Fungal Infections Lead to Shifts in Thermal Tolerance and Voluntary Exposure to Extreme Temperatures in Both Prey and Predator Insects

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Abstract

Pathogens can alter the thermal tolerances of insects; these changes can have cascading impacts across trophic levels in terrestrial food webs. However, the effects of fungal infections on thermal tolerances and behavioral responses to extreme temperatures (ET) across trophic levels have rarely been studied. We examined how a fungal pathogen, *Beauveria bassiana,* affects the thermal physiology and behavior of an herbivorous insect, *Acyrthosiphon pisum*, and a predator beetle, *Hippodamia convergens*. We compared thermal tolerance limits (CT_{Min} and CT_{Max}), thermal boldness (voluntary exposure to ET), energetic cost (ATP) posed by each response (thermal tolerance and boldness) between healthy and infected insects with two fungal loads. Results showed that fungal infection reduced upper thermal tolerance (CT_{Max}) of aphids and beetles, as well as lower thermal tolerance (CT_{Min}) of beetles. CT_{Min} of aphids was not altered by infection. Healthy aphids and beetles crossed warm ET zones (ETZ). Fungal infection modified the tendency, or boldness, of aphids and predator beetles to either cross warm or cold ETZ. ATP levels increased with pathogen infection in insect species, and the highest ATP levels were found in individuals that crossed cold ETZ. Fungal infection narrows the thermal tolerance range and inhibited thermal boldness behaviors to cross ET which may have implications for predator-pay interactions, food web structures, and species distributions. As environmental temperature rises, response to thermal stress will be asymmetric among members of a food web at different trophic levels.

Introduction

Fungal pathogens and temperature can constrain the physiology and behavior of insects, with implications for predation strategies that can ultimately affect populations across trophic levels and food webs[1, 2]. Several insect species are known to regulate body temperature both physiologically and behaviorally, for example, by selecting adequate environmental temperatures. However, behavioral thermoregulation may be affected in complex ways by interspecific relationships, including those of a pathogenic nature. For instance, exposure to temperatures near thermal limits may alter foraging behavior, survival, and reproduction in individuals representing several ectothermic lineages [3-5], but temperature also affects pathogens. In turn, pathogens may affect the host by altering physiological pathways and patterns of behavioral thermoregulation [6]. Under this framework, episodic exposure to temperatures outside thermal limits (extreme temperatures, ET hereafter) may be detrimental, and in insects is known to reducing immune response, thus increasing susceptibility to pathogens [7]. Thus, the effects of ET on insects and their pathogens may be intricate and not yet described, particularly regarding the effects of fungal pathogens on the behavioral responses of insects exposed to ET [8]. Here, we test the hypothesis that fungal infection may reduce maximum thermal tolerance (CT_{Max}) and increase the low temperature counterpart (CT_{Min}) in insects, as well as inducing shifts in voluntary exposure to ET (thermal boldness) [9]. Because these effects are critical for ecological interactions between species, we chose as a model system the predator-prey system composed by *Hippodamia convergens,* and *Acrytosiphom pisum*, and the fungal pathogen *Beauveria bassiana*, which can affect both insect species. At a higher level of organization, we expect fungal infection to alter the predator-prey system, through

differential impacts on each species thermal behavior and the energetic cost associated with exposure to critical thermal limits.

Predator beetles and aphids can be profoundly affected by abiotic and biotic factors, as well as ecological interactions, including the prevalence of fungal pathogens and the expression of ET in thermal landscapes. Both pathogens and exposure to ET can alter insect physiology in ways that influence behavior and life cycles of both predators and prey, as well as fungal infection dynamics, which in turn may be influenced by physical variables [10]. Differences in ET between host and pathogens are expected. For example, the thermal limits of *B. bassiana* growth are between 8°C and 33 °C [11], with an optimal range between 25 °C and 28°C [12], whereas adults of *A. pisum* display 10°C lower and 40°C upper tolerance [13], and the counterparts for the predator beetle are 6.5 °C and 50 °C [14, 15].

Because ET are relative to physiology and not absolute, exposure to ET, either imposed or voluntary, may differ among prey and predators even if individual animals navigate the same thermal landscape. Possible impacts of ET exposure resulted in reduced foraging, attack, and rates of prey consumption in rotifers, dragonfly larvae, and fishes [16-18], but also disruption of pathogen infection. Temperature affects survival and virulence of fungal pathogens [19] and other aspects of pathogen-insect interactions [6]; for example, insects infected by *Metarhizium anisopliae* select temperatures believed to reduce pathogen virulence (behavioral fever) [6]. Facing suboptimal thermal variation may affect cell homeostasis by reducing the energy supply and affecting ATP synthesis [20]. Although the energetic cost of insects thermal tolerance has been well studied, our understanding of the impacts of multiple stressors (fungal infection and thermal variation) is only partially understood.

