Effects of Temperature on Host-microbe Dynamics and Infection Outcomes in the Red Flour Beetle, *Tribolium castaneum*

By

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DEDICATION

To my dearest brother, Abdus Sabur, whose sudden departure has left an irreplaceable void in our lives.

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First and foremost, I express my deepest gratitude to the Almighty for surrounding me with some amazing people and for granting me the strength, perseverance, and guidance throughout this journey.

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INTRODUCTION

Temperature exerts a profound influence on organisms, impacting essential biological processes such as metabolism, growth, development, reproduction, and immune responses (Hafez, 1964; Atkinson, 1994; Cui *et al.*, 2018; Petrov and Razuvaeva, 2018; Sun *et al.*, 2019; Zhang, Ding and Wei, 2021). One potential coping mechanism for these temperature-induced changes is adaptation to shifting climates. However, abrupt temperature fluctuations pose significant challenges for organisms, as they may not have adequate time to adapt, leading to heightened stress levels (Rahman and Rahman, 2020). The concept of optimal temperature is pivotal, representing the temperature range to which organisms are best adapted and can achieve maximal fitness. Conversely, temperatures beyond an organism's threshold of tolerance can diminish fitness and increase susceptibility to infections or diseases, potentially leading to population or species extinction (Rahman and Rahman, 2020). This susceptibility is particularly pronounced in ectothermic organisms, which have narrow thermal tolerance ranges and may struggle to adapt to abrupt temperature changes (Rinehart, Yocum and Denlinger, 2000; Zhu *et al.*, 2017a; Rahman and Rahman, 2020). In this context, ectotherms, such as insects, serve as prime examples of organisms highly sensitive to temperature fluctuations. The intricate interplay between temperature and insect physiology extends to their immune responses. For instance, studies have shown that temperature variations can profoundly impact insect immune function (Ferguson & Adamo, 2023), thereby influencing their ability to combat infections and maintain population viability. Understanding the intricate relationship between temperature and insect immunity is crucial for predicting the consequences of climate change on insect populations and disease dynamics.

Insects possess a diverse array of immune defenses to combat microbial pathogens, employing intricate molecular pathways and mechanisms for pathogen recognition and response (Lemaitre and Hoffmann, 2007). Central to insect immunity is the recognition of conserved microbial-associated molecular patterns (MAMPs) by pattern recognition receptors (PRRs) (Buchon, Silverman and Cherry, 2014). These receptors, including the Toll and Immune Deficiency (IMD) pathways, initiate signaling cascades upon MAMP detection, culminating in the transcriptional activation of antimicrobial peptides (AMPs) and other immune effectors (Buchon et al., 2014).

The Toll pathway, principally activated by gram-positive bacteria, orchestrates a signaling cascade involving the translocation of transcription factors, such as Dorsal and Dif, to the nucleus (Lemaitre and Hoffmann, 2007). Once translocated, these factors bind to specific promoter regions of AMP genes, thereby inducing their expression (Fig.1). Conversely, the IMD pathway, activated predominantly by gram-negative bacteria, involves the nuclear translocation of the transcription factor Relish, which drives the expression of AMP genes (Buchon, Silverman and Cherry, 2014). These immune mechanisms can be constitutively expressed at low levels or rapidly upregulated upon infection, reminiscent of the innate immune responses observed in mammals (Buchon, Silverman and Cherry, 2014). Further complexity arises from the regulation of AMP expression by diverse internal and external factors. Metabolic changes, environmental stressors, and aging have been implicated in modulating the expression levels of AMPs in insects (Stączek, Cytryńska and Zdybicka-Barabas, 2023). This multifaceted regulation underscores the intricate interplay between immune responses and physiological conditions.

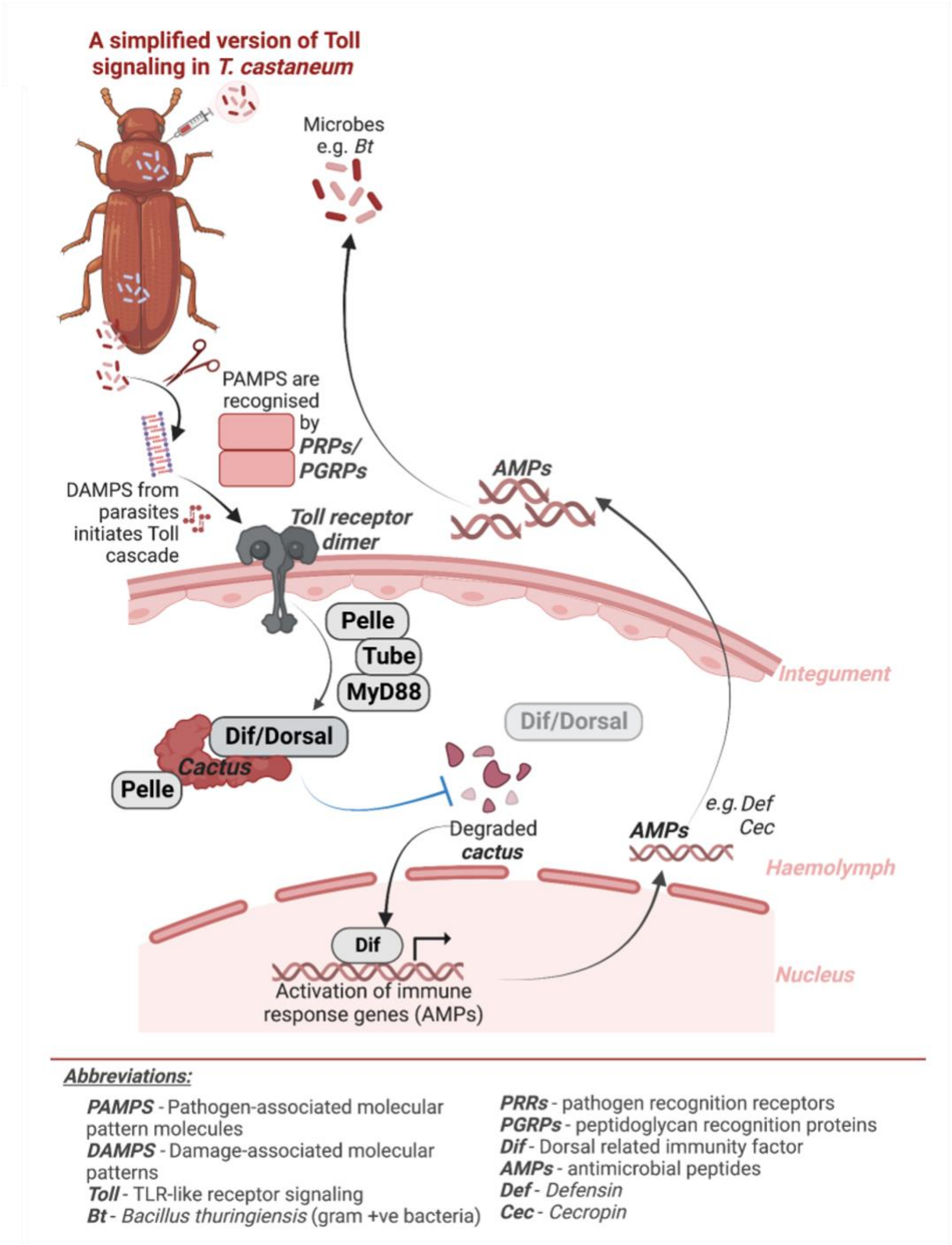

Figure 1. Toll signaling pathway adapted from (Critchlow *et al*., 2024) When pathogen breaks the first line of defense, the integument, pattern recognition receptors (PRRs) such as the toll receptor identify the pathogen or damage-associated molecular patterns (PAMPs or DAMPs) and triggers a cascade of events involving Pelle, Tube, and MyD88 where Pelle phosphorylate Cactus. The subsequent degradation of Cactus leads to the release of Dif and Dorsal that translocate to the nucleus and activate the transcription of effector AMPs such as Cecropin, and Defensin then take part in the clearance of infection.

As ectotherms, insects cannot regulate their internal body temperatures like mammals or birds. Their body temperature is completely dependent on the external environment. Therefore, even a little change in the environmental temperature greatly affects their internal body functions including the immune response (Ferguson and Adamo, 2023). For example, higher temperature increases the rate of metabolism leading to faster growth and development along with an overproduction of reactive species in ectothermic insects that makes them age faster and consequently deteriorates their immune response against the pathogen as they get older (Zuo *et al.*, 2012; Kelly *et al.*, 2013; Colinet *et al.*, 2015; Hillyer, 2016). Studies showed a clear indication of temperature-mediated change in the metabolism and immune gene expression in insects like ants, butterflies, and fruit flies(Pylayeva-Gupta, 2011; MacMillan *et al.*, 2016; Stucki, Freitak and Sundström, 2017; Wojda, 2017; Chen *et al.*, 2019).

Now the question is, why is it important to understand the effect of temperature on host-microbe dynamics and what significance does it bear for the future biodiversity? The temperature of our planet is increasing day by day due to climate change. The mean atmospheric temperature of our planet has increased by 0.75°C in the 20th century (Seney *et al.*, 2013) and scientists have projected that if the current trajectory of rising temperature continues, it will cause another 2-5°C rise in the mean atmospheric temperature by the end of 21st century which will be the number one reason of biodiversity loss in that period (Morris *et al.*, 2018). Arguably, most of those extinct species would be invertebrates, particularly the insects that makeup over 50% of the described species on earth (Mayhew, 2007). Unfortunately, very few studies have been conducted on terrestrial invertebrates such as insects, the most diverse class of organisms, which requires more attention from the scientific community. These insects are an integral part

of the global food web, and many insects have economic importance (Aidoo *et al.*, 2023). Therefore, it is imperative to know how temperature variation can affect the insects directly or indirectly by affecting the host-pathogen dynamics. An increase in the atmospheric temperature could weaken the host immune system and make the organism more susceptible to infection. Since temperature can affect the immune response and potentially compromise the ability of insects to combat infections, investigating the effect of temperature on immune function is paramount for understanding the implications of climate change on insect populations and disease dynamics. Moreover, the evolutionary conservation of key immune pathways and regulatory mechanisms between insects and mammals underscores the relevance of insect models in biomedical research. By elucidating fundamental principles of immune function in insects, researchers can glean insights into the mechanisms underlying immune-related diseases and develop novel therapeutic strategies.

