

The influence of immune activation on thermal tolerance along a latitudinal cline

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Abstract

Global change is shifting both temperature patterns and the geographic distribution of pathogens, and infection has already been shown to substantially reduce host thermal performance, potentially placing populations at greater risk than previously thought. But what about individuals that are able to successfully clear an infection? Whilst the direct damage a pathogen causes will likely lead to reductions in host's thermal tolerance, the response to infection often shares many underlying pathways with the general stress response, potentially acting as a buffer against subsequent thermal stress. Here, by exposing *Drosophila melanogaster* to heat-killed bacterial pathogens, we investigate how activation of a host's immune system can modify any response to both heat and cold temperature stress. In a single focal population, we find that immune activation can improve a host's knockdown times during heat shock, potentially offsetting some of the damage that would subsequently arise as an infection progresses. Conversely, immune activation had a detrimental effect on CT_{max} and did not influence lower thermal tolerance as measured by chill-coma recovery time. However, we also find that the influence of immune activation on heat knockdown times is not generalizable across an entire cline of locally adapted populations. Instead, immune activation led to signals of local adaptation to temperature being lost, erasing the previous advantage that populations in warmer regions had when challenged with heat stress. Our results suggest that activation of the immune system may help buffer individuals against the detrimental impact of infection on thermal tolerance; however, any response will be population specific and potentially not easily predicted across larger geographic scales, and dependent on the form of thermal stress faced by a host.

KEYWORDS

CT_{max} , disease, *Drosophila melanogaster*, global change, host-pathogen interactions, immune challenge, local adaptation, thermal adaptation, thermal limits, thermal stress

1 | INTRODUCTION

With both pathogen prevalence and thermal stress predicted to shift together under scenarios of global change (Altizer, Ostfeld, Johnson, Kutz, & Harvell, 2013; Lafferty, 2009; Metcalf et al., 2017), research has increasingly emphasized the detrimental impacts of infection on a population's critical thermal limits (Greenspan et al., 2017; Hector, Sgrò, & Hall, 2019; Linderman, Chambers, Gupta, & Schneider, 2012). This focus is understandable due to the inevitable physiological damage that occurs to a host during infection (i.e. virulence, Cressler, McLeod, Rozins, van den Hoogen, & Day, 2016; Day, 2002), and the negative impacts this will have on a host's ability to buffer against further stress (e.g. Cohen, Civitello, Venesky, McMahon, & Rohr, 2019; Hoang, 2001). Infection in the water flea *Daphnia magna*, for example, has been shown to cause reductions in upper thermal limits of up to 2°C as disease progresses (Hector et al., 2019). However, not all interactions between a host and pathogen will necessarily lead to a reduction in thermal tolerances. By sharing common pathways with the response to thermal stress itself (Ferguson, Kortet, & Sinclair, 2018; Sinclair, Ferguson, Salehipour-shirazi, & MacMillan, 2013; Wojda, 2017), an encounter with a pathogen, particularly those that do not elicit any pathogenicity or are rapidly cleared, may actually act to boost a host's thermal limits.

In invertebrates, the innate immune response is tied to a more general stress response, resulting in correlated responses to various biotic or abiotic stressors (Lazzaro & Little, 2009; Sinclair et al., 2013), including both the presence of pathogens, and heat and cold temperature stress (Ferguson et al., 2018; Sinclair et al., 2013; Wojda, 2017). In *Drosophila*, for example, temperature stress has been shown to cause an up-regulation in expression of immune-related genes, including those related to the antimicrobial peptide pathways (Telonis-Scott, van Heerwaarden, Johnson, Hoffmann, & Sgrò, 2013; Zhang, Marshall, Westwood, Clark, & Sinclair, 2011), whilst exposure to infectious disease increases the expression of temperature stress-related genes, such as heat shock proteins (e.g. Merklings et al., 2015). Exposure to stressful temperatures has also been shown to improve the outcome of infection in multiple host and pathogen systems, whereby cold or heat stress can increase resistance and tolerance to infection in many cases (Le Bourg, Massou, & Gobert, 2009; Marshall & Sinclair, 2011; Wojda, Kowalski, & Jakubowicz, 2009; Xu & James, 2012). Attempts by a host to clear pathogen infection, therefore, may better prepare an individual for exposure to other stressors such as heat or cold shock, at least in the period shortly after pathogen exposure.