In this study, we experimentally infected aphids and predator beetles with a fungal pathogen using spore solutions at different concentrations in the field. We then collected infected and uninfected individuals in the field and assessed thermal tolerances (C_{TMax} and $_{CTMin}$) of healthy and infected aphids and predator beetles in the lab. Next, we examined whether infection alters thermal boldness using species-specific extreme temperatures in order to characterize the impacts of infection on thermal boldness (voluntary crossings of ET). Finally, we determined energetic cost (ATP levels) associated with each physiological and behavioral response in healthy and infected insects as an indicator of energetic cost of reaching or exploring critical thermal limits

Material And Methods

Field trials. Field trials were conducted in three raised beds (1 x 2 x 0.6 m) on the Penn State University campus from July to August 2020. The raised beds were separated by at least 8 m to avoid treatment cross-contamination. Faba bean (*Vicia faba* L.) seeds were planted at a density of 20 seeds/ m2 (50 plants per bed), and each bed was caged using a metal-framed tent. Oudoor Widerness Fabric (Noseeum) was draped over the frame and the edges buried in the soil of the bed. The sides of the cages were fastened closed with zippers to allow access.

Insects. Aphid and predator beetle colonies were raised separately on faba bean plants in cages (Bugdorm 20 cm x 40 cm x 20 cm) in the field. Larvae and adults of predator beetles were fed with a combination of *A. pisum* and *Rhopalosiphum padi* every other day (Supplementary information Fig. S1). Trials involving plants, insects, and entomopathogenic fungi were conducted according to institutional, national, and international guidelines and legislation.

Fungal inoculations (*Beavueria bassiana*): We released first instar aphid nymphs on each faba bean plant on the raised beds (~1100 aphids) by gently shaking plastic containers with groups of 20 nymphs and placing them on the plants using a paintbrush. They were allowed to grow and reproduce for fifteen days. During the night, we sprayed spore suspension of the *Beauveria* strain GHA (BotaniGard ®, MT, USA) at 1.4 x 10^{6} and 1.4 x 10^{12} spore ha^{-1}, low and high load respectively. Two days after inoculation, we collected adult aphids (~ 4-5 days old) from the experimental plots and measured physiological parameters (see details below). Next, we released 300 adult beetles inside each aphid–fungal inoculated cage, allowed them to feed for 2-3 days in our experimental cages, and then collected beetles for physiological measurement.

Identification of critical thermal limits (CT_{Max} and CT_{Min}) of healthy and infected insects. To determine critical thermal maximum for locomotion (CT_{Max}) of healthy and infected individuals of each species, we employed a protocol modified from Ribeiro et al.[21], using a hotplate with a programmable heating rate controlled by a computer interface (Sable Systems, LV, USA). The temperature was monitored by independent thermocouple channels connected to a Hobo 4-channel data logger. One thermocouple was attached to the surface of the hotplate, and the other sensor was attached inside the glass tube plugged by a cotton ball in which we placed an individual insect. This equipment was located inside an automated thermal chamber (interior dimensions: width 40.5 cm × 35 cm length × 40 cm height). We transferred an adult aphid (4-day-old) into the glass tube and exposed it to increasing temperatures at a rate of 0.3 °C min−1 until its locomotion stopped. CT_{Max} was recorded when the insect turned upside down and could no longer return to the upright position within 5 s. The insect was returned to a faba bean leave for recovery.

To measure the critical thermal minimum for locomotion (CT_{Min}) of healthy and infected individuals of each species, we used an insulated incubator where the temperature was monitored by independent thermocouple channels connected to a Hobo 4-channel data logger. The sensors were attached inside three glass tubes, each tube with an adult (3 to 4-day-old), and plugged by a cotton ball. The glass tube was exposed to decreasing temperature at a rate of 0.3 °C min−1 until its locomotion stopped. CT_{Min} was recorded when no movement was recorded within 5 s. The insect was returned to an aphid-infested faba bean leave for recovery. Data were only considered valid if the insect displayed normal activity 2 h after a CT_{Max} or CT_{Min} test.

Impacts of infection on voluntary exposure aphids and predator beetles to extreme thermal zones. To examine how voluntary exposure to ET zones (Z)was affected by fungal infection, we collected aphids and predator beetles (3 to 5 day-old) from our field plots and transferred them to a dark plastic bottle.

Next, a bottle containing the insects was attached to a choice test following a modified protocol from Navas et al.[9]. This prototype allows insects to freely move across extreme temperatures to access food in containers located at each end of the device. To reach food, individuals had to cross an ETZ, either warm or cold. The location of each insect was recorded after 60 minutes, and it was classified as: exploration for individuals that left the initial black bottle, warm or cold ETZ crossings. The experiment was simultaneously replicated ten times for each species and treatment condition [aphid: healthy, infected (low and high spore load); predator beetle: healthy, infected (low and high spore load)].

Effects of fungal infection and thermal conditions (critical thermal limits and voluntary exposure to ETZs) on longevity of aphids and predator beetles. To examine whether fungal infection and thermal conditions alter longevity in aphids and beetles, we isolated three individuals from each factor combination (low, high fungal load, CT_{Min}, CT_{Max}, behavior: crosses to ETZ cold, warm, and no cross) from previous experiments, and counted the number of days the adults survived after the exposure to the thermal condition (n=3 factor combination).

