosen via information criterion evaluation (bbmle::ICtab; Bolker and R Development Core Team, 2023).

I used a linear mixed effects model to assess the impacts of heat and pathogen stress on individual thermal tolerance. This model used thermoregulation (calculated as difference between core thoracic body temperature and ambient temperature (ºC)) as the response variable, ambient temperature and infection treatment as categorical fixed effects and as an interaction, body size as a covariate and parent colony and day of experiment as random effects. For the behavioral analysis, I employed a bottom-up modeling approach to generate the best fit models for the likelihood of observing moving and fanning behaviors. Including body size in the model resulted in overfitting such that all linear mixed effects models were singular. To create a model that still addressed my hypothesis, I removed random effects from my analyses and employed linear models instead. For each model, I performed a binary logistic regression using moving and

fanning vectors as binary response variables and ambient temperature and infection treatment as categorical fixed effects.

Finally, to assess my hypothesis on metabolic demands, I calculated respiration rate as milligrams of O_2 per second per gram of body mass. To calculate the body size in grams based on wing size, I calculated a linear regression using size data from previous work: $y = 2.9425x + 1.4054$, $R^2 = 0.6418$, where x is the body size in grams and y is the wing length (mm) (Van Wyk et al., 2021). I used a linear model with respiration rate as the response variable and infection treatment as a categorical fixed effect. I used an additional linear model with the same response variable to assess the effect of infection intensity amongst the subset of infected replicates. Since body size is accounted for in the respiration rate, I excluded size as a covariate in these models to avoid redundancy. I performed these analyses on the entire respiration dataset, as well as on subsets based on temperature treatment (variable versus stable).

RESULTS

Eighty percent of replicates (n = 48/60) survived to the end of the ramping experiment. The thermoregulatory capacity– the difference between thoracic and and ambient temperatures (ºC) – of the bees decreased with increasing ambient temperature ($\chi^2 = 986$, n = 717, df = 1, $p < 0.001$); was greater in uninfected than infected bees ($\chi^2 = 3.88$, n = 717, df = 1, $p = 0.048$) and in larger bees than smaller ones ($\chi^2 = 9.86$, n = 717, df = 1, $p = 0.001$). Furthermore, there was a significant interaction effect between infection treatment and body size such that thermoregulatory capacity was lower in smaller, uninfected bees ($\chi^2 = 13.35$, n = 717, df = 1, $p < 0.001$; Fig. 1). When the thoracic temperatures are compared to a homeostatic baseline of 32 ºC rather than the ambient temperature, the relationships between thermoregulation and infection status and between thermoregulation and ambient temperature remain consistent, with a significant interactive effect of infection status and body size on the difference between body temperature and a homeostatic baseline ($\chi^2 = 13.35$, n = 717, df = 1, $p < 0.001$).

The likelihood of observing moving behavior was not impacted by ambient temperature ($F_{(1, 719)} = 0.163$, $p = 0.687$) or infection treatment ($F_{(1, 719)} = 0.036$, $p = 0.85$). Infection treatment also had no effect on the likelihood of observing fanning behavior; however, there was a significant relationship between the likelihood of fanning and ambient temperature such that the likelihood increased at higher temperatures ($F_{(1, 719)} = 49.247$, $p < 0.001$; Fig. 2).

Across all respiration data, larger bees exhibited significantly higher respiration rates than smaller-bodied bees ($F_{(1, 169)} = 16.221$, $p < 0.001$). Amongst the respiration trials performed with ambient temperature ramping, there is a non-significant trend where the respiration rate decreases with increasing ambient temperature ($F_{(1, 77)} = 1.183$, $p = 0.28$). Furthermore, the respiration rate did not differ significantly between infected and uninfected individuals ($F_{(1, 77)} =$

1.688, $p = 0.198$). When held at a constant ambient temperature, the respiration rate was not significantly different between infected and uninfected individuals ($F_{(1,88)} = 0.02$, $p = 0.886$). However, amongst infected replicates, the respiration rate decreased significantly with increasing infection intensity ($F_{(1, 43)} = 7.564$, $p = 0.009$; Fig. 3).