The potential for beneficial plastic shifts in the thermal limits of populations, as mediated by pathogen exposure, could dramatically alter predictions for survival and adaptation to further environmental changes (Chown et al., 2010; Hoffmann, Chown, & Clusella-Trullas, 2012; Hofmann & Todgham, 2010). Provided that underlying pathways are shared and respond in the same direction (Merklings et al., 2015; Telonis-Scott et al., 2013; Zhang et al., 2011), immune activation upon infection may provide a buffer against the loss of

thermal tolerance associated with further pathogen proliferation (Hector et al., 2019). This may be especially true for populations locally adapted to be more resilient to thermal stress. If such locally adapted populations, which are already better able to cope with thermal stress, also see increases in stress resistance in response to pathogen exposure, species may be able to overcome the simultaneous impacts of disease and global change. As of yet, however, generalities around the influence of any immune challenge on upper or lower thermal limits remain unknown, especially across populations that already vary in their thermal limits and possibly also in their responses to infection (e.g. Juneja, Quinn, & Jiggins, 2016; Tinsley, Blanford, & Jiggins, 2006). Whereas changes in thermal limits with latitude and the broad impact of pathogen proliferation have proven to independently result in variation across populations (Hector et al., 2019; Hoffmann, Anderson, & Hallas, 2002), there have been no attempts to isolate the host's physiological response from the damage caused by pathogen proliferation across populations.

In this study, we first sought to experimentally test how activation of a host's immune system affects a host's resistance to both heat and cold thermal stress. A common approach for simulating a systemic infection and eliciting an immune response in insects, in isolation from the subsequent damage a pathogen might cause, is to introduce heat-killed pathogen cells into the host via an injection through the cuticle (e.g. Bashir-Tanoli & Tinsley, 2014; Khalil, Jacobson, Chambers, & Lazzaro, 2015; Kubiak & Tinsley, 2017). We assessed both the direction and intensity of any response to varying intensities of simulated infection, as well as the rate of decay of any signal from the time since the immune system was stimulated. We then sought to generalize our findings by measuring the thermal limits of individuals across a range of populations originating from along a latitudinal cline that are known to vary predictably in their tolerance to thermal stress (Hoffmann et al., 2002; Lasne, Hangartner, Connallon, & Sgrò, 2018). By doing so, we sought to test whether there were predictable impacts of an immune challenge on thermal limits across locally adapted populations, and whether populations adapted to higher temperatures could benefit more, or rather less, from the shared pathways involved in both immune activation and the thermal stress response.

To do so, we used experimental infections of *Drosophila melanogaster* with heat-killed bacterial pathogens. Immune activation was accomplished either by wounding individuals with a pinprick to the thorax, or by introducing a combination of heat-killed Gram-positive and Gram-negative bacterial cells (similar to Juneja et al., 2016; Lazzaro, Flores, Lorigan, & Yourth, 2008; McKean, Yourth, Lazzaro, & Clark, 2008; Nystrand, Cassidy, & Dowling, 2016). We measured upper thermal limits under static and dynamic ramping assays (Lasne et al., 2018; Sgrò et al., 2010), and chill-coma recovery (Clemson, Sgrò, & Telonis-Scott, 2016; Lasne et al., 2018) as a measure of cold tolerance across several simulated infection intensities. Next, we investigated whether there were consistent effects of immune activation on upper thermal limits (using the thermal assay that showed the clearest response to immune challenge) across 12 *D. melanogaster* populations originating from along a large geographic cline (from

tropical to temperate locations) along the east coast of Australia. Through these experiments we sought to unravel: how activation of the immune system affects the response of ectotherms to stressful temperatures; whether it can mitigate some of the negative impact of infection on thermal limits due to correlated stress responses; and finally, whether any effect is consistent and generalizable across genetically distinct locally adapted populations.

2 | METHODS

2.1 | *Drosophila melanogaster* maintenance

All populations of *Drosophila melanogaster* were originally collected along a latitudinal cline in eastern Australia and had been maintained under standard laboratory conditions for up to 70 generations (see Table S1 for sampling locations for each population, and Lasne et al., 2018 for details on original population collection, laboratory culture and maintenance). To control for density effects, populations were subjected to a series of short laying periods (18–24 hr each in 250 ml bottles containing 60 ml of agar–dextrose–potato–yeast medium) for three generations prior to thermal assays described below, and maintained at 25°C under a 12:12 light/dark cycle. To further control for density effects in the experimental generations, adults from parental generations were placed in 250 ml bottles containing 60 ml of medium for laying periods of 4 hr. For experimental animals, virgin females were collected under light CO₂ anaesthesia within 6 hr of eclosion and maintained in small groups (15–18 individuals) in 40 ml vials containing 7 ml of normal medium until their respective assays.

Only female flies were used in the experiments described below, although there is known variation between the sexes in thermal tolerances in *Drosophila* (e.g. Hangartner & Hoffmann, 2016; Lasne et al., 2018; Mitchell & Hoffmann, 2009), and often sexual dimorphism in the response to infection more generally (Gipson & Hall, 2016, 2018; Hall & Mideo, 2018), investigating both sexes was beyond the scope of this study. We acknowledge, however, that the relationship between a host's response to any immune challenge (or infection more broadly) and its subsequent thermal tolerance may wholly depend on its sex. We will be addressing this interesting point in an upcoming study (Laidlaw, Hector, Sgrò and Hall, in preparation).

2.2 | Experiment 1: the impact of simulated infection intensities on upper and lower thermal limits in a focal population

For the first experiment, we used a single population (Melbourne) from the original cline to test the effects of immune activation over different simulated infection intensities on upper thermal limits during both heat shock and two dynamic ramping assays, and chill-coma recovery time as a measure of cold tolerance (See: Lasne et al., 2018; Sgrò et al., 2010, for similar approaches). *Drosophila melanogaster*

maintenance and experimental animal collection was carried out as described above.