The insect immune system serves as a valuable model for studying the impact of environmental factors on immune function, with implications extending to both basic biological research and biomedical applications (Mukherjee, Twyman and Vilcinskas, 2015; Adamski *et al.*, 2019). Through interdisciplinary approaches, researchers can leverage the unique attributes of insect immunity to advance our understanding of immune regulation and disease pathogenesis. Ongoing scientific inquiry continues to unravel the complexities of insect immune physiology, shedding light on adaptation strategies and the impacts of environmental factors, as previously demonstrated in insects. Linder et al., (2007) showed that a lower temperature (17°C) facilitates a better immune response (*pgrp-LC*, *cactus*, *spatzle* gene expression) in *Drosophila melanogaster* leading to a higher survival compared to 25°C and/or 29°C (Linder, Owers and

Promislow, 2008). Similarly, mosquitos housed in 18°C experimental temperature also showed higher melanization, phagocytosis, and expression of defensin genes (Buchon, Silverman and Cherry, 2014). On the contrary, colder temperatures have been discovered to inhibit the expression of virulence factors in numerous mammalian pathogens; however, once these pathogens reach the standard mammalian body temperature of 37°C, there is a notable surge in their virulence factor production (Lam, Wheeler and Tang, 2014). This implies that a warmer climate could prove unfavorable for ectotherms, while simultaneously offering a conducive environment for the thriving of pathogens. Further studies also suggest that rising temperature could harm the host by making the pathogens more virulent by turning the commensal microbes into pathogenic ones, or by increasing their transmission rate (Marcogliese, 2008; Pylayeva-Gupta, 2011; Lam, Wheeler and Tang, 2014; Guijarro et al., 2015). Therefore, if the atmospheric temperature increases significantly, the host-microbe relationship could be thrown off balance and infections could lead to the demise of the host populations.

In this study, we used the red flour beetle, *Tribolium castaneum*, as our model organism to determine the effects of temperature variation on host survival when they are infected. The beetle's development closely resembles that of other insects making it a good representative of many insect species (Richards *et al.*, 2008). Moreover, its sequenced genome, short life span, quick immune response to infection, and ease of handling make it an ideal model species for infection studies (Richards *et al.*, 2008). The red flour beetle is considered one of the most common secondary pests of stored grain products, including food grain, cereal, flour, pasta, and beans, and can be found in storehouses and silos causing damage to the stored products (Sallam, 2013). Although it has a worldwide distribution, the beetle can be found predominantly in

warmer regions. Nonetheless, they can be exposed to different temperatures in their habitat with seasonal change and within the silos based on their location. Alabadan and Oyewo (2005) showed that the inside temperature greatly varies with seasonal change in both wooden and metal silos (Alabadan and Oyewo, 2005), whereas Ajayi et. Al., (2016) reported location-based temperature variation inside the silos ranging from 24-36°C, being the highest temperature on top and lowest at the bottom (Ajayi *et al.*, 2016). Insects, including red flour beetles, can be infected by a variety of pathogens and the relationship can have different outcomes due to temperature change (Burges and Weiser, 1973; Janovy *et al.*, 2007; Agarwal and Agashe, 2020). To explore that, we used the bacteria, *Bacillus thuringiensis* (Bt), to immunologically challenge the host *T. castaneum* in our experiments. Bt is a gram-positive bacterium that produces pore-forming toxins (known as Cry toxin) in the form of the crystal when ingested. Cry toxins destroy the gut epithelium by lysing the epithelial cells leading to insect death (Bravo *et al.*, 2013) which could be aggravated at higher temperatures. Rising temperatures can negatively affect the red flour beetle by affecting its immune response and inducing heat stress. On the contrary, it can have a positive effect on the pathogen in the form of an increased rate of multiplication and toxin production leading to extensive damage and eventual death of the host.

The thermal mismatch hypothesis implies that the largest performance gap for cold and warm-adapted insect hosts would be in warm and cold conditions, respectively (Cohen *et al.*, 2017). However, for infected hosts, the outcome also depends on the thermal tolerance of the pathogen. A pathogen with high heat tolerance would be favored by the temperature rise whereas lower heat tolerance could inhibit its normal function and eventually benefit the host. Although both the host and the pathogen in our study are optimized at 30°C in laboratory conditions; as an

ectotherm, the red flour beetle is susceptible to heat stress whereas the pathogen, Bt, is resistant to high heat and desiccation (Heini *et al.*, 2020; Abdel-Hady *et al.*, 2021). Therefore, we hypothesized that higher temperatures would deteriorate the host's optimal immune response and increase the growth and activity of the pathogen, *B. thuringiensis*, leading to an increased mortality rate in the red flour beetle, *T. castaneum*.

Studies show that with the change of global climate, parasite distribution as well as their infectiousness are also changing (Ryan *et al.*, 2019; Cohen *et al.*, 2020) which is causing the host species to fight a two-fronted battle against extreme heat stress and virulent infection (Claar and Wood, 2020; Hector *et al.*, 2022). Hence, our objective was to unravel the intricate relationship between environmental temperature, host immune response, and the virulence of the pathogen that would ultimately determine host survival in a warming world. For this, we acclimated adult beetles in five different temperatures for 12 days and septically injected them with heat-killed Bt to quantify the immune response. To assay survival, we infected them with live Bt and recorded their mortality every hour from the 4^{th} to the 24^{th} hour post-infection. The effect of temperature on Bt growth was observed by growing the Bt *in vitro* for 24 hours at the experimental temperatures. We demonstrated that rising temperatures not only increase the susceptibility of beetles to infection by facilitating pathogen growth but also have the potential to induce immunopathology by suppressing crucial components of the immune signaling pathway. These findings underscore the complexity of host-pathogen interactions in the face of climate change, highlighting the urgent need for a more nuanced understanding of how temperature variations impact disease dynamics in insect populations. By unraveling these complexities, we can better anticipate and mitigate the consequences of climate-driven shifts in parasite distribution and

infectiousness, ultimately aiding in the development of more effective strategies for insect pest management and conservation efforts.

MATERIALS AND METHODS

The model system

For this experiment, we used adult beetles from the Snavely *T. castaneum* population that was initially collected from Pennsylvanian feed stores and grain elevators in June 2013 (Tate and Graham, 2015). The stock colony is routinely kept in the dark at 30°C and 70% humidity, and the beetles are reasonably adapted to this temperature. We followed most of the steps in the experimental design (Fig. 2) according to Jent et al. 2019 (Jent *et al.*, 2019) with some modifications to infection and survival protocols.

We used *Bacillus thuringiensis* (Bt), a ubiquitous Gram-positive, spore-forming bacterium, and a natural pathogen of beetles, as the infectious agent. It can kill the host within 12-24 hours upon septic infection (Raymond *et al.*, 2010; Nielsen-LeRoux *et al.*, 2012). The insecticidal activity of Bt stems from Cry toxins that bind to insect gut receptors, causing pore formation and lysis (Soberón, Gill and Bravo, 2009; Bravo *et al.*, 2013). Remarkably, even heat-killed Bt elicits immune responses in beetles (Khan, Prakash and Agashe, 2016). While Bt naturally infects beetles orally, septic injection offers a reliable alternative for research, bypassing the natural route and enabling precise dose control (Schulz, Stewart and Tate, 2023).

Preparing experimental beetle population

To produce the virgin, adult, experimental beetles, we set up five replicates of ~200 beetles from the adult breeding group into round Petri dishes (100 mm diameter) half-filled with wheat flour and 5% yeast. We kept the beetles in flour (MP Biomedicals) for two days for egg