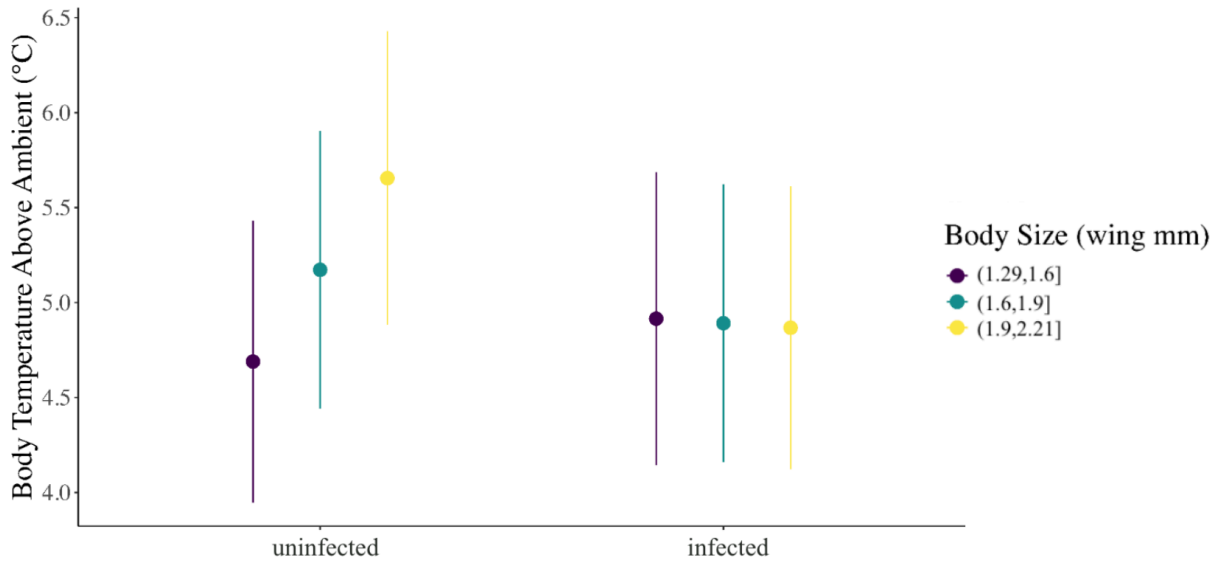

Figure 1. Mean ± 95% confidence interval thermoregulation of uninfected (left) and infected (right) *Bombus impatiens* workers across a temperature gradient (ºC). Here, thermoregulation is calculated as the difference between the ambient temperature (ºC) and the core body temperature (ºC) of individual bees. The length of the marginal cell of the forewing (mm) is a proxy for body size.