Energetic cost associated with fungal infection of aphid and predator beetles under critical thermal limits and voluntary exposure to ET. Intracellular ATP content was determined in neutralized perchloric acid extracts and by a spectrophotometric coupled enzyme assay, based on modified protocol from Churchill and Storey [22]. An insect was ground to powder using a mortar and pestle cooled in liquid nitrogen, and then weighed into 1.5 mL microcentrifuge tubes (Eppendorf). Powder was dissolved with 0.1 mL ice-cold TE buffer (50 mM Tris-HCl, pH 7.5 plus 1 mM EGTA) and homogenized by sonication (15 seconds, three times), using a Q500 Sonicator system (QSonica, Newtown, CT, USA). An aliquot (10 µL) of the well-mixed homogenate was removed for protein determination. Cells were lysed by adding 6% (v/v) ice-cold perchloric acid, strongly vortexed for 2 min and incubated at 4 °C for 10 min. Next, the cell homogenate was centrifuged at 14 462 g and 4 °C for 5 min. The resulting supernatant was neutralized by adding KOH/Tris (3 M/0.1 M) and centrifuged again to discard the perchlorate salts. Extracts were kept at 4 °C for their immediate utilization. ATP content was determined spectrophotometrically by following the production of NADPH at 340 nm (ε= 6.22 mM-1 cm-1) and using CARY WinUV-Vis Spectrophotometer (Agilent, Santa Clara, CA, USA). The following reagents were used for the spectrophotometric coupled enzyme assay: 5 U Hexokinase, 10 U Glucose 6-phosphate dehydrogenase, 1 mM NADP+, 5 mM MgCl2 and 10 mM Glucose in HE buffer (100 mM Hepes-HCl plus 1 mM EGTA, pH 7.0) at 25 °C. Chemicals were purchased from Roche (Manheim, Germany) and Sigma (St Louis, MO, USA).

Infection status: Infection of each experimental insect was confirmed following protocols, 1) counting spores from body washes (sterile deionized water) of insects used for ATP measurements using Neubauer hemocytometer (spore ml^{-1}: total spore counted/total number of cells; Table S1); 2) placing each individual in wet towel paper inside a Ziploc bag to observe hyphal growth (Supplementary information Fig. S1) [23].

Data analysis. All data were tested for statistical test assumptions using a qqplot, Levene's homogeneity test and the Shapiro-Wilk normality test at alpha=0.05 significance level. For critical thermal limits (CT_{Max}

and CT_{Min}) experiments, the data sets were non-normal and transformation did not normalize the residuals, so we used nonparametric ANOVAs (Kruskal–Wallis followed by post-hoc nonparametric pairwise comparisons with Wilcoxon tests. For voluntary exposure to ETZs, we used a Generalized linear model with treatment (healthy, low and high spore load) with Poisson distribution, followed by comparisons within each treatment group. For healthy insects, we used a t-test to compare crosses between warm or cold ETZs; for infected-insects, we conducted ANOVAS for comparisons among 23 °C, warm or cold ETZs.

ATP data: Data for CT_{Max} of *A. pisum* were non-normal, and transformation did not normalize the residuals, nonparametric ANOVAs (Kruskal–Wallis) were then used and followed by post-hoc nonparametric pairwise comparisons with Wilcoxon tests. ATP data sets from voluntary exposure to ETZs were analyzed following the same protocol as described previously for in crosses analysis of ETZ experiment. Longevity was analyzed using a two-way Anova with fungal load and thermal condition (critical temperature and behavior) as factors. Analyses were performed in the R programming environment (v. 3.4.3., CRAN project).

Results

Critical thermal tolerance. Fungal infection reduced heat tolerance of *A. pisum* and *H. convergens* by an average 7°C and 4°C, respectively, regardless of the pathogen load, compared to healthy individuals (Fig. 1 a,d). Regarding cold tolerance, the pathogen effect was species-specific, with infected aphids having comparable levels of cold tolerance relative to healthy ones, but predator beetles' ability to withstand cold was reduced according to pathogen load (Fig 1).

Impacts of infection on voluntary exposure of aphids and predator beetles to extreme thermal zones Healthy aphids and predator beetles were very bold and crossed ETZs, but in both species crosses through the warm ETZ were more frequent (> 60% of individuals in samples) than crosses through the cold ETZ (< 36%; Fig. 2). However, thermal boldness of both species was reduced by fungal infections in a dose-dependent manner. For instance, almost 50% of aphids infected with low fungal load opted not to cross ETZs and stayed inside the home bottle, and the remaining 50% crossed the warm (27%) or the cold (24%) ETZs in similar fashion. When infected with the high fungal load, more aphids did not cross ETZs (70%), and less crossed through the warm ETZ (8%), while crosses through the cold ETZ remained comparable to those at the low fungal load. Similarly, 47% of infected beetles with a low fungal load crossed through the warm ETZ, 30% crossed through the cold ETZ, and 21% did not perform any cross. Most beetles infected with the high fungal dose did not cross any ETZ (66%), whereas 23% crossed the warm ETZ and only 13% crossed through the cold ETZ.