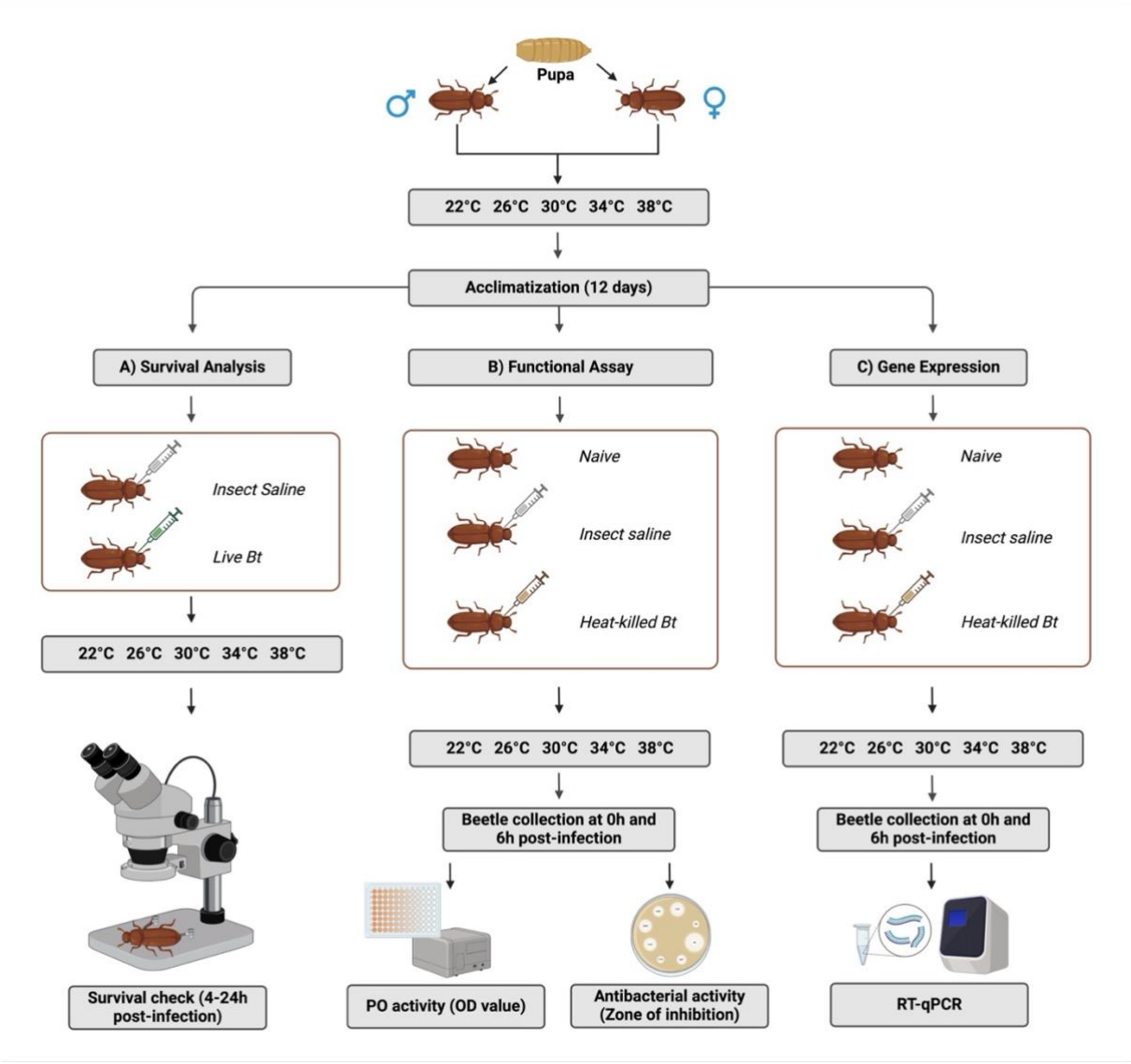

Figure 2. Experimental design. To produce the experimental group of beetles, around ~200 beetles from the breeding population were put in a petri dish containing standard diet and separated the eggs after 48 hours which developed into larva and eventually into pupa at 30°C. Then pupae were collected from the breeding group and separated into male and female. When the pupae developed into adults, the young adults were exposed to experimental temperatures (22, 26, 30, 34, and 38°C) for 12 days. (A) For survival analysis, control, and experimental groups were stabbed with sterile insect saline and live Bt, respectively. Infected beetles were put back to their respective temperature and mortality was recorded from 4-24 hours post-infection. (B-C) For functional assay and gene expression analysis, beetles were stabbed with insect saline and heat-killed Bt and collected at 0 hours (naïve, insect saline stabbed beetles) and 6 hours (insect saline and heat-killed Bt stabbed) time points. (B) PO activity was measured by measuring the darkening of the collected hemolymph at OD490 in a microplate reader, and the AB activity was measured through the zone of inhibition assay. (C) For gene expression analysis RNA was extracted from collected beetles, used to make cDNA, and run RT-qPCR.

laying and removed the adults afterward. To avoid a difference in developmental time leading to a difference in age of the experimental beetles we incubated all the Petri dishes containing eggs at 30°C until pupation. Once the pupae developed, we separated into males and females, kept them in separate petri dishes containing standard diet, and incubated at 30°C until adults emerged from the pupae. When the adults emerged, we divided them into five experimental temperature groups (22, 26, 30, 34, and 38°C; 100 beetles/group/sex) and exposed them to respective temperatures for 12 days.

Preparing the infectious agent, *Bacillus thuringiensis*

Bacillus thuringiensis (Bt) is a rod-shaped, gram-positive, spore-forming entomopathogen that relies on insect-killing for transmission. In this experiment, we used a Bt strain isolated from another lab population and identified by the presence of Cry protein genes, for septically infecting the beetles. For this, we prepared bacterial culture from a glycerol stock of Bt, stored at -80°C. First, using a 200 µl pipette we dipped the tip in the glycerol stock, flushed it in a 5 ml Luria–Bertani liquid medium, and incubated overnight (16-17 hours) in a shaker at 30°C and 200 rpm at 30° angle. The overnight stationary culture was then used for making logistic growth culture by adding 200 µl of stationary culture into 3 ml fresh LB which was then incubated in the shaker at 30°C, 200 rpm at 30° angle for two hours. We measured the optical density (OD) of both the stationary and log cultured Bt (4 replicates each) using a spectrophotometer at 600 nm wavelength and used the recorded ODs to normalize the culture by combining the stationary and log culture in a 2:1 ratio (500/OD µl of stationary culture + 250/ OD µl of log culture). We centrifuged the combined culture at 4°C and 5000 rpm, discarded the supernatant, and washed the pellet with 1 mL sterile insect saline two times by repeating the

centrifugation. Then we collected the washed Bt pellets by resuspending them in 150 µl of sterile insect saline and combining them in one tube. The live Bt culture was diluted 1:10 into sterile insect saline to achieve an LD50 dose that we used to infect beetles for survival checks. For the functional assays (phenoloxidase and antibacterial activity assay) and gene expression experiment, we used heat-killed (HK) Bt which was achieved by incubating the Bt culture in a heat-block for 30 minutes at 90°C.

Bt infection procedure

To determine the effect of temperature on host survival upon infection, we separated the beetles from each temperature into sham (insect saline) and live Bt treatment groups. For the control group, we separated 8 beetles/sex/temperature and for live Bt treatment we separated 32 beetles/sex/temperature into different Petri dishes, making a total of 40 beetles/sex/temperature. We stabbed the beetles between the head and first segment/thorax using an ultrafine sterile needle which was dipped only in insect saline for the control group before pricking, whereas for live Bt infection, every time the needle was first dipped in insect saline to clean and then in Bt solution (Schulz, Stewart and Tate, 2023b) We kept the treated beetles in clear 96 well plates and put them back in incubators at the respective temperatures. We monitored the beetles for 24 hours and recorded the number of dead beetles every hour from 4 hours post-infection to 24 hours post-infection.

To determine the effect of temperature on host immune response, we separated the beetles from each temperature into naïve, sham (insect saline), and heat-killed Bt treatment groups. We sacrificed the naïve beetles by flash-freezing them at -80°C halfway through the

stabbing of the other two groups to minimize the effect of the circadian rhythm on the immune response and stabbed insect saline and HK Bt group with sterile insect saline and HK Bt, respectively, using the above method. After stabbing, we kept the beetles in clear 96 well plates, put them back in respective temperatures, and flash-frozen them 6 hours post-infection at -80°C for further assays.

Bacterial plating

To determine the number of colony-forming units/mL, we serially diluted the Bt culture (10^6X and 10^7X dilutions) into sterile insect saline, plated 100 µl for each dilution on LB agar, and counted the colony-forming units (CFU) after overnight incubation at 30°C. We used the mean CFU of both dilutions to estimate the number of CFUs/mL in 1:10 dilution of Bt culture. To ensure heat-killing worked, we also plated 100 µl of the heat-killed Bt culture in an agar plate, incubated overnight at 30°C, and checked (manually) for any CFUs the following day.

***In vitro* Bt growth**

Temperature is positively correlated with bacterial growth rate which in turn could affect host survival. To determine the correlation between *T. castaneum* survival and Bt growth rate, we recorded Bt growth rate at different temperatures for 24 hours. For *in vitro* Bt growth, we used a single colony of Bt which was attained through streak plating the glycerol stock Bt on LB plate and incubating for 16 hours (5 PM to 9 AM) at 30°C. To make the overnight culture we used 3 different colonies (technical replicates) and only a small portion of each colony was put in 3 different 15 ml tubes containing 5 ml of LB and cultured at 30°C inside a shaker until the OD value reached 1. Then we made a fresh log culture by adding 30 µl of the overnight Bt culture

into a 15 ml tube containing 2.7 ml of LB and incubated at 30°C in a shaker for 3 hours and 30 minutes. We kept the caps a bit loose to ensure airflow inside the tubes for Bt growth. Then we measured the OD of the log culture and adjusted it to an OD of 0.4. We added 1µl of the log culture Bt to a well of clear 96 well plate containing 200µl of LB, placed the plate in a microplate reader, and took OD reading at 0-minute time point. Then the OD was measured at 600 nm wavelength every 30 minutes for 24 hours at 22, 26, 30, 34, and 38°C. The microplate reader was set to continuous shaking (Fastest shake, 1096 cpm) and validated before running.

Phenoloxidase activity assay

We investigated the activity of phenoloxidase (PO), an enzyme crucial for insect defense, in beetles across different temperature and infection groups, following the methods of (Li *et al.*, 2013). First, we homogenized individual beetles in 120 microliters (µl) of ice-cold Bis-Tris buffer and centrifuged three times at 6,200 revolutions per minute (rpm) for 5 minutes at 4 degrees Celsius (°C) using an Eppendorf centrifuge model 5810R. After each centrifugation, we carefully transferred the supernatant to a new chilled tube. To measure PO activity, we prepared a 96-well plate on ice containing 50 µl of sterile deionized water and 50 µl of Bis-Tris buffer in each well. Subsequently, we added 20 µl of beetle extract to each well except for the control/blank wells where we added 20 µl of Bis-Tris buffer instead. Then we added the substrate, 50 µl of 3, 4-Dihydroxy-L-phenylalanine (L-DOPA) solution (4 mg/ml in Bis-Tris buffer, filter-sterilized) to each well, and immediately placed the plate into a Microplate Spectrophotometer. We measured the absorbance at 490 nm and 37°C every minute for 90 minutes. We measured the PO activity as Vmax of the linear reaction phase in each sample well

after correcting for background darkening using the blank values, following the method described by Li et al. (2013).

Antibacterial activity assay

To evaluate the antimicrobial (AB) activity of the beetle hemolymph, we adhered closely to the protocol outlined in reference (Khan, Prakash and Agashe, 2016). We measured inhibition zones produced by the whole-body homogenate of beetles from different temperature groups on a lawn of Bt bacterial growth. We prepared individual beetle whole-body homogenates using 30 µL of sterile filtered Bis-Tris buffer (0.1 M, pH 7.5, Sigma-Aldrich Ltd), supplemented with 0.01% phenylthiourea acid (PTU; Sigma-Aldrich ltd) to impede melanization. After centrifuging the homogenates at 7500 rpm at 4˚C for 10 minutes, we isolated the supernatant on ice. We created LB agar plates by using an overnight Bt culture in LB Broth at 30˚C until it reached an optical density of 1.0. Simultaneously, we autoclaved LB broth with 1% agar and allowed it to cool in a water bath at 45˚C. The Bt bacterial culture was added to the agar medium and thoroughly mixed, resulting in a final OD concentration of 0.001. We poured 6 mL of the mixture into each 75 mm Petri dish, swirling constantly for a homogeneous distribution of the bacterial culture. Next, we punched five wells (2x2 mm diameter) into each plate and added 10 µl of beetle homogenate to each well. As a positive control, 2 µl of Kanamycin (2 mg/ml) was added to every plate. The plates were incubated at 30˚C for 16 hours, after which we measured the clear zones of inhibition around the wells. The mean value, calculated by averaging the horizontal and vertical diameters around each well, served as a proxy for AB activity. Due to the non-normal distribution of the data, we employed a non-parametric Wilcoxon rank sum test for analyzing AB activity.