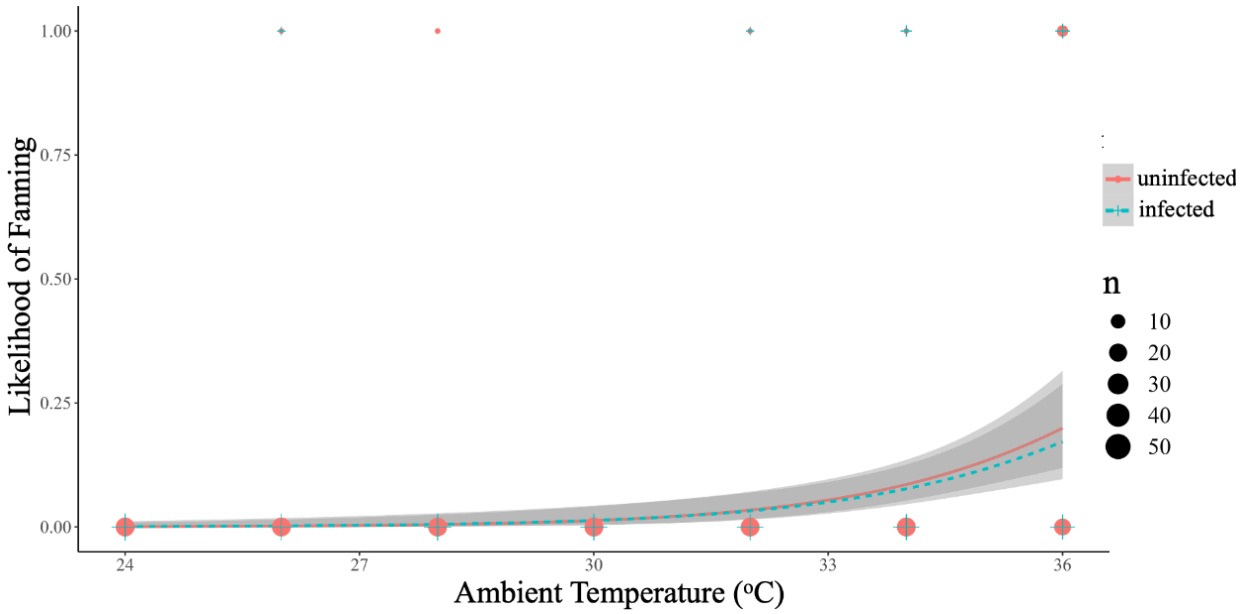

Figure 2. The likelihood of observing fanning behavior in infected versus uninfected *Bombus impatiens* workers across an ambient temperature gradient (ºC). The pink circles and solid line represent the likelihood of observing fanning in uninfected workers, while the blue plus-signs and dotted line represent the likelihood of observing fanning in infected workers. The size of the points represents the number of behavioral observations at each temperature. The shaded polygons represent 95% confidence limits of the binomial models.

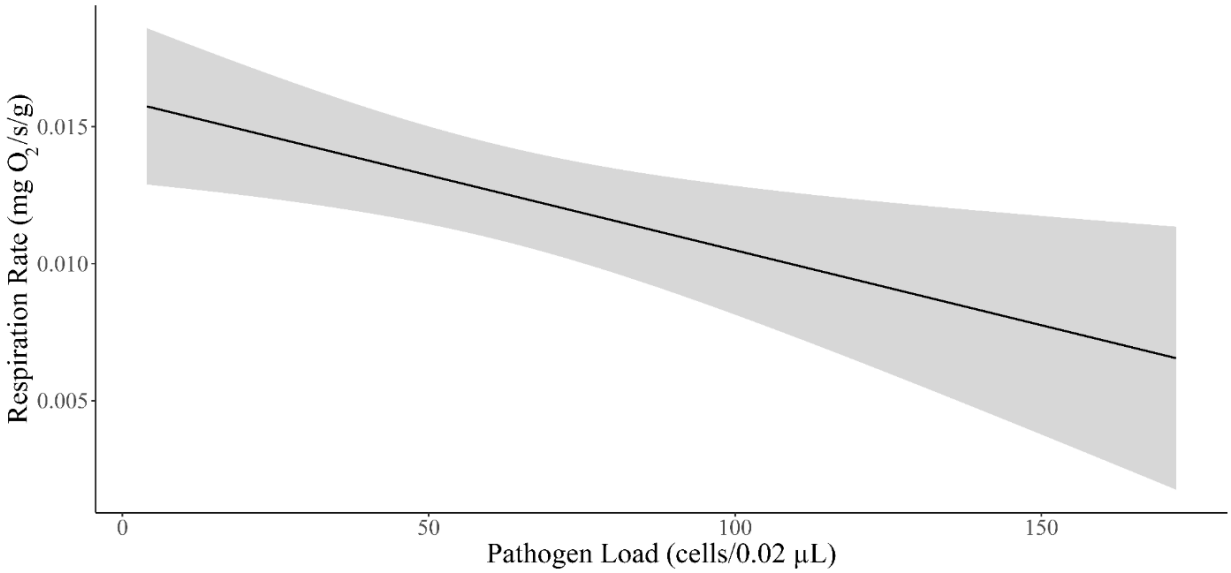

Figure 3. The respiration rate, expressed as milligrams of O_2 per second per gram of body size, of infected *Bombus impatiens* workers decreases with increasing infection intensity. Infection was determined by counting live *Crithidia* on a hemocytometer. The shaded polygon represents 95% confidence limits of the binomial model.