Energetic costs associated with fungal infection of aphid and predator beetles under critical thermal limits and voluntary exposure to ET. Although ATP levels of infected aphids at their CT_{Max} nominally increased with fungal load relative to healthy individuals, these differences were not statistically significant (Fig 3A). Nevertheless, ATP levels of beetles exposed to their CT_{Max} significantly increased

with fungal load, relative to healthy beetles (Fig. 3B). ATP levels at the CT_{Min}, of aphids and predator beetles followed a similar trend, it was highest in infected individuals with the high fungal load, intermediate in infected aphids with low fungal load, and lowest in healthy ones (Fig. 3C-D).

ATP levels of healthy aphids and predator beetles were significantly highest in individuals that crossed cold ETZs (aphid: t= 5.51, DF=3.49, P=0.0039; beetle: χ^2=3.85, DF=1, P=0.0495) (Fig. 4). In infected aphids with low fungal loads, the highest ATP levels were found in individuals that crossed ETZs, but those levels did not significantly vary between cold and warm ETZs. Infected aphids that did not cross ETZs or remained at 23°C showed the lowest ATP levels (F=12.60, DF=2,6, P=0.0111). ATP levels in infected aphids with high fungal followed the same trend (F=24.27, DF=2,6, P=0.0013).

ATP levels did not significantly vary in infected beetles with low fungal doses (χ^2=5.42, DF=2; P=0.0665). However, we found significant differences in ATP levels of beetles infected with high fungal loads. ATP levels were significantly higher in infected beetles that crossed cold ETZ, followed by ATP levels of beetles crossing the warm ETZ. Infected beetles that remained at 23 °C showed the lowest ATP levels (χ^2=5.42, DF=2, P=0.0665).

Effects of fungal infection and thermal conditions (critical thermal limits and voluntary exposure to ETZs) on longevity of aphids and predator beetles. The longevity of infected aphids was significantly reduced by fungal infection, thermal response, and interaction (CT limits and voluntary exposure to ET) by an average of 13.5 days (F=152.11, DF=17,36, P<0.0001; F=1253.64, DF=2, P<0.0001; F=7.44, DF=5, P<0.0001; interactions infection*thermal respone F=4.15, DF=10, P=0.0007, Supplementary Table 1). Beetle longevity followed a similar trend, both factors reduced longevity by average of 11.3 days (F=31.17, DF=17,90, P<0.0001, F=253.15, DF=17,90, P<0.0001; F=2.40, DF=5, P=0.431; factor combination F=4.15, DF=10, P=0.3192; Fig. 5, Supplementary Table 2).

Discussion

The results of our field experiments demonstrate that fungal infection reduced the heat tolerance limits of aphids and predator beetles asymmetrically, while cold tolerance was only reduced in *H. convergens* (Fig. 1). Infection reduced voluntary exposure to ET in both aphid and predator (Fig. 2). Our results indicate that ATP levels are high in adult insects infected with high fungal loads and exposure to cold ET (Figs. 3 -4). Survival was significantly reduced with infection and thermal conditions (Fig 5). Fungal infections in insects are expected to be compensated by behavioral fever [10]; however, this is the first time, to our knowledge, that infection shifts behavioral response for both predator and prey in ways that reduce exposure to heat.

The maximum heat tolerance was reduced by fungal infection, the limits were narrowed with increasing fungal load in both aphids and predator beetles, suggesting that *B. bassiana* may have induced the opposite of host behavioral fevering. Fungal infection may block ion transfer in cells challenging the homeostasis of insects. Although, behaviors that elevate body temperature are expected in infected

insects [6], our results indicated infection altered boldness to cross ET. Reduced heat tolerance was confirmed in behavioral experiments, the majority of infected aphids and beetles were not able to cross either warm or cold ETZ, while the majority of healthy insects crossed either ETZ (Fig. 1-2). This suggests that fungus might manipulate host physiology and behavior in ways that favor the fungal virulence [24] since its thermal thresholds are narrower than any of the insect hosts [11, 12, 25]. However, infection is costly, and insects redirect energy to immune function to battle it. In that sense, less energy is available to face thermal challenges.

The energetic cost of infection for both insect species at minimum thermal limits significantly increased with fungal load (Fig.3-4). This suggests that both factors, thermal condition and infection, may disrupt metabolic pathways resulting in increased ATP synthesis. ATP levels can increase following cold exposure in aphids [26] and other insects, including flies [27]. Changes in ATP levels might be explained by a mismatch among metabolic pathways, since many cellular functions are ATP-dependent. For example, ion pumps, proteolysis, synthesis of substances used to prevent cell damage from cold (i.e., glycerol, trehalose, or proline) [28], and expression of heat shock protein genes require ATP synthesis. Our results suggest that fungal infection and short exposure to critical limits or ETZ did not critically disrupt metabolic pathways since aphids and beetles were able to synthesize ATP, but might have long-lasting impacts on other parameters such as longevity and fitness. Further, ecophysiological studies are needed to identify the mechanisms underlying the ATP synthesis under infection and ET.