2.2.1 | Immune challenge

Focal flies were administered bacterial cells via a septic pinprick to the thorax (as in Khalil et al., 2015) containing one of the three bacterial dilutions, or sterile PBS as a wounding treatment, whilst non-wounded controls were treated identically except from receiving a pinprick. The bacterial challenge involved exposing focal flies to a solution containing equal amounts of heat-killed *Staphylococcus aureus* (a Gram-positive bacteria) and *Pseudomonas aeruginosa* (Gram-negative), diluted in PBS to concentrations of either 10¹⁰, 10⁹ or 10⁸ cells per ml (heat-killed bacterial cells supplied by InvivoGen, San Diego, CA, USA). Experimental flies were exposed to immune treatments at five to six days old, with 37 to 40 individuals per treatment in each thermal assay. All individuals received their immune challenge under light CO₂ anaesthesia (control individuals were also exposed to CO₂) in the morning between 8 and 9 a.m., and given at least 3 hr to recover before their respective assays.

2.2.2 | Static heat resistance

To see how the response to an immune challenge on upper thermal limits depended on the time since an immune challenge occurred, we measured knockdown times under static heat shock on *D. melanogaster* at four time points (3, 6, 12 and 24 hr) following the immune challenge. Each fly was placed in a 5 ml glass vial and acutely exposed to 39°C by submersion in a preheated water bath (as in Lasne et al., 2018). Heat knockdown time was scored as the time taken for individual flies to become immobilized. All treatments were tested over four runs (9–10 flies per treatment per run), split over two blocks.

2.2.3 | Ramping (dynamic) heat resistance

Two thermal ramping rates were used to measure critical thermal maxima (CT_{max}) of *D. melanogaster* across the five immune treatments. Starting at 25°C, individuals were exposed to gradually increased temperatures at ramping rates of 0.1°C/min or 0.06°C/min (as in Sgrò et al., 2010). The temperature at which individuals became immobilized (CT_{max}) was recorded. All treatments were tested over four runs (9–10 flies per treatment per run), split over two blocks.

2.2.4 | Cold resistance

Chill-coma recovery time was measured by placing individuals in 1.5 ml eppendorf tubes and submerging them for five hours in a 10% glycol cooled water bath set to 0°C (Clemson et al., 2016; Lasne et al., 2018).

Flies were then returned to 25°C to recover, and the time taken for them to right themselves was recorded for each individual. All treatments were tested over two runs (18–20 flies per treatment per run).

2.3 | Experiment 2: clinal variation in immune and thermal stress response

Having established that static knockdown times are most sensitive to an immune challenge and that the 3-hr window is when any effect is most likely to be seen, we next sought to explore the generality of these findings across multiple populations. Twelve populations of *D. melanogaster*, originally collected from along the east coast of Australia from temperate to tropical locations (Lasne et al., 2018; see Table S1), were used to investigate how the impact of immune activation on thermal stress resistance varies across genetically distinct populations known to vary in multiple stress resistance traits including upper thermal tolerance (Lasne et al., 2018). All populations and experimental animals were maintained as described above.

2.3.1 | Immune challenge

At five to seven days old, experimental flies were exposed to one of three different immune treatments, with 39–42 individuals per treatment and population. The immune challenge involved exposing focal flies to a solution containing equal amounts of heat-killed *S. aureus* and *P. aeruginosa*, diluted in PBS to a concentration of 10^{10} cells per ml (heat-killed bacterial cells supplied by InvivoGen, San Diego, CA, USA). Focal flies were administered bacterial cells via a septic pinprick to the thorax (as in Khalil et al., 2015) containing either the bacterial dilution or the sterile PBS as a wounding treatment, whilst nonwounded controls were treated identically except from receiving a pinprick. All individuals received their immune challenge under light CO₂ anaesthesia (control individuals were also exposed to CO₂) in the morning, and given approximately 3 hr to recover before their respective assays.

2.3.2 | Thermal limit assays

Static heat shock at 39°C (as described above) was used to measure heat knockdown times across the three immune treatments and 12 populations. All treatments were tested over 17 runs (2 flies per treatment per run with all treatments and populations included in each run) split over five blocks. Each block was used for two assay days, and in total, all blocks spanned three generations.