DISCUSSION

We are already living with the consequences of climate change– 2023 experienced the highest global surface temperatures on record (NOAA, 2023). Given current practices and policies surrounding greenhouse gas emissions, global annual temperatures are projected to increase by an additional 1.5 ºC before the middle of the 21st century and the frequency, intensity, and duration of heat waves is expected to rise (IPCC, 2023). While thermal stress from changes in maximum temperatures alone is a cause of concern, this work demonstrates that it is also crucial to consider the effects of temperature variation on ectotherm fitness. Insect species such as *Bombus impatiens* are economically important as crop and native plant pollinators, and are important for the biodiversity of many ecosystems across the temperate United States (Lozier et al., 2011; Goulson et al., 2011). The experimental design of this study offered an opportunity to collect specific data relating to size, behavior, and metabolic processes for *Bombus impatiens* while maintaining field-realistic conditions, thus allowing us to gain a better understanding of the effects of heat and pathogen stress on pollinators. In turn, these results can help to guide conservation efforts that should be taken for bumble bees. In this discussion, I will assess my results in the broader context of the literature surrounding paradigms of heat and pathogen stress in ectotherms, behavioral response thresholds, and the metabolic costs of stress. Understanding bumble bee ecophysiology in response to thermal variability and disease is critical to our understanding of how bee populations may shift in response to the ever-intensifying effects of climate change. Such information is essential for protecting the biological diversity of bees and the wildflowers that they pollinate, as well as the food security and livelihoods associated with bumble bee-pollinated agriculture.

Temperature variation from environmental phenomena such as heat waves is an established threat for ectotherms, and one that will continue to grow in severity due to climate change. For example, studies in mosquitoes have demonstrated that as average temperatures rise, daily fluctuations in temperature shift thermal reaction norms compared to thermally stable baselines. The consequent reduction in survival probabilities and development rates for these insects indicates that short-term environmental variability incurs fitness costs at warmer average temperatures (Paaijmans et al., 2013). Disease with heat waves poses a perhaps more dangerous threat. A study in Spencer's river tree frog, *Litoria spenceri,* found that infection with a fungal pathogen lowered the critical thermal maximum of individuals, making them more vulnerable to daily heat pulses (Greenspan et al., 2017). In line with these instances of the relationships between environmental factors and fitness, the thermal mismatch hypothesis posited by Cohen and colleagues describes how climate change shifts environments away from ectotherms' thermal optima and towards their CT_{max}, which may increase their susceptibility to disease. This hypothesis builds on the idea of size differences between hosts and parasites, where parasites are capable of a broader thermal range due to their higher metabolic rates, which are attributed to smaller body mass (Cohen et al. 2017). Overall, the interplay between temperature, hosts, and parasites is complex and context-specific, but general trends point to heat and pathogen stress acting synergistically on hosts.

As temperatures continue to warm, pathogens may present decreased sensitivity to temperature variability as compared to the hosts that they infect due to differences in size and thus metabolic rate (Koelle et al., 2005). Previous work suggests that this may be the case for *Crithidia bombi* and *Bombus impatiens*– infection outcomes were robust to thermal variability within naturally experienced ranges (21–29 ºC) in the host gut (Tobin et al., 2019). However, it is

crucial to understand how temperature variability during extreme heat events will shift *Bombus impatiens* host-pathogen dynamics given their pollination services in natural and commercial settings. This work demonstrates an interactive effect of body size and infection status on thermoregulation in *Bombus impatiens* workers such that smaller, uninfected bees exhibit the smallest difference between thoracic temperature and ambient temperature. Conversely, this metabolic scaling with size does not occur in infected bees. Interestingly, these relationships between body temperature, size, and infection are maintained when thermoregulation is calculated as the difference between thoracic temperature and a homeostatic baseline (32 ºC). This pattern of body size and infection impacting thermal tolerance is paralleled in recent work with *Bombus impatiens* and *Crithidia* across a similar thermal gradient: body size demonstrated a positive rescue effect where larger bees had longer life spans, but this effect disappeared when the bees were infected with *Crithidia* (Fallman Tirado, 2022). This data points to the potential for a pattern of *Crithidia* infection neutralizing size-based differences in fitness.