Our field experiments indicate that exposure to the maximum thermal limit reduced longevity by about 3 days in aphids, and fungal infection significantly reduced longevity for both aphids and predator beetles (Fig. 5) exposed to any of the thermal conditions; 100% of insects used in the experiments that were infected died early. Previous studies have suggested that the broad host range of *B. bassiana* [29], which includes earthworms, beetles, parasitoids, and honey bees, [30], reported similar lethal and sublethal effects. For example, *B. bassiana* alters foraging and prey handling in the predator mite, *P. persimilis* [31]. The findings of this study suggest that longevity might be reduced by disruption of ATP synthesis in infected insects during exposure to critical temperatures and ET.

We demonstrated that multiple stressors such as ET and fungal infection profoundly affect thermal tolerance in aphids and predator beetles, but in different ways, leading to an asymmetric response. Thermal stress and infection may alter predators' foraging, handling time, and digestion [32]. Altogether, the results indicate the strong effects that infection has on thermal tolerance across trophic levels. This finding suggests that a fungus can modify behavioral plasticity with implications for thermoregulatory strategies in insects with cascading impacts on the strengths of predator–prey interactions and food webs. Additional studies are needed to understand the molecular and physiological mechanisms underlying reduced thermal boldness or voluntary exposure to ETZ in infected insects. This information has critical implications for understanding the physiological and behavioral mechanisms by which organisms respond to biotic and abiotic stressors. This work provided a change of paradigm. In overall, the findings suggest that we cannot expect to understand how an organism responds to the environment by studying the insect species alone, as pathogen infections can alter insect thermal physiology and

behavior. This new dimension opens a wide array of research avenues with fundamental and applied implications to the management of insect species.

Declarations

Acknowledgments

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Author Contributions

MP and ER conceived the idea and designed experiments; CN, VL, MP, GAC, and JS designed thermal arenas; MP conducted the experiments; GS analyzed ATP levels; MP, GAC, and JS analyzed data. MP, CN, ER, GAC, JS, and VL wrote the main text. All authors approved the final version of this manuscript.

Competing interest

The authors declare no competing interests.

Data availability

The source data underlying Figs. 1-5, and Supplementary Table 1 will be deposited in AEKOS data repository.