2.4 | Statistical analyses

All analyses were performed in R (v. 3.3.3; R Development Core Team, www.R-project.com). For experiment 1, the three types of thermal assay were analysed separately using one- or two-way analysis of

variance (ANOVA, Type III). Prior to analysis, responses were corrected for run effects by multiplying each individual's response by the ratio of the grand trait mean divided by the run mean (as in Sgrò et al., 2010). For the static heat assay, immune treatment (control, wounded, high dose, medium dose and low dose), time since immune challenge (3, 6, 12 and 24 hr) and their interaction were fitted as fixed effects. For the dynamic ramp assays, immune treatment, ramping rate (0.06°C/min and 0.1°C/min) and their interaction were fitted as fixed effects. Finally, for the cold resistance assay, immune treatment was treated as a fixed effect. The assumptions of normality and homogeneity of variances for each trait were assessed via inspection of the standardized residual plots, and accordingly, we transformed CT_{max} and chill-coma recovery times using square-root transformations. As CT_{max} was negatively skewed, we first reflected this trait by subtracting the original value of each observation from a constant that is calculated by adding one to the largest CT_{max} score observed (e.g. transformed $CT_{max} = \sqrt{([largest\ observed\ CT_{max} + 1] - original\ CT_{max})}$).

For experiment 2, we modelled the change in knockdown times along the latitudinal cline as a second-order polynomial as estimated via a linear mixed-effects model (lme4 package; Bates, Maechler, Bolker, & Walker, 2015) with latitude and latitude² as continuous covariates and immune treatment (control, wounded and immune challenged) included as a fixed effect factor (including their interaction), and assay run fitted as a random effect. The significance of fixed effects was then tested using ANOVA (Type III; car package, Fox & Weisberg, 2018), where a significant interaction term between the covariates and immune treatment would be evidence that the trends in knockdown times varied by immune treatment. We then tested the effects of latitude on each treatment level (control, wounded and immune challenged) independently, using second-order polynomial regression, to more directly test how knockdown times varied with latitude under each treatment.

Finally, using the predicted average control knockdown times for each population (obtained from the mixed model described above), we then partitioned the relative effects of wounding and immune challenge on heat knockdown. To do so, we first estimated the effects of wounding by subtracting the predicted control times from those of the wounded individuals (separately for each population). We then partitioned the effects of immune challenge by subtracting the sum of the control times and the relative effect of wounding for each population. These two data sets were then modelled separately using second-order polynomial linear regression to allow us to examine the relative impact of wounding and immune challenge on knockdown times across latitude.

3 | RESULTS

3.1 | Experiment 1: Immune activation may improve thermal limits in a focal population

In our initial focal population, we found an overall positive effect of the different immune challenges on static heat resistance across

FIGURE 1 Knockdown times (min) under 39°C static heat shock for *Drosophila melanogaster* from different immune treatments across time (a), and the main effects of immune treatment (b) and time since the immune challenge (c) in isolation. Knockdown times were measured on flies exposed to one of five different immune challenge treatments administered via a septic pinprick to the thorax (high: 10^{10} bacterial cells per ml, medium: 10^9 bacterial cells per ml, low: 10^8 bacterial cells per ml, wounded and control). Knockdown times were measured for flies from all immune treatments at 3, 6, 12 and 24 hr following their immune challenge. Significance levels represent pair-wise post hoc analyses between each immune challenge and unwounded controls. Shown are mean ($\pm$ SE)