Given that bees infected with *Crithidia* demonstrated similar thoracic body temperatures regardless of size in this experiment, it appears possible that the burden of both heat and pathogen stress could detract from their thermoregulatory abilities. Experiencing additive stressors in this way may confer a fitness disadvantage as it requires more energy than dealing with just one stressor or with neither. Alternatively, this result could be demonstrative of behavioral fever in *Bombus impatiens*. Behavioral fever is a method of behavioral thermoregulation in response to infection that has been identified in many ectotherms. Behavioral fever entails an acute increase in preferred temperature in response to pathogen infection, and often involves purposeful movement of a host to temperatures generally considered to be above their thermal optimum (Reynolds and Casterlin, 1982). In the

Panamanian golden frog (*Atelopus zeteki*), for example, individuals can modify their body temperature above its optimal set point and consequently decrease their odds of infection with the fungal pathogen *Batrachochytrium dendrobatidis* (Richards-Zawacki, 2010). Other insect species have demonstrated changes in behavior such as increased basking time or movement up a temperature gradient in their environment in order to fight off pathogen infections (Adamo, 1998; Catalán et al., 2012). Importantly, maintaining febrile temperatures to either prevent or clear infections is costly. Other insect species have demonstrated that while behavioral fever increases chances of surviving infection, it decreases their growth rate (Boorstein and Ewald, 1987). A critical avenue for future study, then, is to determine if *Bombus impatiens* or other bumble bee species exhibit behavioral fever in response to prevalent pathogens such as *Crithidia*. Exploring concepts such as behavioral fever allows for the consideration of hosts, their pathogens, and the ambient conditions that they share.

Concepts such as behavioral fever are representative of the triangle of disease, a paradigm that describes this interplay between a host, a pathogen, and their shared environment (McNew, 1960). This study exemplifies an application of this framework by studying host-pathogen dynamics between *B. impatiens* and *Crithidia* in a variable thermal environment. Here, I found that infected bees displayed body temperatures significantly closer to that of ambient temperatures compared to uninfected bees. Furthermore, this paradigm is demonstrated in recent work with solitary bees and another protozoan in the genus *Crithidia*, where infection increases host thermal sensitivity, measured as CT_{max}, during extreme heat events (Porras et al., 2023). This particular study also suggests that extreme heat events have analogous effects on the pathogen, such that growth is limited as temperatures approach the host's CT_{max} (Porras et al., 2023). These findings point to the idea that climate change alters host-pathogen dynamics, and

that the consequences of disease dynamics depend on the effects of heat on hosts and pathogens. Recent work with the *Bombus impatiens* and *Crithidia bombi* system also demonstrates the triangle of disease. One study found that in the wake of simulated heatwaves, workers' ability to mount an immune response to infection was limited (Tobin et al., 2024). This is further evidence that changes in temperature can have deleterious effects on bumble bee immunity and population viability in the face of pathogens such as *Crithidia*.

Bumble bees exhibit physiological adaptations to suboptimal or harmful temperatures. In this study, we collected behavioral data from the bees to understand how different behaviors may impact their body temperature. Here, the likelihood of an individual fanning increased significantly with ambient temperature, which is consistent with ectothermal responses to temperature (Weidenmüller et al., 2004). Similarly, these data indicate that bees begin fanning at temperatures above their homeostatic baseline of 32 ºC, further strengthening this result. Conversely, the likelihood of an individual moving was not impacted by ambient temperature or infection treatment. This is not a surprising result given the time scale resolution for this study, the binary quantification of movement, and the biological relevance of movement as a thermoregulatory behavior given the lack of backing in the literature and that the bees were housed in small plastic cups. Overall, while fanning is an established thermoregulatory mechanism and the fanning observed here aligns with established trends regarding behavior and ambient temperature, it appears that movement is not a biologically relevant thermoregulatory mechanism.