Immune gene expression via RT-qPCR

To extract whole-body RNA from each pooled beetle sample (3 individuals per pool), we used the Qiagen RNeasy kit, with a final elution volume of 50 µL of nuclease-free water. For cDNA synthesis, we added 1 µL of RNA into a 9 µL reaction of the SuperScript IV VILO master mix from ThermoFisher Scientific, using the manufacturer-recommended protocol. The resultant cDNA was then diluted with 80 µL of nuclease-free water. Subsequently, we performed RT-qPCR analyses using the PowerUp SYBR Green master mix (Applied Biosystems) on a Biosystems QuantStudio 6 Flex instrument. The thermal cycling protocols commenced with a 2-minute denaturation at 95°C, followed by 40 cycles of 95°C (15 seconds), 55°C (10 seconds), and 60°C (1 minute). Duplicate samples were processed for each specimen, with the average Ct value utilized for subsequent assessments, unless the difference between technical replicates exceeded 1 Ct. Discrepancies beyond this threshold necessitated repeated measurements.

We measured the expression level of AMPs including *cecropin-2 (cec-2), defensin-2 (def-2)*, *defensin-3 (def-3)*; Toll pathways negative regulator,*cactus*, and heat-stress associated protein, *heat shock protein 27* (*hsp27*) **(**Table 1**)** using RT-qPCR. Genes were selected based on their association with different immune pathways in *T. castaneum*. Reference gene expression was determined using the ribosomal protein S18 (*rsp18*) primer pair, as established by (Lord *et al.*, 2010) which demonstrated consistent *rps18* expression throughout infections. To assess the gene expression, we used the Δct method where we quantified the relative expression of each gene on a log2 scale by subtracting the mean ct value of the target gene from the mean ct value of the reference gene (Schmittgen and Livak, 2008). The Δct method keeps the value in a log scale, and thus any effect sizes need to be interpreted accordingly.

Table 1. Primers used to determine immune gene expression in *T. castaneum*

Primer Set	Full Name	Function	Forward Primer Sequence	Reverse Primer Sequence	AT (°C)
18S	*rps18s*	*Ribosomal protein*	CGAAGAGGTCGAGAAAATCG	CGTGGTCTTGGTGTGTTGAC	55
Cec-2	*Cecropin 2*	Toll AMP	TAATGTGTTTTGTTCAAGTGATGGC	CAGCTCCTTCGGCCCACT	55
Def-2	*Defensin 2*	IMD AMP	GCTTTTCCTACAGATGGAGAACAC	AAAGAGGCAATGGGTCGCAC	55
Def-3	*Defensin 3*	Toll/IMD AMP	TTGCAATCACTGCTTACCCAC	ACCCGGATATGTGCCACTTG	55
Cact	*Cactus*	Negative regulator	TGGAACTACGAAGGTCAGACG	CTATGGAGTAGTGGAGGGCG	55
HSP27	*Heat-shock Protein 27*	Chaperone protein	CATGACGTGAATCGGGTGGA	TCTCCTGCTCCTTCCCATCG	55

Data analysis

For each batch of beetles, across various temperatures, sexes, and treatment (Bt. infected & sham infected) groups, we analyzed the survival data with a mixed-effects Cox proportional hazards model, using the R package 'coxme' (Therneau, 2024). We specified the model as: survival ~ temperature x sex x treatment + (1|replicate numbers), with temperature and sex as a fixed effect and replicate numbers as a random effect.

We found that residuals of AB & PO activity data were non-normally distributed when tested using Shapiro–Wilks's test. Hence, we first log-transformed the data and then confirmed that the log-transformed residuals were still non-normally distributed. Subsequently, we used a non-parametric one-way ANOVA (Kruskal-Wallis test) to test the effects of temperature, on male and female adult beetles separately following Sham & heat-killed Bt exposure. We specified the model as: survival ~ temperature * treatment * sex * (1|replicates), with 'temp', 'treatment' and 'sex' and their interactions as fixed effects, and 'replicate' as a random effect.

To examine whether exposing beetles to different temperatures was associated with the lower expression of some genes (including AMPs, negative immune regulator and stress-related), we estimated their relative expression level in naïve beetles, beetles exposed to heat-killed Bt and sham-infected individuals at 6 hours post-exposure, by using qPCR (as outlined in Critchlow et al., 2024) (n= Total 15 beetles in a group of 3 homogenized in 350 µl reagent/ treatment/ temperature and sex combination). We ensured the normality of data by examining the standardized residuals using histograms and Q-Q plots. We performed a general linear model analysis (GLM) on gene expression using treatment, temperature, sex, and their interaction as factors with the lme4 package in R (v. 4.3.0). A significant effect for treatment indicates a difference in gene expression magnitude between treatments. To investigate the impact of temperature on each immune gene such as target AMPs, negative regulator *cactus*, and a heat-shock protein we employed a linear model (expression ~ temperature x treatments x sex), where "temp" is a continuous variable, and the treatment group ‘naïve’ serves as a reference point (baseline expression).

RESULTS

Elevated temperature increases the overall mortality of *T. castaneum* upon infection by *B. thuringiensis*

To determine the effect of temperature on host survival we acclimatized virgin male and female beetles at 22, 26, 30, 34, and 38°C for 12 days. To check their survival, we infected them by stabbing them, using a micro needle, with an LD50 dose of live Bt which usually kills the beetles within 12 hours. We recorded the mortality of beetles 4-24 hours post-infection. The overall survival percentage of Bt-infected *T. castaneum* was temperature dependent, being the highest at 22°C (53.13%) and the lowest at 38°C (10.94%); however, control groups (insect saline stabbed) in all five temperatures showed 100% survival (Fig. 3). This shows a positive correlation between temperature and beetle mortality where raising the temperature significantly increased beetle mortality ($\chi^2 = 84.1236$, df = 1, $p < 0.001$). Moreover, the beetles at lower temperatures died at a significantly lower rate compared to higher temperatures. At 22°C the beetles started dying 12 hours post-infection and continued dying at a slower pace until the 24th hour (Fig. 3). On the other hand, beetles at higher temperatures, such as 34 and 38°C, started dying from the earliest time point, 6-7 hours post-infection, and the peak mortality was observed between the 6th and 10th hour at 34°C and between 6th to 9th hours post-infection at 38°C. Although, these trends were similar in both sexes, a considerably larger proportion (80-90%) of females died at 30, 34, and 38°C compared to the males (55-60%) at 30 and 34°C, suggesting that females beetles are more susceptible to infection at higher temperatures (Fig. 4). Further analysis also showed that sex marginally significantly influenced beetle survival ($\chi^2 = 3.7514$, df = 1, $p = 0.052$), supporting the observation of heightened mortality in female *T. castaneum*.

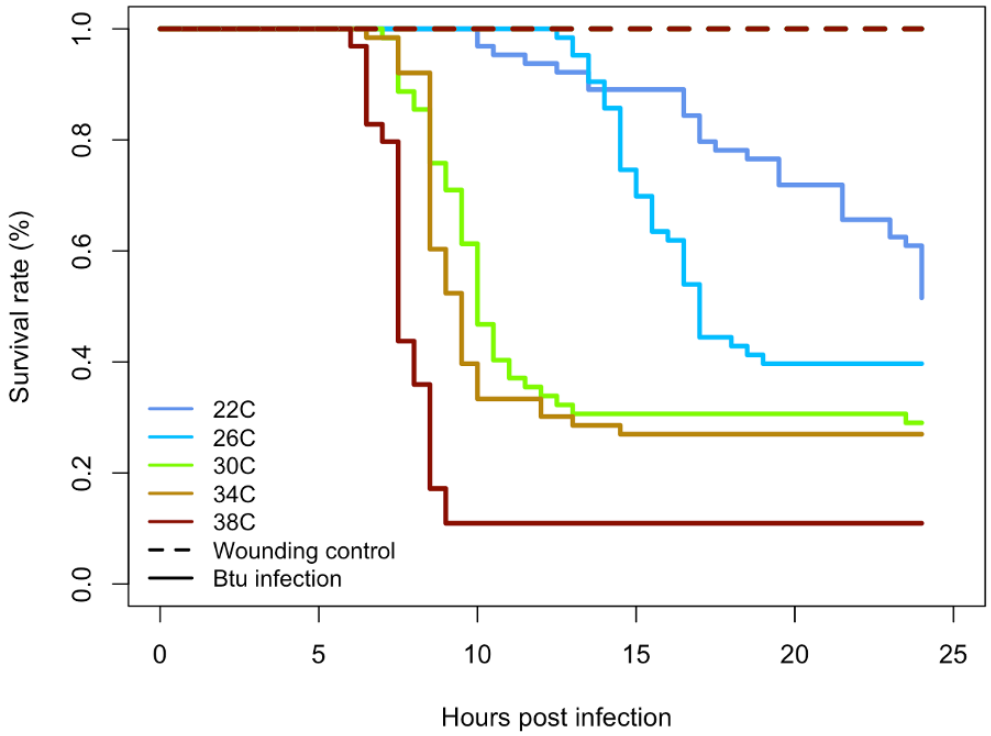

Figure 3. The survival rate of Bt infected *T. castaneum* at different temperatures. Cornflower blue, deep sky blue, light green, gold, and dark red indicate survival at 22, 26, 30, 34, and 38°C, respectively. Bt infected group and wounding control (insect saline stabbed) were indicated by the solid and dashed line, respectively. N = 400 (40 beetles/sex/temperature).

