University of Nevada Reno

Microhabitat, microbiota, mitochondria and the epigenome shape the biparental legacy of heat exposure in a tropical arthropod

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By

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Abstract

As technology and policy advance towards sustainable energy sources, humanity is reaching a critical climax regarding climate change. Average global surface temperatures have risen by 1ºC since pre-industrial levels, and it is estimated that temperatures will continue to rise by at least 1.5ºC until at least 2100, despite mitigation efforts. The effect that such warming will have on biodiversity remains undetermined. Warming will likely have a substantial impact on tropical regions, which are a hotbed for biodiversity, and warming in tropical regions has already been associated with massive declines in species population numbers. Whether biodiversity will continue to decline, culminating in mass extinction, due to climate change depends on the ability of tropical species to adapt to new thermal limits. Tropical regions exhibit low seasonal variability, thus tropical species are adapted to little variation in environmental conditions and rapid increases in temperature may prove unsustainable for life. Tropical arthropods make up the majority of species in the tropics, with 1.3 million species described, and the true estimate at several million species. However, they are at especially high risk, since as ectotherms, arthropod metabolism increases exponentially with increases in ambient temperature. Understanding the effect of temperature on tropical arthropods is crucial to understanding biome dynamics to target conservation efforts as the planet continues to warm. Epigenetic mechanisms, or changes in gene expression without changes in the genetic sequence, provide a short-term plastic way for tropical arthropods to respond to climate change. Here,

I utilize a model ectothermic arthropod, *Cordylochernes scorpioides,* to investigate: (1) behavioral mechanisms for moderation of the effects of high temperature; (2) phenotypic, genetic and epigenetic effects of high temperature exposure on intergenerational inheritance, and (3) the microbial composition of individuals exposed to high temperature. This tropical arthropod has a unique reproductive biology, enabling non-invasive investigation into fitness effects and reproductive capacity, and it also exhibits high variation in the mitochondrial genome, which was exploited to determine how mitochondrial function may influence biological responses to climate change.

In Chapter 1, I present the results of a study designed to determine how microclimate variation in lowland tropical rainforests impacts the abundance in *C. scorpioides* populations. Tropical rainforests exhibit high levels of fine-scale climatic variation, yet climate studies in these regions typically retrieve temperature data from large-scale weather stations. This provides an incomplete view of how tropical arthropods may experience temperature and change their behavior to compensate for warming. This species of arthropod inhabits decaying *Ficus* trees in their native Panamanian rainforests. Several *Ficus* trees occupied by *C. scorpioides* were found and microclimate variation was assessed by placing iButton temperature loggers in three types of microhabitat, frass, side/south, and top/north over two types of trees, fallen and standing, in open canopy or closed canopy habitats. *C. scorpioides* were then collected from the trees to estimate abundance. Reduced abundance was associated with the hottest microhabitats, top/north in open canopy trees, and high abundance was

associated with the coolest microhabitats, frass in closed canopy trees. Thus *C. scorpioides* actively manage exposure to high temperatures through behavioral mechanisms.

In Chapter 2, I investigate whether phenotypes from simulated climate change can be passed to offspring intergenerationally. During nymphal development, *C. scorpioides* were placed in incubators programmed to diurnally fluctuate in correspondence with ambient temperature measurements from rainforest habitats found in Chapter 1. A split-brood design was employed where, upon birth, 40 pseudoscorpions were collected from each family and half were directly exposed to a control temperature regime and the other half to a high (+2.5º) temperature regime. Developmental, survival, and male and female reproductive traits were assayed. An intergenerational effects study was then carried to determine whether direct effects of high temperature exposure are transmitted to offspring maternally, paternally or through both sexes. Females and males directly exposed to the high temperature were mated to control males and females, respectively, to establish female outcross (FOUT) and male outcross (MOUT) families. Developmental, survival, and reproductive traits were then assayed on offspring. As in males directly exposed to high temperature, males with both high-temperature treated mothers and fathers had significantly reduced sperm counts, though this effect was stronger in males with high temperature mothers. Sperm counts were also significantly affected by haplotype, with A2 haplotype males producing the most sperm and B2 haplotype males the most negatively affected by high-temperature treatment of the parent.

Female reproductive traits did not exhibit significant intergenerational effects, suggesting they are more robust to the effects of high temperature. However, high temperature effects on some non-reproductive traits, including reduced survivorship and reduced male offspring size, were transmitted maternally. Interestingly, although males born to high-temperature mothers exhibited reduced body size, their development time was not significantly reduced, as in the directly treated generation. By contrast, female offspring with high temperature fathers had reduced developmental time, but not significantly reduced body size, indicating an uncoupling of size-temperature dependence and developmental rate often associated with arthropod species. This is hypothesized to be due to *C. scorpioides* XX/XO system of sex determination, where epigenetic or genetic mutations acting on the males' lone X chromosome make them more vulnerable to sex-linked effects of high temperature. However, males with high temperature mothers experienced some positive effects of reproduction, producing 22% more offspring than did males with control temperature mothers. The best explanation for this may be selection, where only the most genetically or epigenetically fit males were capable of siring offspring.