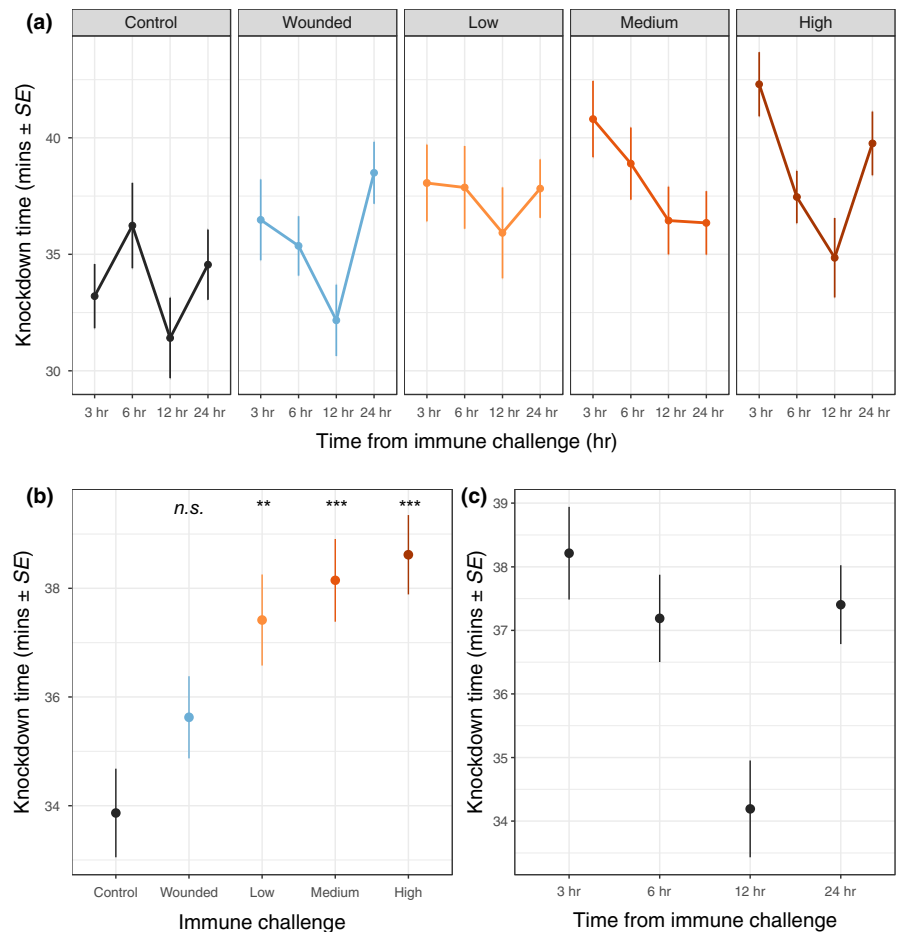


TABLE 1 One- or two-way analysis of variance (ANOVA) for the effects of (a) immune challenge (control, wounded, low, medium or high) and time since immune challenge (3, 6, 12 or 24 hr) on knockdown time during 39°C static heat shock assay, (b) immune challenge and ramp rate (0.06°C/min and 0.1°C/min) on CT_{max} (°C) during two ramping (dynamic) assays and (c) immune challenge on chill-coma recovery time

Trait	Term	df	F	p-Value
(a) Static heat shock	Immune challenge	4, 743	6.644	<.001
	Time	3, 743	7.417	<.001
	Immune challenge × Time	12, 743	1.394	.163
(b) Dynamic ramp	Immune challenge	4, 373	3.452	.009
	Ramp rate	1, 373	499.201	<.001
	Immune challenge × Ramp rate	4, 373	1.701	.149
(c) Chill-coma recovery	Immune challenge	4, 187	1.649	.164

both recovery time and the various doses of heat-killed bacteria (Figure 1, Table 1a). Individuals administered the highest bacterial doses had higher knockdown times and thus greater resistance to thermal stress than lower doses, wounded individuals and controls (Figure 1b). However, the effect of any immune challenge was greatest shortly after the immune challenge (3 hr recovery, Figure 1c), with a general decline in knockdown times occurring over time, until 24 hr after immune challenge when knockdown times generally rose (Figure 1c). There was no significant interaction between the type of immune challenge and the time since a challenge occurred on knockdown times (Table 1a), suggesting that all immune treatments responded similarly over time. We also saw variation across time

in control individuals, which likely represents a circadian rhythm in thermal tolerance throughout the day (Figure 1a).

Ramping rate had a large effect on CT_{max} across all immune treatments, with individuals exposed to the faster ramping rate exhibiting higher CT_{max} than those in the slow ramping rate (Figure 2a, Table 1b). In contrast to the static heat shock experiment, however, the immune challenges led to a general decline in CT_{max} after controlling for the main effect of ramp rate (Figure 2b, Table 1b). There was no overall significant interaction (Table 1b) between ramping rates and the immune challenge treatments indicating that the detrimental influence of immune challenge on CT_{max} was generally consistent across ramping rates. Finally, there was no overall effect of immune

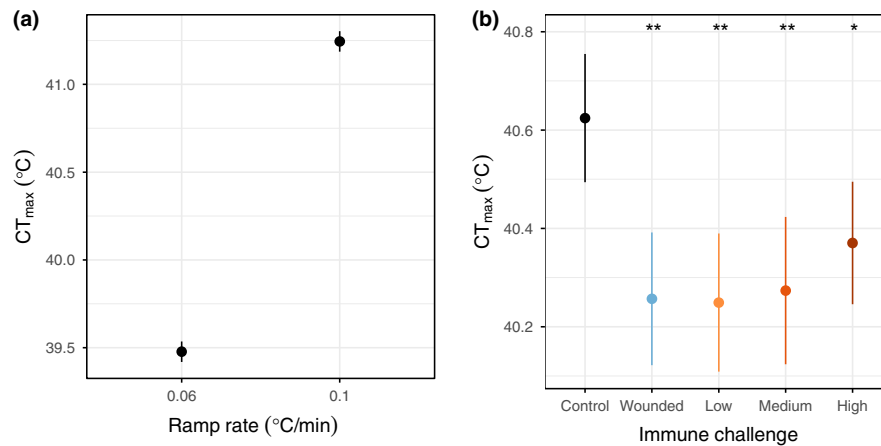


FIGURE 2 Critical thermal maxima (CT_{max} °C) of *Drosophila melanogaster* measured over two dynamic ramping assays (a) after exposure to one of five different immune challenge treatments (b). CT_{max} was measured on flies exposed to one of five different immune challenge treatments administered via a septic pinprick to the thorax (high: 10^{10} bacterial cells per ml, medium: 10^9 bacterial cells per ml, low: 10^8 bacterial cells per ml, wounded and control). Two dynamic assays were used, including a 0.06°C/min and 0.1°C/min ramps, both starting at 25°C. Significance levels represent pair-wise post hoc analyses between each immune challenge and unwounded controls. Shown are mean ($\pm$ SE)

treatment on cold resistance (Table 1c, Figure 3), as measure by chill-coma recovery times. However, from Figure 3, it appears that there may be some benefit to immune activation on chill-coma recovery, but this will at best be limited to the highest bacterial doses.