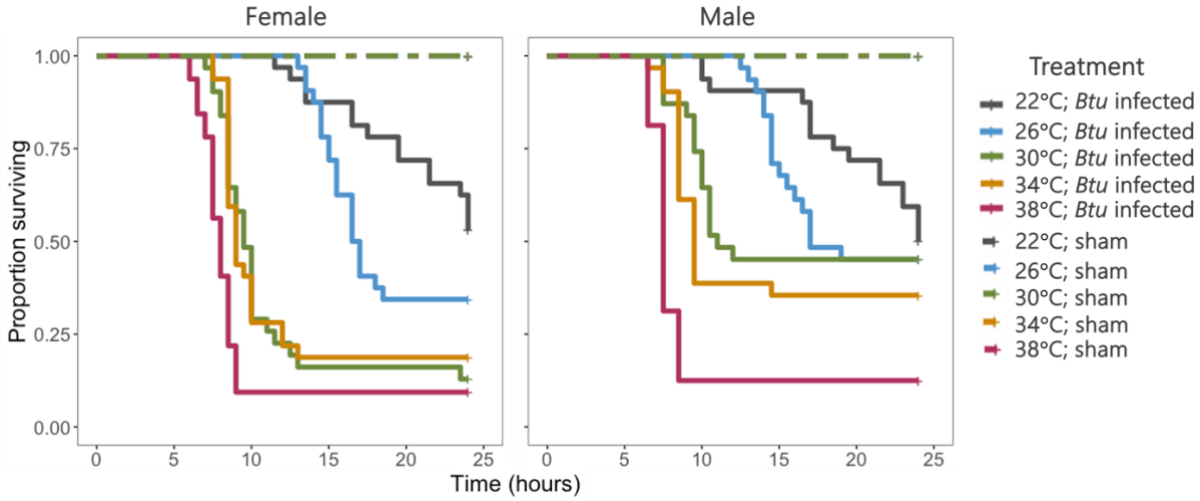

Figure 4. Effect of sex on the survival rate of Bt infected *T. castaneum* at different temperatures. All the beetles survived in wounding control. Black, cornflower blue, green, gold, and magenta color indicate survival at 22, 26, 30, 34, and 38°C, respectively. N = 400 (40 beetles/sex/temperature).

Table 2. Summary of mixed effects Cox model, fitting the model to estimate response to temperature and Bt infection in *T. castaneum* for males and females. We specified the model as: survival ~ temperature*treatment*sex* (1|replicates), with temp & sex as fixed effects and replicate ID as random effects. The table shows model output (ANOVA) for survival post-infection for *T. castaneum* beetles across temperatures following Bt systemic infection.

Source	**loglik**	**chi sq.**	**df**	**p**
treatment	-1231.3	77.3992	1	<0.001
sex	-1229.4	3.7514	1	0.052
temp	-1187.3	84.1236	1	<0.001
treatment:sex	-1187.2	0.2307	1	0.63
treatment:temp	-1185	4.3932	1	0.03
sex:temp	-1185	0.0085	1	0.92
treatment:sex:temp	-1185	0.0005	1	0.98
random effects	***std dev***	***variance***		
Replicate	0.111	0.012		

Effect of temperature on phenoloxidase activity

Phenoloxidase (PO) enzyme is a very crucial component of the insect immune system. Insects use serine proteases to cleave prophenoloxidase into active PO that oxidizes tyrosine to form melanin and clears the infection through encapsulation (Tang, 2009). To determine the effect of temperature on PO activity we incubated beetles at five different temperatures, infected them with sterile insect saline and heat-killed Bt treatment and analyzed the collected hemolymphs for PO activity. We found no significant difference in PO activity of naïve, sham, and heat-killed groups at different temperatures. However, GLM analysis revealed a significant effect of sex on PO activity ($F = 8.306$, $p = 0.004$), indicating that sex has a substantial influence on the enzyme's activity. Surprisingly, there was no significant effect of temperature ($F = 0.102$, $p = 0.74$) or treatment ($F = 1286.67$, $p = 0.9$) on PO activity (Fig. 5). This suggests that variations in temperature and treatment did not significantly impact PO activity in the red flour beetle. The interaction between sex and temperature ($F = 2.048$, $p = 0.15$), sex and treatment ($F = 0.531$, $p =$

0.58), temperature and treatment (F = 1.102, p = 0.33), and the three-way interaction of sex, temperature, and treatment (F = 0.703, p = 0.49) were not statistically significant.

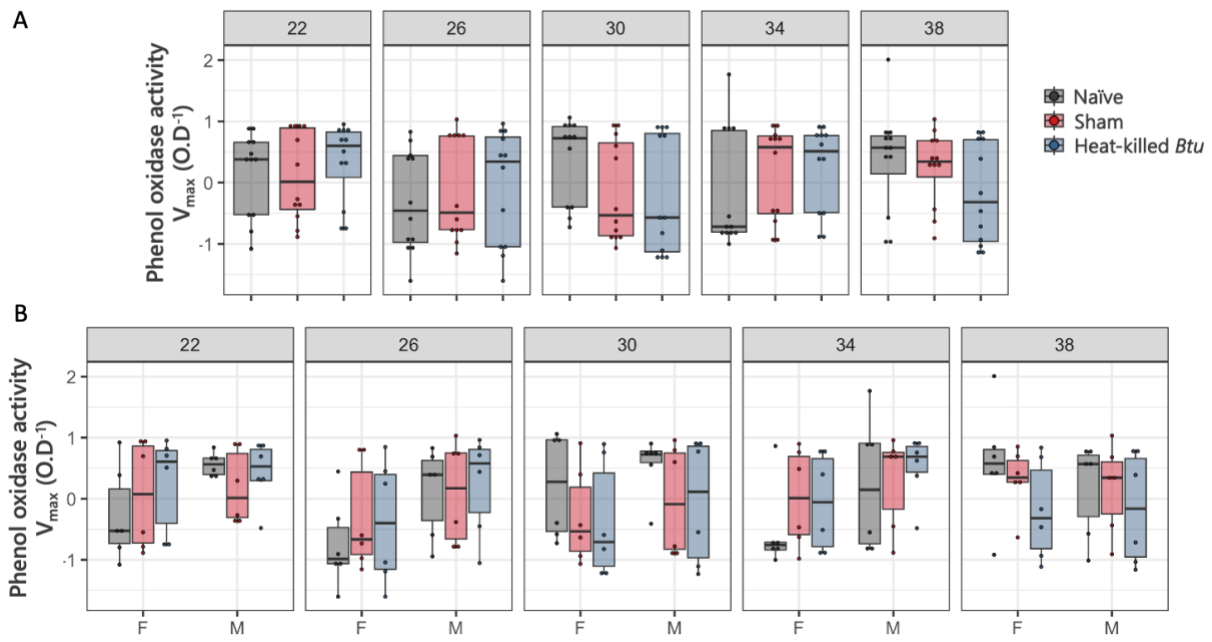

Figure 5. PO activity of naïve, sham (insect saline treated), and heat-killed Bt treated red flour beetle at 22, 26, 30, 34, and 38°C, collected 6 hours post-infection. (A) PO activity in male and female beetles combined and (B) separately. Overall, females had a significantly lower PO activity compared to males. Sample size: 6 individual beetles/sex/treatment.

Table 3. Effect of temperature on PO activity in *T. castaneum*. We used GLM to analyze the PO activity data using a negative binomial distribution (due to the non-normal distribution of our data tested using Shapiro test). Subsequently, we used a non-parametric one-way ANOVA (Kruskal-Wallis test) to test the effects of temperature, on male and female adult beetles separately following Sham & heat-killed Bt exposure. We specified the model as: survival ~ temperature*treatment*sex*(1|replicates), with 'temperature', 'treatment' and 'sex' and their interactions as fixed effects, and 'replicate' as a random effect.

Source	Nparm	df	Sum of sq.	f ratio	p
sex	1	1	51595.2	8.306	0.004
temp	1	1	633.765	0.102	0.74
sex*temp	1	1	12720.9	2.048	0.15
treatment	2	2	1286.67	0.103	0.9
sex*treatment	2	2	6602.14	0.531	0.58

temp*treatment	2	2	13700.2	1.102	0.33
sex*temp*treatment	2	2	8740.29	0.703	0.49

Effect of temperature on antibacterial activity

To determine the effect of antibacterial (AB) activity in the insect hemolymph, we exposed the beetles to different temperatures and used hemolymph to determine its ability to prevent Bt growth in a solid media through a lytic zone assay. The AB activity data, assessed through the lytic zone assay, underwent a full model GLM analysis to examine the effects of temperature, treatment, sex, and their interactions on the observed activity. Surprisingly, we did not find a significant effect of temperature on the AB activity of the beetles ($F = 0.2065$, $p = 0.65$), suggesting that temperature variation did not significantly influence AB activity (Fig. 6), although there was variation in AB activity at different temperatures. Similarly, neither treatment alone ($F = 1.1639$, $p = 0.31$), nor the interaction between temperature and treatment ($F = 0.1055$, $p = 0.89$) yields a significant effect suggesting antibacterial activity does not differ in naïve, sham, or heat-killed Bt treated beetles (males & females) exposed to varying temperature (Fig. 6) which is contradictory to other studies. Although sex alone did not show a significant effect on antibacterial activity ($F = 0.0445$, $p = 0.83$), there was a marginal interaction between temperature and sex ($F = 2.8068$, $p = 0.095$), suggesting a potential trend worth further exploration.

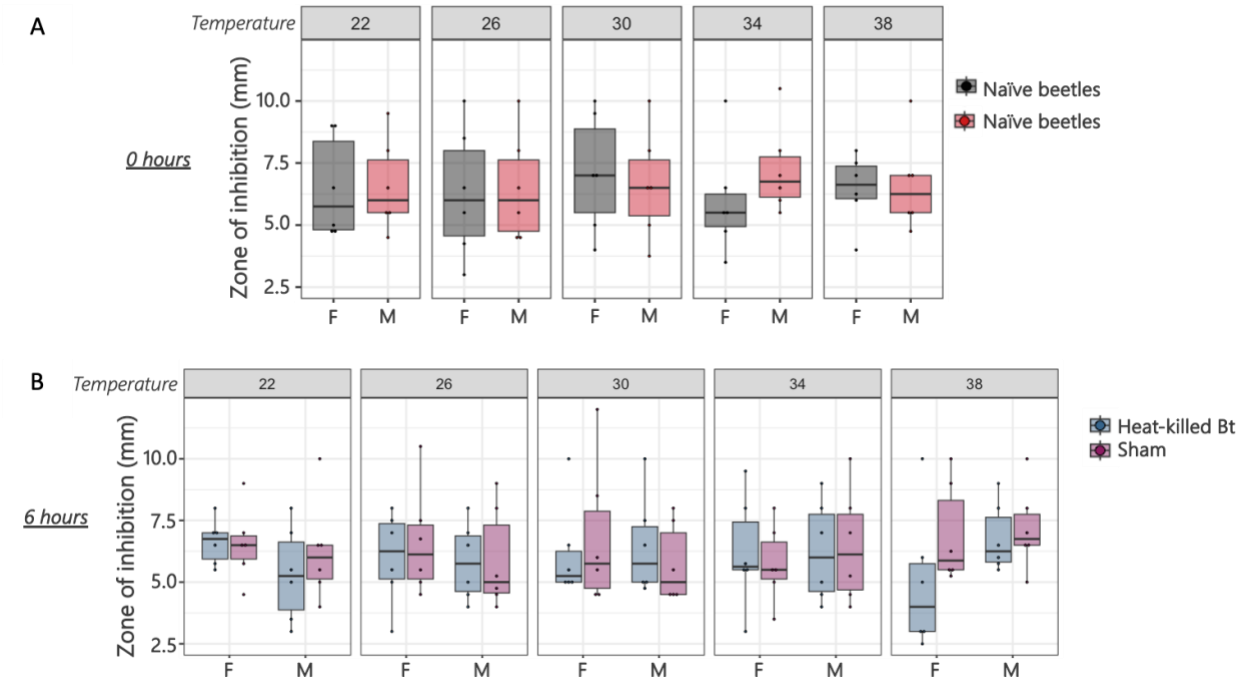

Figure 6. AB activity of naïve, sham (insect saline), and heat-killed Bt treated red flour beetle at 22, 26, 30, 34, and 38°C. (A) AB activity in male and female naïve beetles. (B) AB activity of male and female sham (insect saline) and heat-killed Bt treated beetle at the 6 hours post-injection timepoint. Sample size: 6 individual beetles/sex/treatment.