In Chapter 3, I determined the potential for intergenerational epigenetic effects in males exposed to high temperature by assaying gene expression of protein-coding genes, transposable elements, and noncoding RNA of testicular and spermatic tissue. Transposable elements are mobile genetic elements able to excise themselves and move throughout the genome, capable of inducing genomic instability and disrupted gene expression, but they are typically

controlled by epigenetic modifications to silence their expression. They are highly expressed in the germline, where suppression is achieved through targeted destruction by non-coding RNAs known as PIWI-interacting RNAs. Approximately 70% of the *C. scorpioides* genome consists of transposable elements/highly repetitive regions, an unusually high level for an arthropod species, making transposable element expression a likely factor in intergenerational inheritance of alternative phenotypes in males exposed to high temperature. Males exposed to high temperature were either dissected for removal of the testes or had their mating sequence interrupted to collect sperm, and RNA sequences annotated to determine levels of differential expression. Testicular protein-coding genes and transposable elements were significantly up-regulated, and testicular and spermatic piRNA expression were significantly down-regulated in males exposed to high temperature. Hence, high temperature exposure likely induces transposable element activity in the germline, and causes dysregulation of the epigenetic control mechanisms designed to silence them.

Finally, in Chapter 4, I conducted microbial diversity analysis to determine whether other mechanisms may be responsible for alternative phenotypes intergenerationally inherited from exposure to high temperature. Diverse and healthy gut microbiomes play a key role in host metabolism, physiology, nutrition, immune function, and pathogen defense for many species, with differing microbial profiles associated with environmental factors, such as temperature and nutrition, and host factors, such as genotype or mitochondrial haplotype. On

the other hand, dysbiosis, or imbalances in the gut microbiome, have been associated with a variety of diseases, including metabolic disorders, immune disorders, neurodegenerative disease, and psychological disorders. Growing evidence also suggests that crosstalk between the gut microbiome and the host is mediated by microbial-derived molecules that induce changes in the epigenetic mechanisms that regulate host gene expression. Microbial diversity of control temperature and high temperature *C. scorpioides* were analyzed using a factorial design of two temperature treatments and three haplotypes to investigate temperature, haplogroup, and interaction effects on the diversity and community composition of the *C. scorpioides* microbiome. Elevated temperature was associated with increased microbial diversity, which was a counterintuitive result as increased microbial diversity is often correlated with a healthy microbiome, yet elevated temperature induced maladaptive states in *C. scorpioides*. The most variation between microbial profiles was due to haplotype, with A1/A2 haplotypes having higher diversity than B2 haplotypes. Lack of appreciable temperature effects on the *C. scorpioides* microbiome coupled with significant haplogroup effects suggest that microbial composition changes are not responsible for detrimental phenotypic effects of a 2.5ºC temperature increase found in Chapter 2, and that epigenetic mechanisms and loss of epigenetic control over transposable elements, as discovered in Chapter 3, are implicated instead.

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Microclimate variation matters for a tropical ectotherm in decaying Ficus tree habitats

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Abstract

As ectotherms adapted to already high and relatively constant temperatures, tropical terrestrial arthropods may be vulnerable to even the modest climate warming occurring at tropical latitudes. Tropical forests appear to exhibit heterogeneity in fine-scale microclimates, yet climate change studies commonly rely on ambient air temperature data measured by weather stations at a coarse-scale, macroclimate level. Here, we investigated temperature variation between decaying *Ficus* tree microhabitats as a potential buffer of climate warming for the harlequin beetle-riding pseudoscorpion, *Cordylochernes scorpioides*. This pseudoscorpion has proved to be a model organism for experimental investigation of the phenotypic, genetic, and epigenetic effects of climate change. The study demonstrated significant microclimate variation within and between fallen and standing decaying *Ficus* tree habitats in open and closed canopy environments. Mean temperature, maximum temperature and diurnal temperature ranges were greatest on the top sides of fallen/open canopy trees where, on the hottest days, maximum temperature and daily temperature range exceeded 40^0C and 20^0C, respectively. These values surpassed those for ambient air temperature by 10^0C to 15^0C. Across all tree types, mean ambient air temperatures were consistently 1^0C to 1.5^0C cooler than any of the pseudoscorpion's microhabitats. While tree type effects on diurnal temperature regimes appear to have a major impact on *C. scorpioides* abundance, it seems

unlikely that microhabitat selection could mitigate the long-term effects of global warming on this tropical ectotherm and the highly diverse community of saproxylic invertebrates that exploit decaying *Ficus* tree habitats in the Neotropics.