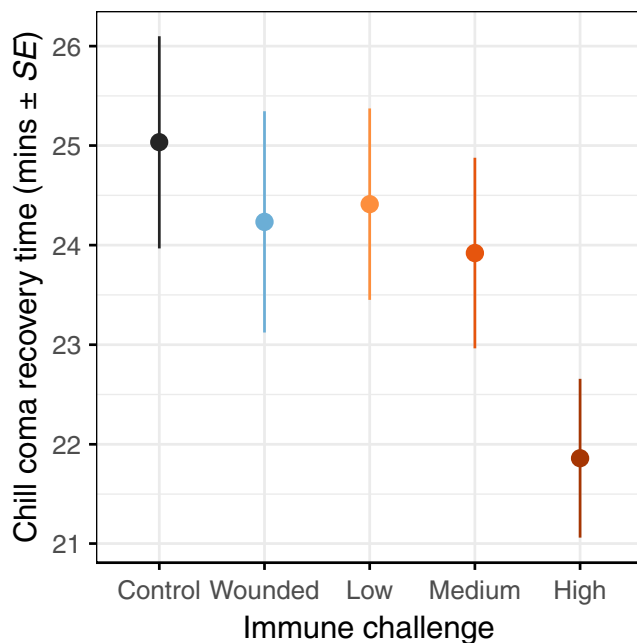


FIGURE 3 Chill-coma recovery time (min) for *Drosophila melanogaster* exposed to one of five different immune challenge treatments. Flies were exposed to one of five different immune challenge treatments administered via a septic pinprick to the thorax (high: 10^{10} bacterial cells per ml, medium: 10^9 bacterial cells per ml, low: 10^8 bacterial cells per ml, wounded and control). Chill come recovery was measured as the time taken for flies to right themselves at 25°C after a five-hour exposure at 0°C. Shown are mean ($\pm$ SE)

3.2 | Experiment 2: clinal variation in immune and thermal stress responses

When modelled as a second-order polynomial, there was a significant interaction between the quadratic and linear terms for latitude and the immune treatments on heat knockdown times (Table 2), indicating that the change in knockdown times with latitude differed significantly between the various immune challenges. The knockdown times of control individuals, for example, were highest for populations from lower latitudes and steadily declined until plateauing or even slightly improving for populations from latitudes 30°C and higher (Table 3, Figure 4a). This result is consistent with the previously reported nonlinear relationship between latitude and heat knockdown times for uninfected individuals (Lasne et al., 2018). However, after wounding or the immune challenge this response was diminished, resulting in a loss of clinal variation, and no linear or nonlinear effect of latitude on knockdown times in either immune challenged group (Table 3, Figure 4a).

Accounting for the observed loss of clinal trends in heat knockdown times upon immune activation appears to be a range of population-specific responses to both wounding alone and the immune

TABLE 2 The effects of treatment (control, wounded and immune challenged), latitude of origin and, their interaction on knockdown times under 39°C heat shock

Term	χ^2	df	p-Value
Treatment	9.782	2	.008
Latitude	17.591	1	<.001
Latitude ²	14.448	1	<.001
Treatment $\times$ Latitude	8.949	2	.011
Treatment $\times$ Latitude ²	7.702	2	.021

TABLE 3 Parameter estimates for the effect of latitude on heat knockdown times for control, wounded and immune challenged *Drosophila melanogaster*, and the relative effects of wounding and immune challenge. Linear models were constructed individually for each treatment group

Treatment	Term	Estimate	SE	t-Value	p-Value
Control	Latitude	-3.058	0.692	-4.419	<.001
	Latitude ²	0.052	0.013	4.054	<.001
Wounding	Latitude	-0.886	0.598	-1.481	.139
	Latitude ²	0.014	0.011	1.296	.196
Challenged	Latitude	-0.629	0.607	-1.037	.300
	Latitude ²	0.010	0.011	0.929	.354
Wounding effect	Latitude	2.189	0.579	3.782	<.001
	Latitude ²	-0.038	0.011	-3.541	<.001
Challenged effect	Latitude	0.277	0.596	0.465	.642
	Latitude ²	-0.004	0.011	-0.386	.700