Table 4. Effect of temperature on AB activity in *T. castaneum*. We used GLM to analyze the AB activity data using a negative binomial distribution (due to the non-normal distribution of our data tested using Shapiro test). Subsequently, we used a non-parametric one-way ANOVA (Kruskal-Wallis test) to test the effects of temperature, on male and female adult beetles separately following Sham & heat-killed Bt exposure. We specified the model as: survival ~ temperature * treatment * sex * (1|replicates), with 'temperature', 'treatment' and 'sex' and their interactions as fixed effects, and 'replicate' as a random effect.

Source	Nparm	df	Sum of sq.	f ratio	p
temp	1	1	0.7793	0.2065	0.65
treatment	2	2	8.784	1.1639	0.31
temp*treatment	2	2	0.7961	0.1055	0.89
sex	1	1	0.168	0.0445	0.83
temp*sex	1	1	10.591	2.8068	0.095
treatment*sex	2	2	1.9298	0.2557	0.77
temp*treatment*sex	2	2	4.5899	0.6081	0.54

Effect of temperature on immune gene expression

The main defense against pathogens is the immune system that recognizes and immediately responds to infection by activating immune pathways to initiate AMP production. To determine the effect of temperature on host immune response and stress level, we exposed the beetles to different temperatures, challenged them with insect saline control and heat-killed Bt, and determined the expression of *cec-2, def-2, def-3, cactus,* and *hsp27* using RT-qPCR. *Cec-2, def-2,* and *def-3* did not show a significant difference between the expression of sham and heat-killed Bt treatment. Though *cactus* expression remained the same between the two treatments (sham and heat-killed Bt) from 22-34°C, a marginally significant downregulation ($\chi^2 = 3.6923$, df = 1, p = 0.054) was observed in heat-killed treatment compared to the sham at 38°C. Despite not showing any significant effect of temperature between treatments, *def-3* showed a trend of better immune response at lower temperatures which declined with increasing temperature. On the contrary, *hsp27* expression was significantly upregulated in naïve beetles with the increase of temperature, particularly at 38°C ($\chi^2 = 18.1720$, df = 1, p = 0.0011), indicating increasing heat stress in beetles exposed to a higher temperature (Fig. 7).

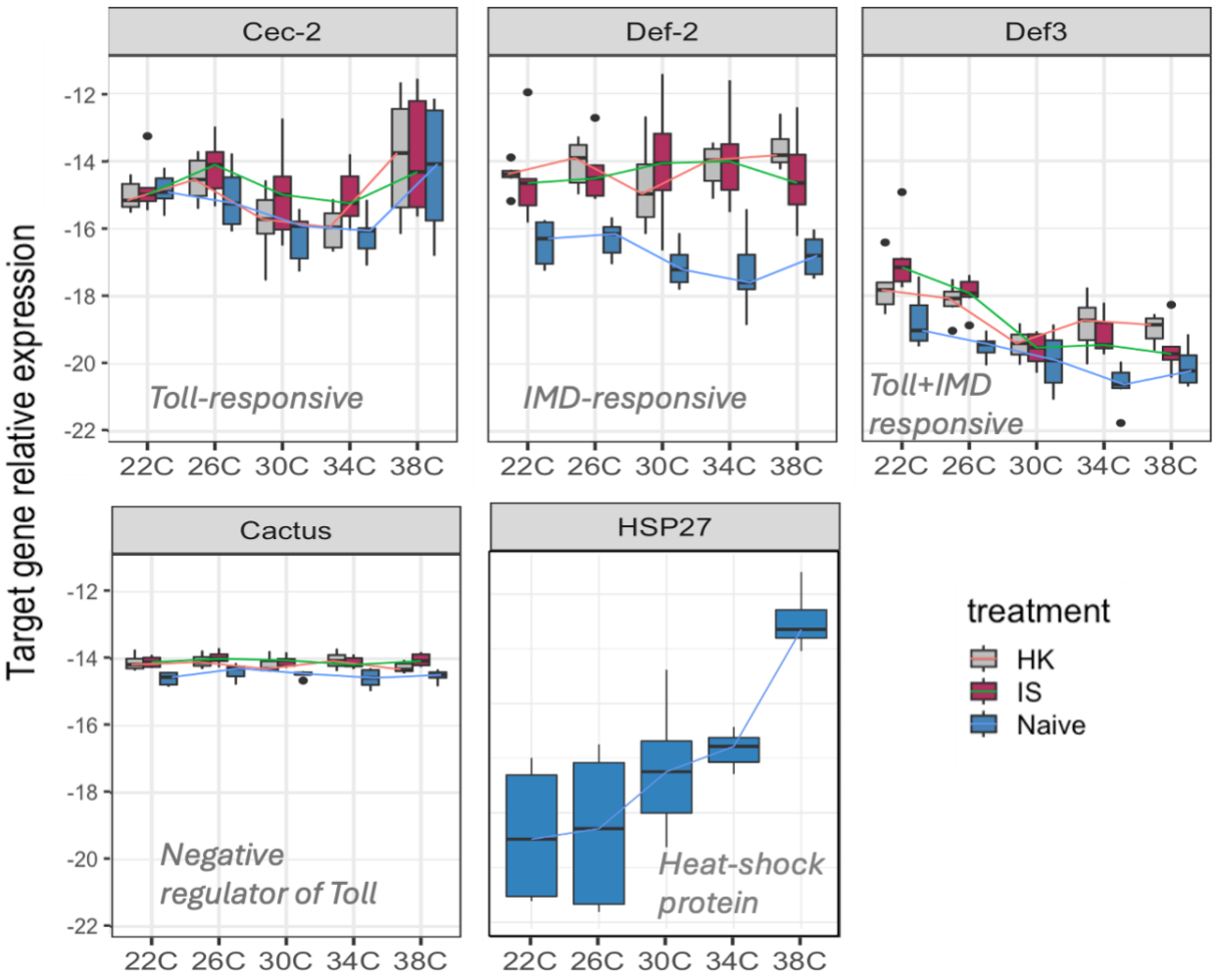

Figure 7. Expression level of *cecropin-2*, *defensin-2*, *defensin-3*, *cactus*, and *hsp27* of naïve, sham (insect saline) and heat-killed Bt treated beetle at 22, 26, 30, 34, and 38°C. The figure shows a relative expression of the target genes measured by ΔCt value. Blue, red, and gay bars represent expression levels of naïve, insect saline, and heat-killed Bt treatment groups, respectively, at different temperatures. Sample size: 3 (each a pool of 3 beetles)/sex/temperature.

Table 5. Effect of temperature on gene expression of *T. castaneum*. We used GLM to analyze the gene expression data using a negative binomial distribution (due to the non-normal distribution of our data tested using the Shapiro test). We analyzed the data using 'treatment' (sham-infection vs. heat-killed) as a fixed effect across 5 different temperatures following infection. We used gene expression of only naïve beetles for *hsp27* to show comparative heat-stress at different temperatures.

Target gene	Treatment	Temperature	chi sq.	df	p
cactus	IS vs HK	22°C	0.641	1	0.423
		26°C	0.641	1	0.42

		30°C	1.641	1	0.2
		34°C	0.4103	1	0.52
		38°C	3.6923	1	0.054
cecropin-2	IS vs HK	22°C	0.2308	1	0.63
		26°C	0.641	1	0.423
		30°C	0.641	1	0.42
		34°C	2.0769	1	0.14
		38°C	0.1026	1	0.74
defensin-2	IS vs HK	22°C	2.0769	1	0.14
		26°C	1.2564	1	0.26
		30°C	0.4103	1	0.52
		34°C	0.0256	1	0.87
		38°C	1.641	1	0.2
defensin-3	IS vs HK	22°C	2.0769	1	0.14
		26°C	0.9231	1	0.33
		30°C	0.1026	1	0.74
		34°C	0.641	1	0.42
		38°C	2.5641	1	0.1
hsp27	Naive	All	18.1720	1	0.0011

Temperature rise facilitates faster Bt growth by decreasing the log phase doubling time

Temperature is an abiotic factor that can significantly affect the growth rate of bacteria or make them more virulent. To test that we cultured the Bt in vitro at different temperatures and determined the growth rate of the bacteria by analyzing the OD value collected every 30 minutes for 24 hours. We found that temperature rise greatly increases bacterial growth by reducing the doubling time of Bt. The log phase doubling times of Bt were 64, 42, 32, 25, and 21 minutes at 22, 26, 30, 34, and 38°C, respectively (Fig. 8). Moreover, the required time to reach OD value 1 was 14 and 11 at 22 and 26°C, respectively, and in rest of the temperatures (30, 34, and 38°C) it reached to OD value 1 at 7^{th} hour (Fig. 9), which roughly matches the starting point of beetle death in respective temperature. This suggests that the Bt density at OD value 1 is the possible threshold level that initiates host killing. We used GLMs to analyze the effects of time (in hours)

and temperature (in degrees Celsius) on the growth of Bt. The GLM analysis revealed significant main effects for both temperature and time on Bt growth where the Bt growth is significantly increased ($F = 41.6168$, $p < 0.001$) with increasing temperature and time ($F = 1393.07$, $p < 0.001$). Additionally, the interaction between time and temperature was found to be statistically significant ($F = 3.7252$, $p = 0.0003$), indicating that the relationship between time and temperature is not additive but rather dependent on each other. Further analysis of the temperature data showed significant effects of temperature on growth when considering time as the predictor variable. At 22°C, there was a significant increase in growth over time ($F = 395.78$, $p < 0.001$), followed by similar trends at higher temperatures, including 26°C ($F = 234.97$, $p < 0.001$), 30°C ($F = 103.15$, $p < 0.001$), 34°C ($F = 163.04$, $p < 0.001$), and 38°C ($F = 44.286$, $p < 0.001$).