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Figures

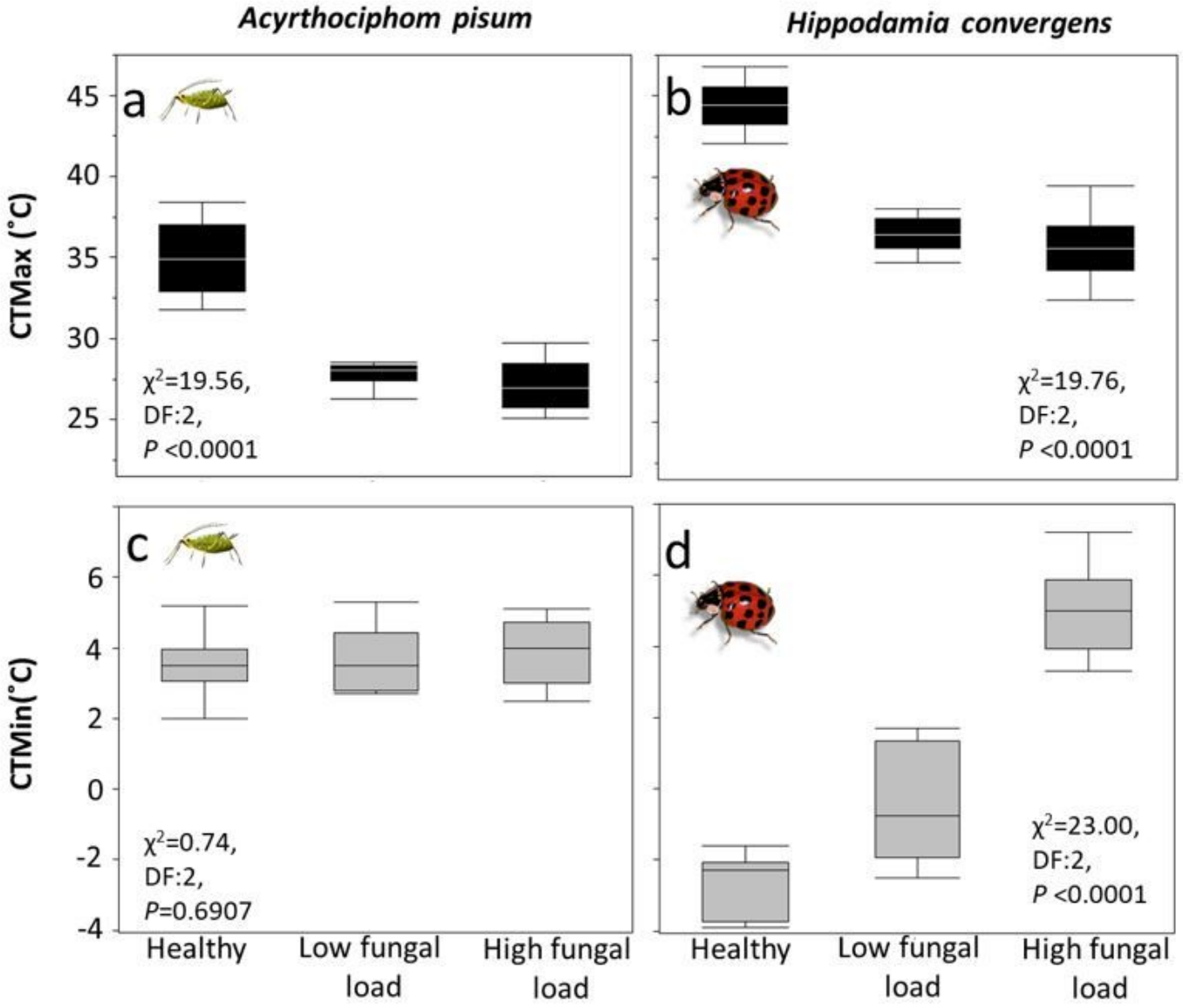

Figure 1

Maximum (CTMax) and minimum (CTMin) thermal tolerances for healthy and infected aphid and predator species. Low spore load= 1.4 x 10 6spores ha-1, high spore load = 1.4 x 1012 spores ha-1. CTMax of (a) aphids (A. pisum) and (b) predator beetles (H. convergens). CTMin of (c) aphids (A. pisum) and (d) predator beetles (H. convergens). Non-parametric Anova test (box plots display median line, interquartile range (IQR) boxes, 1.5 x IQR, n=10 individuals per treatment). Black boxes: CTMax; Gray boxes: CTMin.

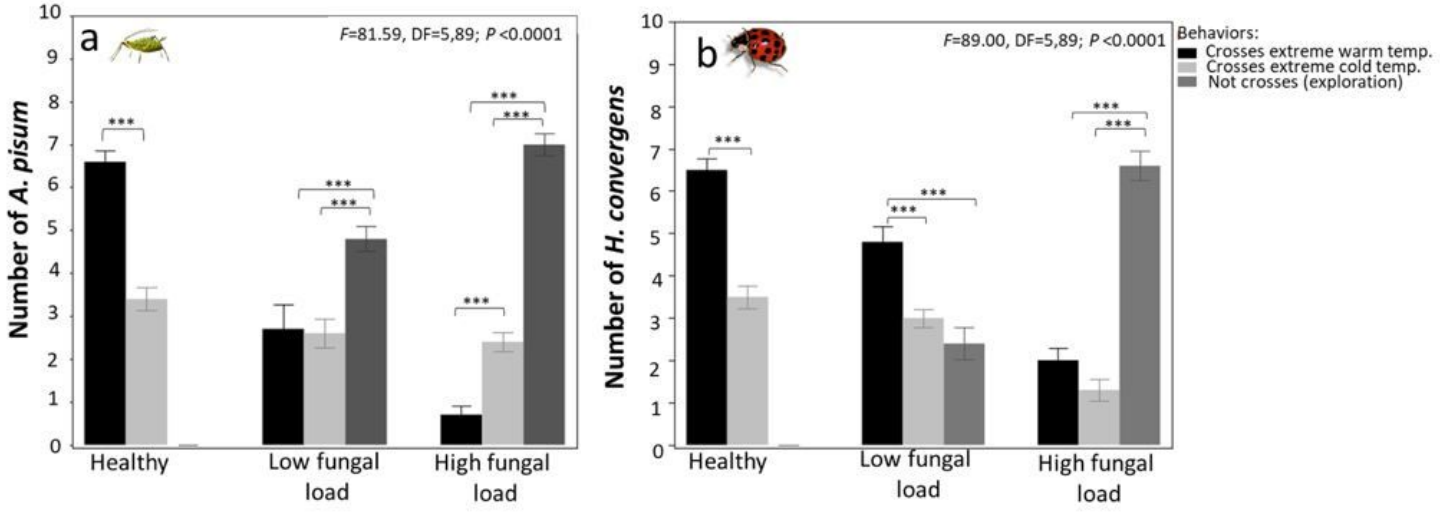

Figure 2

Thermal boldness of healthy and infected aphid and predator beetle species, measured as the number of individuals in a sample that voluntarily crossed through extreme thermal zones (ETZs, cold or warm). Infections: Low fungal load= 1.4 x 10 6 spores ha-1, high fungal load = 1.4 x 10 12 spores ha-1. (a) Aphids (A. pisum). (b) Predator beetles (H. convergens). Warm and cold ETZs for A. pisum were set at 48°C and -4°C, whereas for H. convergens thermal barriers were set at 56°C and -12°C. Two –way Anova. Bars represent mean ± SE, replicates n=10, 10 individuals per replicate, 100 individuals in total per treatment.