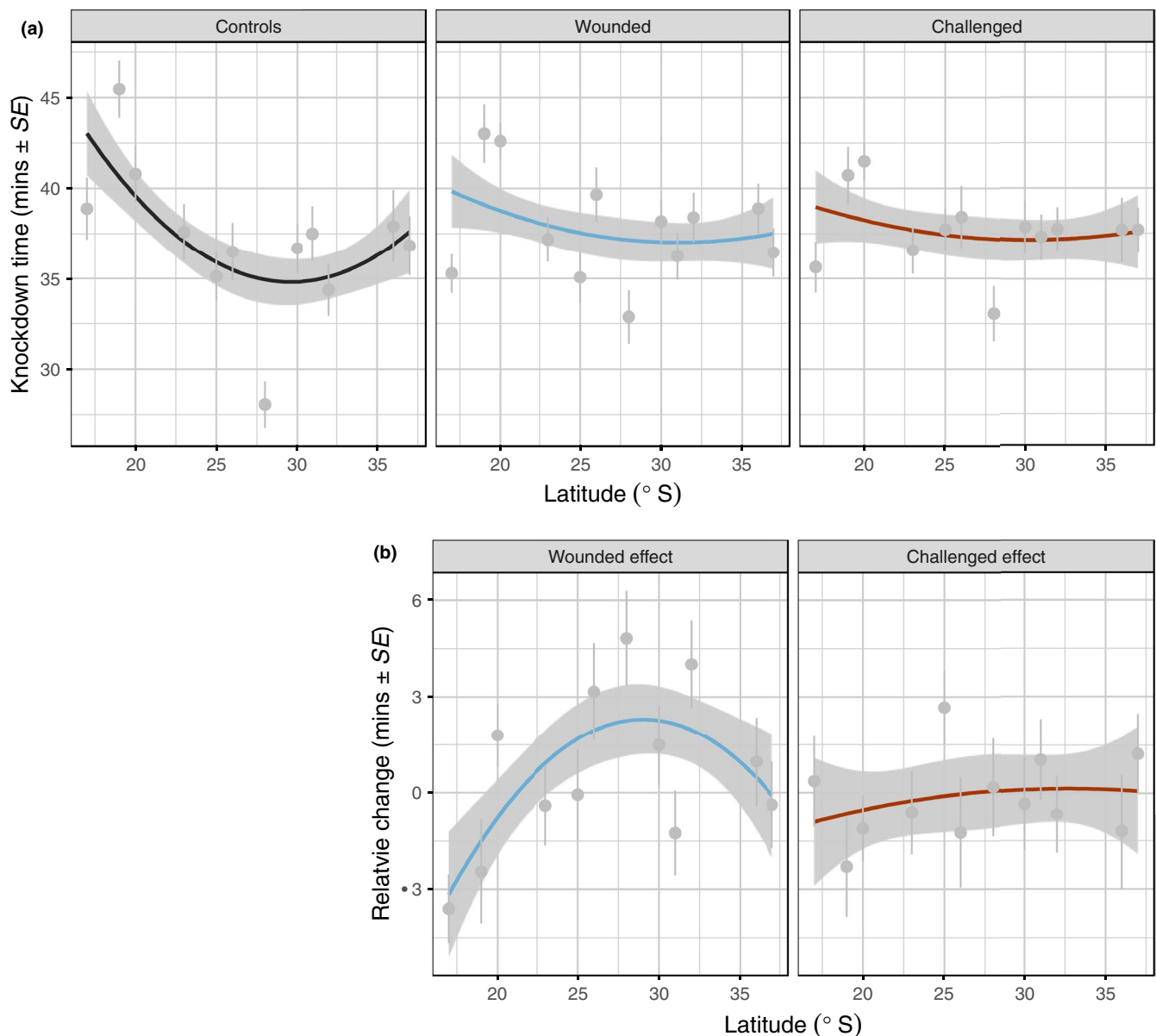


FIGURE 4 (a) Knockdown times under 39°C heat shock for populations along a latitudinal cline (°S) for control, wounded and immune challenged *Drosophila melanogaster*, and (b) the relative effects of wounding (wounded times minus control times for each population) and bacterial immune challenge (challenged times minus control times and the wounded effect for each population). Shown are the population means (±SE) for each metric and the predicted trends across latitude (±SE) as estimated via a second-order polynomial regression

challenge. On isolating the effect of wounding alone (Figure 4b and Table 3), we found a nonlinear relationship across latitude that was opposite to the trend for control individuals, with flies originating from mid-latitudes showing an increase in knockdown times relative to unwounded controls from the same population. In contrast, the effects of an immune challenge on knockdown times, after controlling for wounding and relative to controls, were small resulting in no effect across latitude (Figure 4b, Table 3). This suggests that the loss of clinal variation after wounding or immune challenge was primarily driven by the opposing impact of wounding on heat knockdown times across latitude.

4 | DISCUSSION

In this study, we explored how activation of a host's immune system, via wounding and a bacterial immune challenge, influences their resistance to thermal stress. Focusing first on a single focal population as a test case, we found that the immune challenged flies had improved knockdown times under acute heat stress (Figure 1a). This response was greatest shortly after any challenge (approx. 3 hr, Figure 1c) and increased with the intensity of the immune challenge (Figure 1b). The increases in the knockdown times of immune challenged flies even exceeded the variation in knockdown times known to exist along the entire geographic cline from which this population originated (Hoffmann et al., 2002; Lasne et al., 2018; Sgrò et al., 2010), which consistently varies by around five minutes between the cline ends. In contrast, under dynamic temperature ramps, immune activation had a detrimental effect on upper thermal limits, as estimated via their critical thermal maxima (Figure 2). Finally, we found no overall effect of immune challenge on chill-coma recovery, although there was some evidence of a limited benefit at the highest bacterial dose (Figure 3).