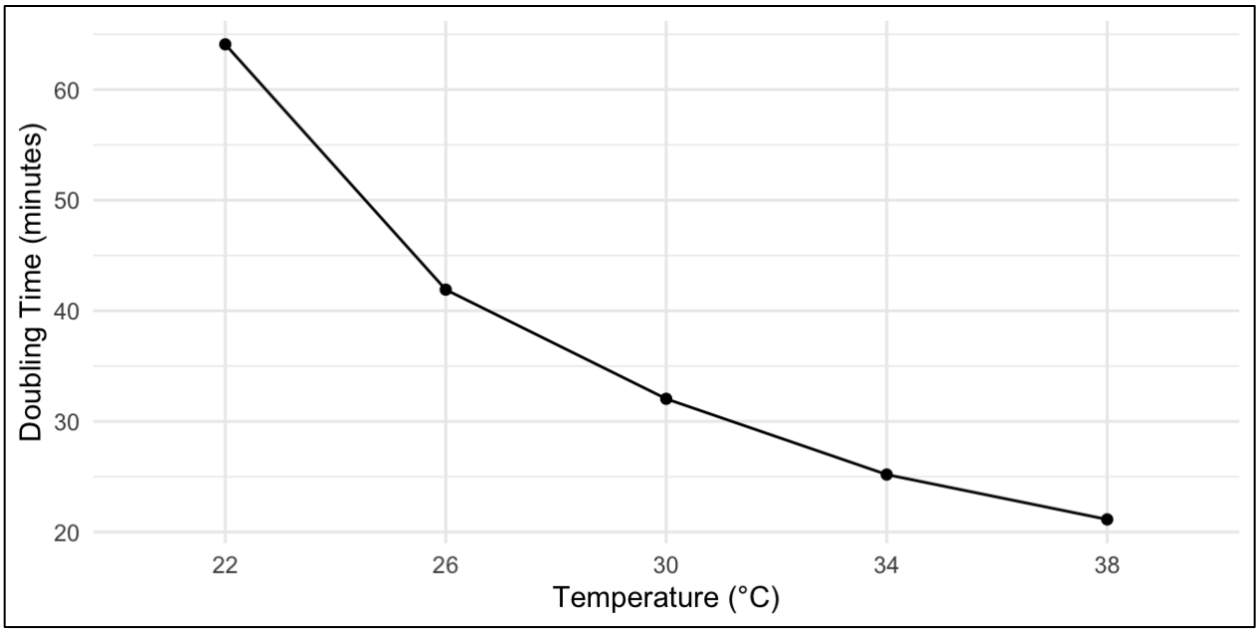

Figure 8. Log phase doubling/replication time of *B. thuringiensis* at different temperatures.

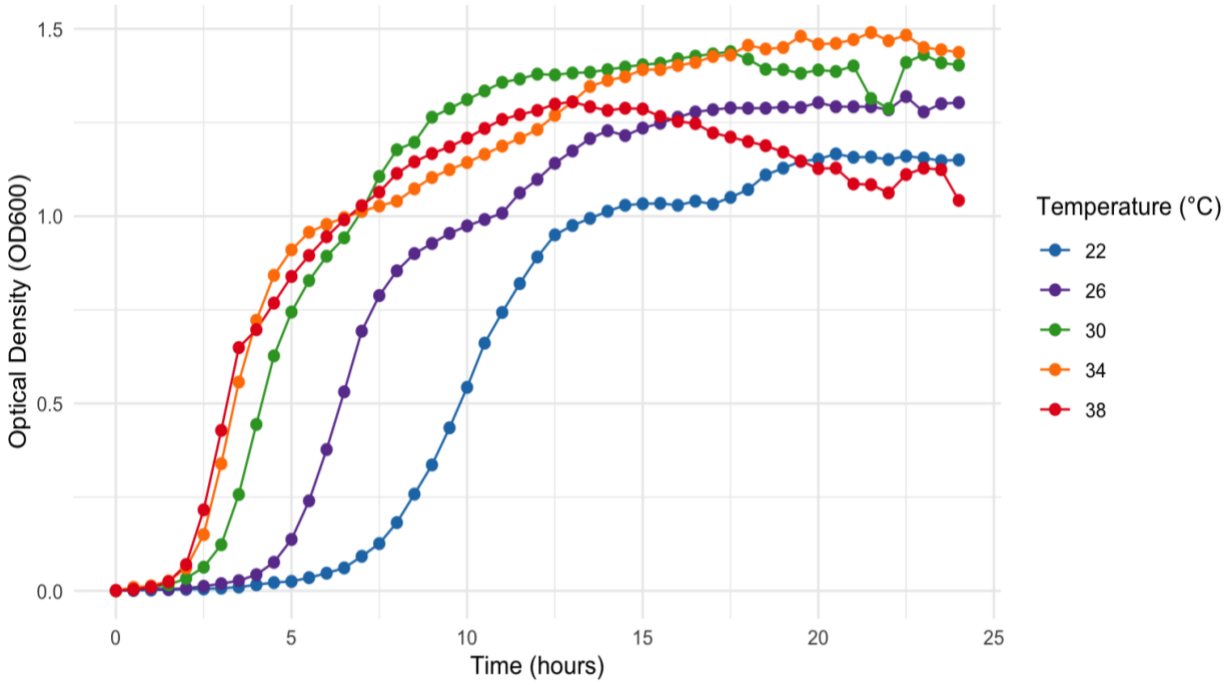

Figure 9. *In vitro* growth of Bt at 22, 26, 30, 34, and 38°C. Each dot represents the OD value recorded in every 30-minute interval.

Table 6. Effect of temperature on in vitro growth of *Bacillus thuringiensis*. We used GLM to examine the relationship between the response variable and the predictors "time" (time in hours), temperature, and their interaction "time*temperature".

Source	df	Sum of sq.	f ratio	p
time	1	72.629	1393.07	<0.001
temperature	8	17.357	41.6168	<0.001
time*temperature	8	1.5537	3.7252	0.0003

Table 7. Effect of temperature on in vitro growth of *B. thuringiensis*. This table displays Bt log phase doubling time at different temperatures. The statistics represent the results of an ANOVA test examining the effect of temperature on the time taken (in minutes) for replication of Bt.

Temperature	Doubling time (minutes)	Source	df	Sum of sq.	f ratio	p
22C	64.09	time	1	10.248	395.78	<0.001
26C	41.91	time	1	10.428	234.97	<0.001
30C	32.04	time	1	8.2621	103.15	<0.001
34C	25.19	time	1	8.1273	163.04	<0.001
38C	21.14	time	1	3.6201	44.286	<0.001

DISCUSSION

Effect of temperature on host survival

Parasites usually have a broad thermal limit (Cohen *et al.*, 2017); whereas insects have a very short tolerance to temperature variation (García-Robledo *et al.*, 2016). Therefore, understanding the host-microbe dynamic in a changing climate, particularly at elevated temperatures is crucial to predict the survival risk of different species in a warmer world. Although some studies explored the effect of temperature on insect immune response, the findings did not show any universal trend in all insects requiring further investigation (Adamo and Lovett, 2011; Hunt *et al.*, 2016; Tang *et al.*, 2016; Adamo, 2017; Liu *et al.*, 2019; González-Tokman *et al.*, 2020). We investigated the effect of temperature variation on the host-microbe interaction dynamics and the ultimate survival of the host, in a *T. castaneum-B. thuringiensis* model. Here, we showed that elevated temperature is not only detrimental to host immune response and exerts heat stress, but also induces disease-induced mortality leading to a significant decline in host survival.

In this study, we exposed the red flour beetle adults to 22, 26, 30, 34, and 38°C and septically infected them with Bt and recorded their survival 4-24h post-infection. According to the thermal mismatch hypothesis, the host would be vulnerable to infection at temperatures where the performance gap between the host and parasites is the greatest (Cohen *et al.*, 2017). This suggests that beetles in either direction (toward 22°C or 38°C) from their initially assumed thermal optima, 30°C for our lab-grown beetles, would have reduced survival. In this study, we showed that higher temperature, particularly 38°C, greatly reduce immune response and consequently the survival of red flour beetles infected with Bt. We also found a significant

increase in mortality rate at each increase in temperature suggesting a positive correlation between temperature and beetle mortality. On the other hand, *in vitro* Bt growth showed maximum growth rate at 38°C which imply that the thermal optima for Bt is possibly 38°C rather than 30°C. These findings partially support the thermal mismatch theory where the largest performance gap between the host immune response and pathogen growth was apparent at 38°C, leading to heightened susceptibility to infection and a significant rise in mortality. Interestingly, we found a marginally significant difference between the survival of male and female beetles where females showed lower survival, in most temperatures, implying that females are more susceptible to infection, particularly at higher temperatures. This could be because females must expend resources toward reproduction activity, such as producing eggs, leading to an insufficient resource allocation toward fighting stress and infection (Schwenke, Lazzaro and Wolfner, 2016). We also found that beetles at the lowest temperature group (22°C) maintained the slowest rate of mortality until the end of 24 hours and had the lowest proportion of death in both sexes, however, in the rest of the temperatures, the beetles not only started to die at earlier time points but also died at significantly faster rate with increasing temperature indicating elevated temperature substantially shortens the survival time of infected beetles. A similar result was also observed in fruit flies, where flies exposed to lower temperatures survived longer than the ones exposed to higher temperatures (Linder et al., 2008). Since temperature did not influence the survival of control beetles (insect saline stabbed, 100% survival), the increased mortality rate at a high temperature can only be attributed to heat stress, a decline in host immune response, and/or the increased growth rate of Bt.