1 | INTRODUCTION

The threat to biodiversity posed by climate warming is most extreme in the tropics which cover only one third of the Earth's land surface (Kricher, 2011; Feeley & Stroud, 2018) but account for more than three quarters of plant and animal species (Barlow et al., 2018). In an analysis of species ranges for ten taxonomic groups, Barlow et al. (2018) found that 91% of terrestrial birds occur within tropical regions, as do over 75% of terrestrial mammals, freshwater fish and amphibians. Terrestrial invertebrates are less well studied but are major contributors to tropical biodiversity (Dahlsjö, et al., 2020). There are 1,359,129 described invertebrate species (IUCN 2019) but the actual number is estimated at seven million (Stork, 2018) and many of these species inhabit tropical rainforests.

As ectotherms adapted to already high and relatively constant temperatures, tropical terrestrial invertebrates may be vulnerable to even the modest climate warming occurring at tropical latitudes (IPCC 2014). On average, temperatures in tropical rainforests have increased by 0.26^{o}C per decade since the mid-1970s (Malhi & Wright, 2004). Analysis of long-term data on climate change and arthropod abundance in the Luquillo Rainforest, Puerto Rico (Lister & Garcia, 2018) found that mean maximum yearly temperature increased by 2.0^{o}C between 1978 and 2015 at an average rate of 0.05^{o}C per year. Although lower than the amplitude of climate warming in polar and temperate regions, this rate of

temperature increase was associated with a massive 10- to 60-fold decline in arthropod biomass compared to the 1970s (but see Lister & Garcia, 2019; Willig et al., 2019). Lister and Garcia (2018) proposed a causal relationship between arthropod decline and temperature increase, consistent with theoretical predictions that physiological consequences of climate warming should be most serious for tropical ectotherms (Deutsch et al., 2008; Dillon, et al., 2010). Because ectothermic metabolic rate increases exponentially with temperature (Gillooly, et al., 2001; Clark, 2006), even a small temperature increase is likely to have detrimental effects on terrestrial ectotherms in tropical regions (Deutsch et al., 2008; Dillon, et al., 2010).

Climate change studies commonly rely on ambient air temperature data measured by weather stations at a coarse-scale, macroclimate level (Potter, et al., 2013; De Frenne & Verheyen, 2016). Responses to climate warming, including elevational and latitudinal range shifts, and declines in abundance, as well as local and global extinctions, have been documented in a variety of tropical species (Sheldon, 2019). Although negative effects of warming could theoretically be mitigated by physiological adjustment to elevated temperatures, ectotherms have limited ability to adjust the upper limit of their thermal tolerance (Gunderson & Stillman, 2015). However, tropical forests exhibit extensive heterogeneity in fine-scale microclimates (Kaspari, et al., 2015; Mau, et al., 2018) and tropical ectotherms could potentially compensate for lack of thermal plasticity by moving between microhabitats (Pincebourde & Suppo, 2016). Here, we

investigated temperature variation between microhabitats as a potential buffer of climate warming for the neotropical pseudoscorpion, *Cordylochernes scorpioides*. Distributed throughout the rainforests of Central and South America, this ectotherm inhabits decaying trees in the families Moraceae and Apocynaceae (Zeh & Zeh, 1994a,b; Zeh, et al., 2003), and disperses under the elytra of the giant harlequin beetle, *Acrocinus longimanus*, which uses the same trees for mating, oviposition and larval development (Zeh, et al., 1997). Locating *Ficus* trees at a prime stage of decay in rainforests is an extremely challenging and time-consuming endeavor with a strong stochastic component. In order to characterize spatial and temporal temperature variation in a study involving simultaneous, replicate sampling, we opportunistically exploited the discovery of multiple standing and fallen *Ficus* trees during the peak season of population growth for the diverse community of saproxylic invertebrates that exploit decaying *Ficus* tree habitats (Zeh, et al., 1992; Zeh & Zeh 1994b).

2 | METHODS

A 10 km^2 search of lowland rainforest conducted between 22 June and 26 July 2017 in and around Parque Nacional Soberanía, central Panamá yielded seven dead and dying *Ficus* trees (Table 1) with evidence of harlequin beetle activity (see Fig. 1). Lack of leaves on most trees precluded species-level identification but they were likely *Ficus insipida* which occurs commonly in secondary forest

throughout the area. Colonization by *C. scorpioides* was confirmed by presence of pseudoscorpions under loose bark and in accumulated frass. To assess *C. scorpioides'* population density, decaying trees were inspected repeatedly over the five-week period and pseudoscorpion nymphs and adults were collected from trunks and the underside of bark fragments arranged on accumulated frass.