Our results show that activation of the immune system does not affect all measures of thermal stress resistance equally. Activation of the immune system improved the host's ability to cope with static heat stress, much like the cross-tolerance commonly observed between desiccation or starvation and thermal tolerance (Benoit, Lopez-Martinez, Elnitsky, Lee, & Denlinger, 2009; Bubliy, Kristensen, Kellermann, & Loeschcke, 2011; Bubliy, Kristensen, & Loeschcke, 2013; Gotcha, Terblanche, & Nyamukondiwa, 2018; Kalra, Tamang, & Parkash, 2017; Sinclair et al., 2013). Under thermal ramping, however, the effect of an immune challenge on CT_{max} was reversed and immune activation additionally had no effect on cold resistance. Collectively, these results suggest that immune activation will have a much more important influence on tolerating heat stress than cold stress. They also imply that during slower and longer exposures to heat stress, which are thought to be more ecologically realistic, a challenge to the immune system will limit the capacity of an individual to cope with the subsequent damage caused by extended exposure to high temperatures (Chown, Jumbam, Sørensen, & Terblanche, 2009; Rezende, Tejedo, & Santos, 2010; Rohr et al., 2018; Terblanche, Deere, Clusella-Trullas, Janion, & Chown,

2007). Such a trade-off could arise if protecting against prolonged heat stress requires mechanisms also involved in the immune response, but a previous immune challenge has exhausted the immune system's capacity to repair the physiological damage caused.

We next explored the effect of immune activation on thermal tolerance across 12 genetically distinct populations of *D. melanogaster* originating from along a large latitudinal cline. For the control individuals, we found a nonlinear decline in heat knockdown times with increasing latitude, a result that has previously been uncovered for these populations (Lasne et al., 2018), and is similar to the linear relationship found along the same cline once more populations from higher latitudes are included (Hoffmann et al., 2002; Sgrò et al., 2010). Our results are thus consistent with previous studies that have used either populations that have spent less time in the laboratory (Cockerell, Sgrò, & McKechnie, 2014; Lasne et al., 2018) or populations that have had minimal exposure to laboratory conditions (Hoffmann et al., 2002; Sgrò et al., 2010). This, combined with the fact that Griffiths, Schiffer, and Hoffmann (2005), found that the thermal tolerance of another *Drosophila* species, *D. birchii*, was not affected by laboratory culturing conditions, suggests that the upper thermal tolerance of our sample populations reflects their natural thermal ecology, and in turn the role that immune activation might play in the wild.

Importantly, after immune activation via wounding or a bacterial challenge, we find that the observed relationship between latitude and knockdown times was lost (Figure 4). Our results indicate that populations vary considerably in their response to different immune challenges, particular wounding, and that this effect is, on average, antagonistic to the normal heat stress response. Rather than universally boosting knockdown times, as we would expect if cross-tolerances between heat stress resistance and immune activation were readily generalizable across populations (Sinclair et al., 2013; See Gotcha et al., 2018 and Table 1 therein), we instead find that the antagonistic response actually erases signals of local adaptation, whereby lower latitude populations no longer exhibit higher knockdown times. This result is more suggestive of immune activation at the clinal scale decreasing knockdown times as a result of a trade-off between the physiological cost of multiple stressors (Manenti, Cunha, Sørensen, & Loeschcke, 2018; Mitchell, Boardman, Clusella-Trullas, & Terblanche, 2017; Nyamukondiwa & Terblanche, 2009; Op de Beeck, Verheyen, & Stoks, 2017; Scharf et al., 2016) and highlights the need to consider more than one population when evaluating how multiple stressors may interact.

Here, we have shown that immune activation and wounding can lead to short term increases in heat knockdown times, but any potential buffer to thermal tolerance is likely to be idiosyncratic across populations. Instead, immune activation may in fact erase signals of local adaptation across populations. The consequences for adaptation and survival of species in light of global change could be significant for two reasons. First, local adaptation in resistance traits that are predicted to buffer populations against future thermal stress (e.g. Bush et al., 2016; Hoffmann & Sgrò, 2011; Razgour et al., 2019) may be lost by even a mild immune challenge. Here, the

populations most resistant to thermal stress when healthy saw the greatest reduction in thermal tolerance after wounding and bacterial challenges, resulting in a sharp reduction in expressed variation across populations. Second, the influence of any pathogen on a population's thermal tolerance may occur even when an infection can be successfully cleared. Yet unlike the reduction in a host's thermal tolerance that a pathogen causes directly (Greenspan et al., 2017; Linderman et al., 2012), which has been shown to be consistent in direction across host genotypes and thermal assay types (Hector et al., 2019), whether or not immune activation will benefit, or instead disrupt, a species thermal tolerance may be unique to a given population and dependent on the form of thermal stress. Ongoing assessments of species risk under global change may therefore need to consider both intra-specific variation in any response and the impact of a pathogen even when infections do not progress, if they are to have accurate predictive value.

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DATA AVAILABILITY STATEMENT

Data sets are available via a figshare repository: <https://doi.org/10.26180/5e2f9beedb577>

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