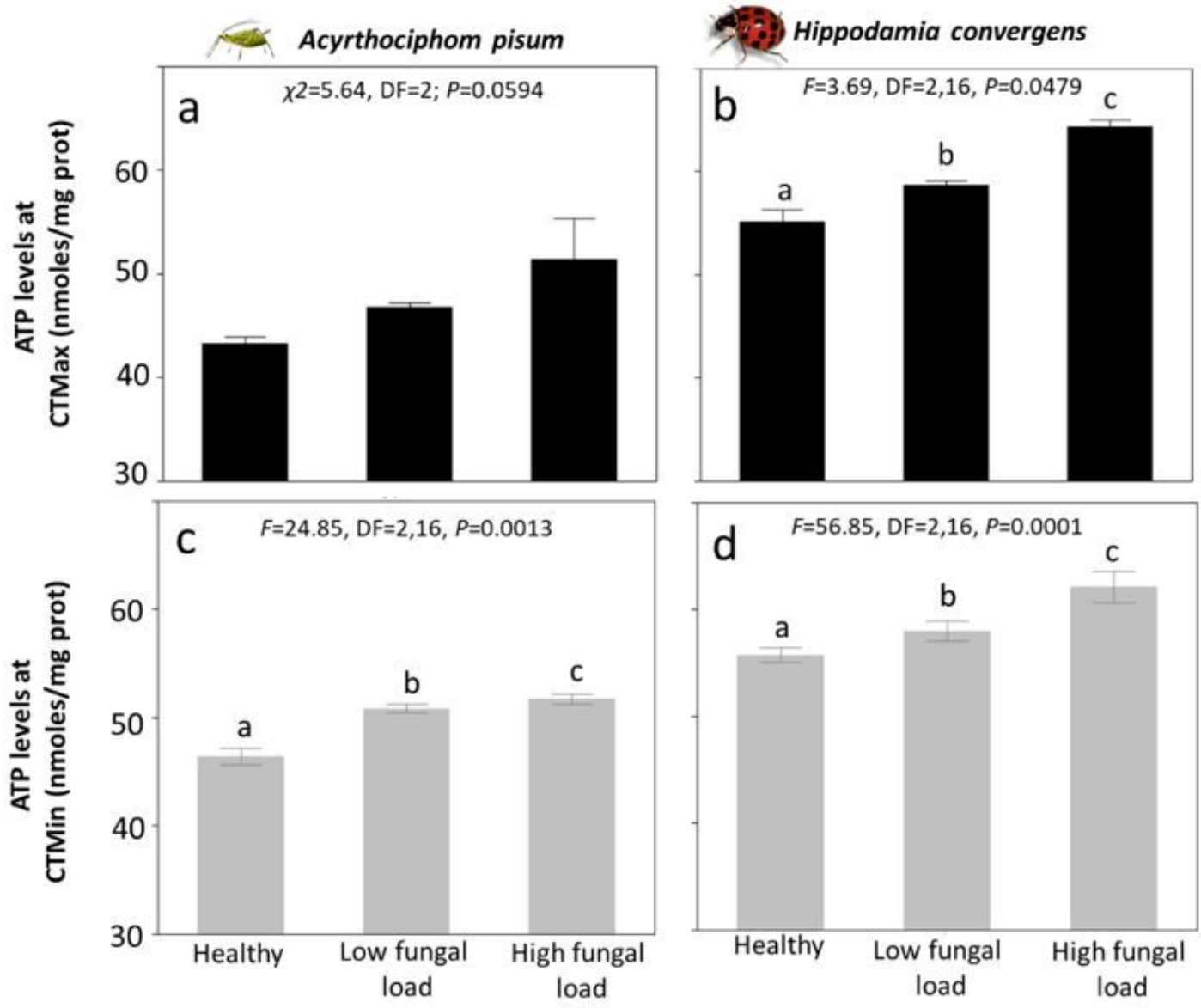

Figure 3

ATP levels of healthy and infected aphid and predator beetle species at upper and lower thermal tolerance (CTMax or CTMin). Infections: Low spore load= 1.4 x 10 6 spores ha-1, high spore load = 1.4 x 10 12 spores ha-1. Anova test, bars represent mean ± SE, n=3 individuals per treatment; black bars: CTMax. Gray bars: CTMin.