Effect of temperature on humoral immune response

To better understand the effect of temperature on the constitutive and inducible immune response, we assessed the PO and antibacterial activity and examined the immune gene expression at different temperatures. Although temperature had no effect on PO and AB activity, we found a significant downregulation of PO activity in females. This finding suggests that females are more susceptible to the infection and possibly one of the reasons why the mortality is significantly higher in female beetles. In insects, PO is a key enzyme for thermoregulation, pigmentation, and immunity that are essential to maintain homeostasis (Gourgoulianni *et al.*, 2023) Therefore, reduced PO activity indicates a weaker immune system that makes the host vulnerable to stress and infection that again supports the heightened mortality in females. We expected a significant rise in PO and AB activity 6h post-infection in heat-killed Bt treatment compared to naïve and sham control (insect saline stabbed); however, we found no difference in PO and AB activity between the three groups. This could be due to the quick upregulation of PO and AB activity that cleared infection (with heat-killed Bt) to come back to its basal level. This is inconsistent with the findings of (González-Rete *et al.*, 2019) where infected *M. pallidipennis* fifth instar nymphs showed significant upregulation in PO activity compared to the uninfected control group, irrespective of temperature (20, 30, and 34°C), although there was a downregulation in PO activity at high temperature. Conversely, the cricket, *Gryllus texensis*, showed enhanced PO and AB activity when exposed to higher temperatures (Adamo and Lovett, 2011) species-specific effect of temperature on the immune-related enzymes.

Effect of temperature on immune gene expression

We quantified the expression of four immune genes (*cecropin-2*, *defensin-2*, *defensin-3*, and *cactus*) to determine the effect of temperature variation on the inducible immune response,

as this could potentially explain the temperature-dependent differences in mortality. As expected, *def-2, def-3,* and *cactus* expressions were upregulated in both the sham and Bt infected group compared to the naïve, indicating the activation of Toll and IMD pathways leading to the production of AMPs. Surprisingly there was no significant difference between sham and heat-killed Bt treatment, except for *cactus* at 38°C, where the expression marginally significantly downregulated. *Cactus* is a negative regulator of the Toll pathway that protects the host from self-damage by controlling the immune response (De Gregorio *et al.*, 2002). However, the downregulation of *cactus* at 38°C indicates lower control over the immune system leading to immunopathology which could aid in resulting the highest mortality at 38°C in both sexes.

We also expected a lower immune response at high temperatures for all other genes; however, only *def-3* expression was downregulated at higher temperatures. Similar heat-induced downregulation in immune gene expression has also been observed in the silkworm, *Bombax mori* (Tang *et al.*, 2016) suggesting a common effect of temperature in many insects. Many insects such as fruit flies and western flower thrips occupy habitats with cooler temperatures to avoid the onslaught by the pathogen, whereas their uninfected counterparts occupy comparatively warmer habitats. This behavior is called as anapyrexia and is known to improve the survival of the infected host (Hunt *et al.*, 2016; Liu *et al.*, 2019). Additionally, as the bacteria multiply and spread throughout the body very quickly at high temperatures, they could initiate an extensive immune response against the pathogen which could cause immunopathology leading to reduced functionality of essential organs (Sadd and Siva-Jothy, 2006) which could contribute to beetle mortality at 38°C. Moreover, at high temperatures resources could be shifted as a response to heat stress in the production of molecules like heat-shock proteins (HSPs) which results in

lesser resources for the immune system (González-Tokman *et al.*, 2020). This implies that heat could be immunocompromising by suppressing the immune response but enhancing the metabolism and stress response network activity in the insect (Adamo, 2017).

Sham and Bt treatment exhibited no difference in expression, which could be due to a quick upregulation of the immune genes that cleared the MAMPs (heat-killed Bt) and came to the level of wounding control (sham). It could also be that the initial dose was insufficient to elicit an immune response in the Bt treatment group, although this dose has proved effective in the past (Critchlow et al., 2024). This suggests that temperature may have different effects on different immune genes. In summary, the RT-qPCR data analysis of immune genes revealed complex interactions between target genes, temperature, and treatment, indicating differential regulation of gene expression under varying experimental conditions. These findings provide valuable insights into the molecular mechanisms underlying gene expression responses in the context of different treatments and temperatures, which are crucial for understanding gene regulation dynamics in biological systems.

Heat stress, a contributing factor to beetle mortality

As heat stress could be an attributing factor to beetle mortality, we measured the expression level of the *hsp27* gene in naïve beetles exposed to different temperatures. HSP27 is a protein chaperone and a biomarker of heat stress (and disease) that also protects organisms from heat-induced oxidative stress by acting as an antioxidant (Wrońska and Boguś, 2020). We found a significant upregulation in *hsp27* expression at 38°C suggesting increased heat stress in beetles. Studies suggest that heat could induce a shift of resources toward a response to heat stress by

upregulating HSP expression resulting in less resource for growth, development, and possibly immune response (González-Tokman *et al.*, 2020; Ferguson and Adamo, 2023) which could be a contributing factor to the heightened mortality at 38°C. Elevated temperature not only induces heat stress but also increases metabolism and consequently, reactive oxygen species (ROS) production leading to oxidative damage (Zhu *et al.*, 2017b). This could be particularly true for 38°C which showed a significantly high mortality compared to other temperature groups. The 100% beetle survival in all control groups in all temperatures indicates that the beetles at high-temperature groups are not dying from heat stress alone, but due to Bt infection, compromised immune response, or a combination of all three. The relative aging at different temperatures could also contribute to the high mortality rate at higher temperatures although the possibility is very low in this case since we used young adult beetles. Studies show that insects including fruit flies and the red flour beetle age faster at higher temperatures which debilitates immune functions (Miquel *et al.*, 1976; Hillyer, 2016). This could also contribute, to some extent, to the high mortality rate of beetles at high temperatures.

Correlation between Bt growth and beetle mortality

At lower temperatures, bacteria grow very slowly and take a longer time to reach the threshold number lethal to the host. Conversely, high temperatures facilitate faster bacterial growth (Qiu *et al.*, 2022) enabling the pathogen to reach the threshold in a short time and kill the host. This is particularly true for the obligatory killer pathogen Bt. The exponential/log phase doubling time showed a significant decrease with increasing temperature, which implies a positive relation between Bt growth and temperature. Moreover, our in vitro growth data of Bt showed the longest lag phase at 22°C which became shorter for each 4°C rise in temperature

being the shortest at 34 and 38°C. Due to the variation of log phase Bt doubling time, the time to reach OD value 1 also varied at different temperatures. The time to reach OD value 1 was close to the starting point of beetle mortality in respective temperatures, suggesting a possible threshold of bacterial density at OD 1 that initiates beetle mortality. This also explains why the beetles at 22 and 26°C had no mortality until the 12th to 14th hour and exhibited the peak mortality between the 14th and 24th hours post-infection whereas at 30, 34, and 38°C mortality started very early at the 7th hour with a peak between 7th to 10th-hour post-infection. This suggests that temperature has a direct effect on Bt growth leading to beetle mortality and we can say that if the host immune system cannot control bacterial proliferation, the exponential growth kills the host faster in higher temperatures due to the increased growth rate of the pathogen. In the case of 30-38°C the Bt reaches a critical number within a short period resulting in a shorter survival time. The hypothesis of slower bacterial growth at low temperatures leading to increased survival time is also supported by another study by Linder et al., (2008) where different groups of fruit fly, *Drosophila melanogaster*, infected with *Pseudomonas aeruginosa* and *Lactococcus lactis* showed similar proportions of fly mortality at 17, 25, or 29°C; however, the flies at 17°C died at a significantly lower rate (slowly), for both bacterial species, compared to 25 and 29°C due to the slower bacterial growth and higher expressions of immune genes at lower (17°C) temperature (Linder, Owers and Promislow, 2008). Together, these results indicate that the higher survival of beetles at 22 and 26°C could be not only due to the slower growth of bacteria but also due to a better immune response by the hosts.

Limitation

Intrinsic factors, such as genetic constituents, could also contribute to resistance and tolerance to bacterial infection and differential gene expression in changing environmental

temperatures (Stucki, Freitak and Sundström, 2017). Therefore, it is important to use different populations of the experimental species to observe the impacts across a wide range of genetically diverse populations of the same species. Since only one population was used in this experiment, these results cannot account for the role of genetic diversity on host immune response and survival. Moreover, developmental acclimation could also influence the effect of temperature on the host immune response (Awde, Řeřicha and Knapp, 2023) and ultimately host-microbe interaction. The environment-matching hypothesis states that matching conditions in the developmental and adult stages would result in the development of the best-performing adults (Scharf *et al.*, 2015; Scharf, Galkin and Halle, 2015), which implies that developing the beetles from the egg to the adult stage in their respective temperatures could provide a more realistic outcome by maximizing the performance of experimental beetles. Given that, the adult beetles which are developmentally adapted to 30°C in our lab, should have performed the best at 30°C, compared to other temperatures. However, they neither showed a better immune response nor survived more than the lower temperature groups (22, 26°C) and thus refutes the assumption of the beneficial effect of developmental acclimation. This indicates a very complicated interaction between temperature, immune response, infectiousness of the pathogen Bt, and possibly other factors that require further investigation.

Future direction

In this study, we showed that a lower temperature facilitates a better chance of survival of an infection in the red flour beetle by slowing down the bacterial growth rate and inducing a better immune response for certain genes. However, to figure out the effect of genetic constituents and whether the immune genes upregulated quickly and then came back to the basal level, another experiment using multiple populations of *T. castaneum* could be conducted where

beetle would be septically infected with live and heat-killed bacteria and examine their comparative immune gene expression at multiple time points including 2-, 4-, 6-, and 8-hours post-infection. Moreover, a mathematical model showing the interaction between temperature, host immune response, and bacterial growth would complement the findings to a better understanding of these processes.

Even though the underlying mechanism still requires further investigation, our study provides comprehensive insights into the complex interplay between temperature, host immune responses, and pathogen virulence, elucidating the mechanisms driving temperature-mediated mortality in insects. By unraveling these intricate interactions, we advance our understanding of the ecological consequences of climate change on host-pathogen dynamics and pave the way for informed conservation and management strategies in a warming world.

CONTRIBUTION

This research endeavor has been made possible through the collaborative efforts and contributions of various individuals. In this research, Rahman and Tate conceived the idea and designed the experiments. Rahman spearheaded the implementation of the research project, data collection, analysis, and interpretation. He was responsible for day-to-day project management, including maintaining experimental beetle populations, conducting experiments, and processing samples. Rahman conducted the survival assay, *in vitro* Bt growth assay, RNA extraction, and RT-qPCR. Morgan Pfeffer assisted during the septic infection process of the beetles. Rahman and Arun Prakash conducted PO and AB activity assays. Prakash crafted the figures and performed statistical analysis. Rahman wrote the thesis with edits from Tate.

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