Given that there was no impact of infection treatment on the likelihood of fanning behavior in conjunction with the high prevalence of *Crithidia* in bumble bee populations, it is promising that these results demonstrate that sick bumble bees can still perform

thermoregulatory behaviors while under thermal stress. Despite this encouraging result, the implications of significant increases in the likelihood of an individual fanning at higher ambient temperatures is still worthy of further consideration. As global climate change impacts temperature extremes as well as variation in temperature, it is possible that an increase in energy allocation for thermoregulatory behaviors such as fanning could take away from other aspects of colony development such as foraging. The ability of bumble bee workers to regulate their own temperature– and perhaps more importantly, that of the brood– is essential for the growth and development of the colony (Stewart et al., 2021). Colonies with higher quantities of workers produce the most reproductive individuals, and thus bumble bees must balance the thermoregulatory demands of the brood with the metabolic demands that fanning or incubating entail (Samuelson et al., 2018; Stewart et al., 2021). It is possible that extreme heat events caused by climate change could disrupt this balance, putting an energetic strain on bumble bee colonies.

Examining the relationship between thermoregulation and behavior introduces a hypothetical feedback loop such that upon reaching a certain temperature threshold, bees will engage in fanning behavior to cool down, and when a low temperature threshold is met, they will incubate to warm up. Such response thresholds have been identified in bumble bee literature, specifically in regards to thermoregulation (Jandt and Dornhaus, 2014). Bumble bees exhibit plasticity in their fanning response thresholds. For example, repeated bouts of fanning behavior decreases the fanning response threshold– that is, the temperature at which the individual initiates fanning– for *Bombus terrestris* workers (Westhus et al., 2013). Furthermore, bumble bees with higher thermoregulation response thresholds have been shown to cool down their nests more quickly (Jandt and Dornhaus, 2014). Overall, these response thresholds are the basis of the division of labor in bumble bee colonies (Beshers and Fewell, 2001; Weidenmüller, 2004). Thus,

examining thermoregulation response thresholds in a simulated heat wave may prove to be a useful future endeavor to elucidate if and how climate change will impact colony structure.

One limitation to consider regarding the behavioral assays in this study is the decision to house the bumble bees individually. Housing replicates in this way for the experiment was critical for associating body temperature and respiration data with body size and infection intensity. However, bumble bees are eusocial organisms with a hierarchical colony structure (Goulson, 2003). Within the colony, the queen is responsible for reproductive output while tasks such as foraging and brood care fall to the non-reproductive worker castes (Goulson, 2003). As mentioned previously, not all workers in a colony complete the same set of tasks, and thus examining individuals offers a much different picture than examining the behavior of the colony as a whole. While the behavior observed in this study may not be an exact parallel for whole-colony behavior, examining entire colonies with this thermal ramping regime would be a valuable avenue for future study.

In addition to assessing individual physiology in regards to thermoregulation, this study design allowed for further exploration of the relationships between environmental stressors, body size, and metabolism in bumble bees. In both of the respiration experiments, there was a positive relationship between respiration rate and body size. This trend is consistent with the allometric scaling of metabolism and body size, in which metabolic rate scales to the ¾ power with body mass (West et al., 1997). Previous studies have already demonstrated interesting trends in regard to the metabolic scaling factor: in a study of marine invertebrates, for example, metabolic scaling demonstrated plasticity in response to environmental temperatures, suggesting that ectotherms' responses to global climate change may be modulated by body size (Carey and Sigwart, 2014). Furthermore, the metabolic scaling exponent changes in accordance with activity level in

ectotherms such that it is higher during active exercise than during resting periods (Glazier, 2009). Thus, replicating the respiration experiments detailed in this study with additional considerations for aspects such as trait-based plasticity and behavior may prove to be informative avenues of future research. Although there were no significant trends in the temperature ramping trials for respiration, it is possible that higher replication would elucidate any trends that may be suggested by this pilot data. Assessing respiration rates of ectotherms in thermally variable environments is more ecologically realistic than holding them at constant temperatures, and thus I am currently conducting this work for analysis in a future publication.