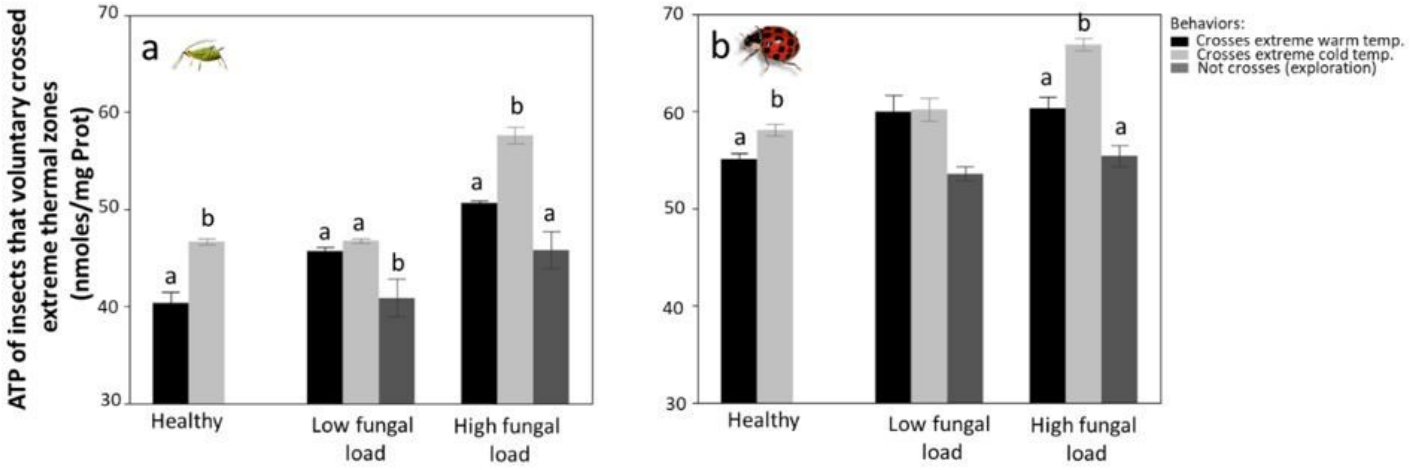

Figure 4

ATP levels of healthy and infected aphids (a) and predator beetles (b) that voluntarily crossed (or not) through extreme thermal zones. A. pisum F=29.13 DF=7,16, P <0.001. H. convergens: F=16.77, DF=7,16; P <0.0001 Infections: Low fungal load= 1.4 x 10 6 spores ha-1, high fungal load = 1.4 x 10 12 spores ha-1. Bars represent mean ± SE, n=3 individuals per treatment.

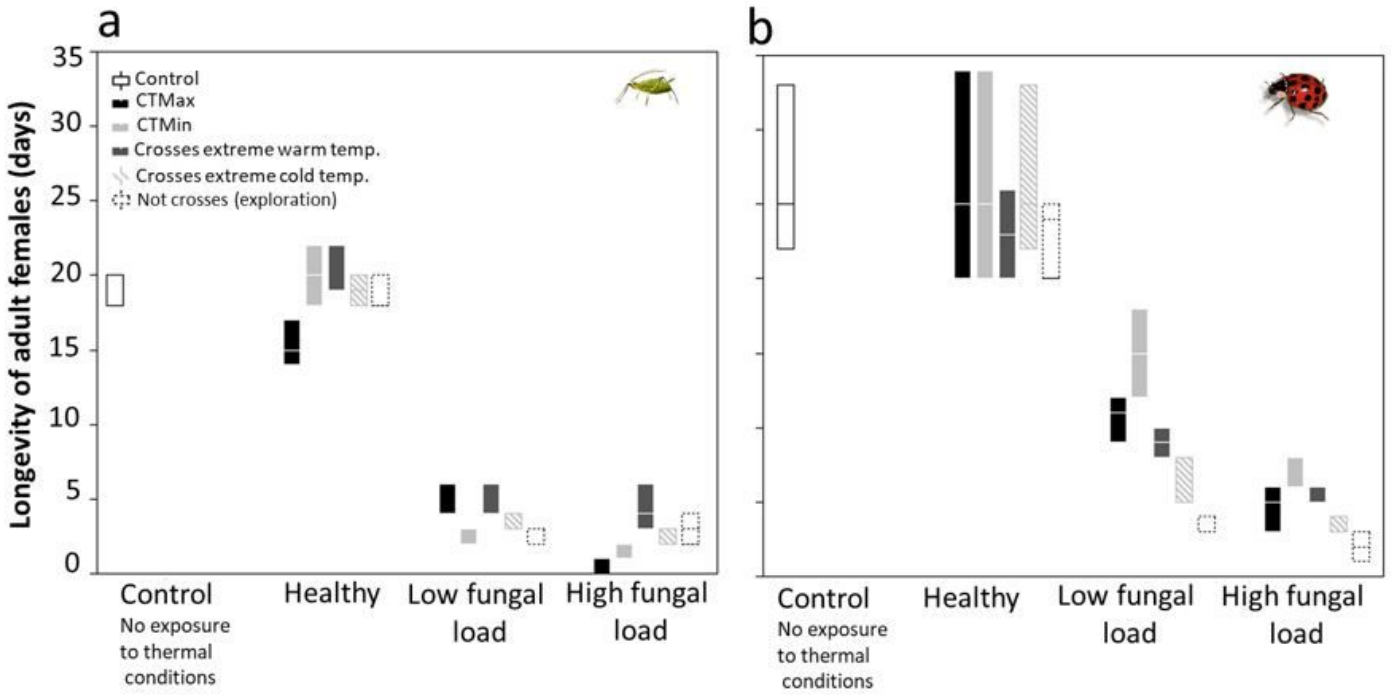

Figure 5

Longevity of adult healthy and infected aphids (a) and predator beetles (b) that experienced thermal conditions: control, healthy and infected individuals exposed to CTMax, CTMin, crosses to warm or cold

ETZ, and exploration but no ETZ crosses. Healthy, low fungal load= 1.4 x 10 6 spores ha-1, high fungal load = 1.4 x 10 12 spores ha-1 (box plots display median line, interquartile range (IQR) boxes, 1.5 x IQR, n=3 individuals per treatment).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementaryinformation1.docx