When held at a constant temperature, infection treatment had no effect on respiration rates; however, across infected individuals, the respiration rate significantly decreased with increasing pathogen load. This relationship may imply a potential metabolic cost for combatting infection. If this is the case, individuals with lower pathogen loads may be evidence of a strong immune response that can fight off some of the *Crithidia* infection at the physiological cost of higher respiration. A previous study with *Bombus impatiens* found an inverse relationship between metabolic rates and lifespans for workers (Kelemen et al., 2019). Although this association was established in the absence of other extrinsic factors such as varying temperatures or pathogen stress, it is important to consider shorter lifespans as a potential tradeoff for combating *Crithidia* pathogen loads. Alternatively, this finding that pathogen load correlates negatively with respiration rate could imply that bees with high pathogen loads are unable to fight off the infection, and thus the disease burden of *Crithidia* confers a physiological cost reflected through lower respiration rates. Ultimately, these hypotheses pose a new question of whether this trend in respiration rate represents an enhanced immune response to infection or a

deleterious physiological symptom of disease. Answering this question requires additional work, as we are not yet able to connect a fitness cost to respiration rate given the data in this study.

In general, insects experience an increase in respiration rate when their ambient environment is suboptimal in some manner. There is very little literature on bumble bees in particular, but examining other ectotherms– especially insects– can help inform the trends observed here. Honeybee workers (*Apis mellifera*), for example, display a depression in respiration rate around their thermal optimum of 32 ºC, below which workers increased oxygen consumption to raise their temperature and above which they struggle with an increasingly harmful thermal environment (Allen, 1959). At upper lethal temperatures, honeybee workers also demonstrated increased respiration rates above those seen at their thermal optima (Allen, 1959). Overall, this experiment quantifies sickness through pathogen load and the physiological cost of disease with respiration. Ascertaining the mechanisms of the immune response to *Crithidia* infection in the context of respiration should be a goal of future work in this host-pathogen system.

Though this experiment demonstrated decreasing respiration in response to increasing infection intensity at a stable temperature, bumble bees in nature face both pathogen and temperature stress. Foraging ectotherms like *Bombus impatiens* are vulnerable to "metabolic meltdown" wherein reduced nutrient intake paired with increased energy costs from higher ambient temperatures creates a cascade of deleterious impacts and reduced fitness for the organism (Huey and Kingsolver, 2019). An additional consideration for this result is the behavior of the bees during the respiration trials. It is possible that increasing *Crithidia* intensity inhibits activity in individuals, thus resulting in lower respiration rates. Future replications of this study should examine individual behavior in conjunction with respiration rate.

In the past century, we have observed a significant decrease in body size for bumble bees in North America (Nooten and Rehan, 2020). Body size is an important determinant of fitness in ectotherms. As previously established, body size impacts transmission probability and infection intensity of *Crithidia bombi* in *Bombus impatiens* (Van Wyk et al., 2021). Furthermore, small body size poses a constraint in foraging range (Westphal et al., 2006). Anthropogenic changes in land use have contributed to limited resource availability for pollinators, thus furthering the problem given that resource limitation begets smaller bees (Gómez-Martínez et al., 2020). In addition to habitat fragmentation posing a selective pressure on body size, previous research in insects has shown that temperature plays a critical role in body size responses (Atkinson, 1994; Forster et al. 2012). The interplay between body size, responses to *Crithidia* infection, and thermoregulation in this work speaks to the complex ecological processes that determine fitness outcomes for bumble bee species. The observation that body size does not drive metabolic rate in infected bees indicates that pathogen stress poses a challenge to the thermoregulatory abilities of *Bombus impatiens*. Further, decreasing body size coupled with range expansions and increased prevalence of disease make a dangerous combination for bumble bees given that smaller individuals can experience higher infection intensity and likelihood of infection for pathogens such as *Crithidia* (Van Wyk et al., 2021).

Since bumble bees rely on floral resources for the energy to grow, reproduce, and fight off pathogens, it is imperative that we focus our conservation efforts on reducing habitat fragmentation and increasing floral abundance and diversity for bumble bee populations (Goulson et al., 2011). In terms of combating the effects of *Crithidia* on *Bombus* species, reducing the long-distance transport of bumble bees for commercial pollination will help to reduce disease spillover between commercial and native populations (Colla et al., 2006;

Williams and Osborne, 2009). As climate change continues to alter the thermal landscape in which bees and their pathogens interact, it is crucial to enact as many adaptive strategies as possible to protect pollinators and the food security and biodiversity that they offer.

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APPENDIX: STATISTICAL MODELS

A full version of the R Studio code for this thesis is available at

https://rpubs.com/lohr22m/1177765.

Thermoregulation Linear Mixed Effects Model

```{r}
m4.1 <- lmer(formula = thermoreg~set_temp_C+infected*body_size+(1|parent_colony)+(1|date), data = ramping4)
Anova(m4.1)
```

```
Analysis of Deviance Table (Type II Wald chisquare tests)

Response: thermoreg
                     Chisq Df Pr(>Chisq)
set_temp_C         986.9217  1  < 2.2e-16 ***
infected             3.8882  1  0.0486252 *
body_size            9.8621  1  0.0016872 **
infected:body_size  13.3467  1  0.0002589 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

Homeostatic Baseline Linear Mixed Effects Model

```{r}
m9 <- lmer(formula = homebase~set_temp_C+infected*body_size+(1|parent_colony)+(1|date), data = ramping5)
Anova(m9)
```

```
Analysis of Deviance Table (Type II Wald chisquare tests)

Response: homebase
                     Chisq Df Pr(>Chisq)
set_temp_C         835.6008  1  < 2.2e-16 ***
infected             3.8882  1  0.0486252 *
body_size            9.8621  1  0.0016872 **
infected:body_size  13.3467  1  0.0002589 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

Moving Behavior Linear Model

```{r}
move_lm2 <- lm(moving~infected+set_temp_C, data=behavior8)
Anova(move_lm2)
```

```
Anova Table (Type II tests)

Response: moving
            Sum Sq  Df F value Pr(>F)
infected     0.007   1  0.0361 0.8494
set_temp_C   0.032   1  0.1626 0.6869
Residuals  139.486 719
```

Fanning Behavior Linear Model

```{r}
fan_lm2 <- lm(fanning_binary ~ infected+set_temp_C, data=behavior8)
Anova(fan_lm2)
```

```
Anova Table (Type II tests)

Response: fanning_binary
            Sum Sq  Df F value    Pr(>F)
infected    0.0048   1  0.1164     0.733
set_temp_C  2.0183   1 49.2470 5.225e-12 ***
Residuals  29.4668 719
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

Respiration and Body Size Linear Model

```{r}
size_resp <- lm(rate~size_g, data=data.rate)
Anova(size_resp)
```

```
Anova Table (Type II tests)

Response: rate
           Sum Sq  Df F value  Pr(>F)
size_g    0.03516   1  16.221 8.5e-05 ***
Residuals 0.36631 169
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

Thermal Ramping: Infection Treatment Linear Model

```{r}
moho<-lm(rate2~infected*set_temp_C, data=rate_mhc)
Anova(moho)
```

```
Anova Table (Type II tests)

Response: rate2
                      Sum Sq Df F value Pr(>F)
infected            0.0000710  1  1.6878 0.1978
set_temp_C          0.0000498  1  1.1829 0.2802
infected:set_temp_C 0.0000487  1  1.1563 0.2856
Residuals           0.0032405 77
```

Stable Temperature: Infection Treatment Linear Model

```{r}
um1 <-lm(rate2~infected, data=rate_umass)
Anova(um1)
```

```
Anova Table (Type II tests)

Response: rate2
             Sum Sq Df F value Pr(>F)
infected  0.0000017  1  0.0201 0.8875
Residuals 0.0073870 88
```

Stable Temperature: Pathogen Load Linear Model

```{r}
um2 <-lm(rate2~Crithidia, data=subset(rate_umass, infected==1))
Anova(um2)
```

```
Anova Table (Type II tests)

Response: rate2
              Sum Sq Df F value   Pr(>F)
Crithidia 0.00025138  1  7.5639 0.008677 **
Residuals 0.00142906 43
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```