Table 1. Location, tree type, canopy type and *Cordylochernes scorpioides* abundance data for the seven decaying *Ficus* trees investigated for spatial and temporal temperature variation in lowland rain forest in central Panamá.

Tree ID	**Latitude**	**Longitude**	**Tree Type**	**Canopy Type**	**Tree Category**	**Collected *C. scorpioides***
GR1	9°5' 22.1994"N	79°40' 29.6394"W	fallen	open	fallen/open	18
MR2	9°6' 58.0710"N	79°37' 6.4770"W	fallen	closed	fallen/closed	35
OD1	9°7' 57.3198"N	79°43' 26.4066"W	standing	closed	standing/closed	66
OD2	9°7' 57.0000"N	79°43' 27.4074"W	standing	closed	standing/closed	75
PLT 1	9°4' 29.7942"N	79°39' 33.7710"W	fallen	closed	fallen/closed	60
PLT 2	9°6' 54.8310"N	79°38' 39.9510"W	standing	closed	standing/closed	36
PLT 3	9°5' 21.9984"N	79°39' 13.9962"W	fallen	open	fallen/open	7

Figure 1. Trunk of standing tree, OD2, showing evidence of harlequin beetle activity, including “ladders’ of oviposition slits in bark, as well as larval holes in the trunk surrounded by circular sections of missing bark chewed out by the wood-boring larvae.

Microclimate variation within and between the four fallen and three standing *Ficus* tree habitats was investigated, using iButton data loggers (DS1921H-F5#, Maxim Integrated, San Jose, USA) to record temperatures at 15-min intervals for a minimum of 72 hr. Tree identification codes are included in Table 1. Data loggers were enclosed in clear, plastic sheaths to protect them from rain damage and were attached to trunks with panel pins. For standing trees, temperatures were recorded on the north and south sides of the tree approximately 1 m above the forest floor, and in accumulated frass/debris at the tree base created by wood-boring insects. For fallen trees, temperatures were recorded on the top and

side of the trunk, and in accumulated frass/debris beneath the trunk. For all tree locations, we also recorded ambient air temperature by suspending a data logger 1 m above the forest floor in full shade within 50 m of the target tree.

To analyze effects of tree type and microhabitat on temperature, we used SAS PROC GLIMMIX, a general linear mixed model (GLMM) procedure (SAS Institute, Cary, NC, USA, 2018). To avoid pseudoreplication from repeated temperature sampling of individual trees, tree identity was included as a random effect, and the denominator degrees of freedom were constrained as number of sampled trees minus one. Temperature data were approximately normally distributed, and the model therefore incorporated a Gaussian distribution, an identity link function and a Laplace maximum likelihood approximation.

Two types of analyses were performed. In the first analysis which focused on temperature extremes, the main and interaction effects of tree type (fallen tree/closed canopy, fallen tree/open canopy, standing tree/closed canopy) and microhabitat (frass, side or south, top or north) were assessed for daily minimum temperature (T_{min}), daily maximum temperature (T_{max}) and diurnal temperature range (DTR), calculated as the difference between daily maximum and minimum temperatures. From a temperature perspective, the top of fallen trunks most closely approximates the north side of standing trunks, since the sun's heading is northward in Panamá in June/July. Ambient air temperature was included as a covariate to adjust for daily temperature differences. For the second analysis, main and interaction effects of tree type and microhabitat on mean temperature

(T_{mean}) were investigated. Both types of analyses incorporated linear contrasts (Rosenthal, Rosnow & Rubin, 2000) for customized hypothesis testing.

Because pseudoscorpion collection data were not normally distributed, the effect of tree type on *C. scorpioides* abundance was analyzed using a generalized Poisson mixed model for overdispersed count data, incorporating a log link function and a Gauss–Hermite Quadrature maximum-likelihood approximation (SAS Institute Inc, 2018: 3925–3932).

3 | RESULTS

A total of 11,760 microhabitat temperatures were analyzed. Diurnal temperature fluctuations involved a steep increase between 7:00 am and 2:00 pm followed by a gradual decline between 5:00 pm and 6:00 am (Fig. 2). For the T_{max} analysis (n = 104 temperature recordings), tree type ($F_{2,6}$ = 42.26, p = 0.0003), microhabitat ($F_{3,6}$ = 44.62; p = 0.0002) and the interaction between tree type and microhabitat ($F_{6,6}$ = 29.85, p = 0.0006) were all highly significant. Averaged across microhabitats, fallen/open canopy trees were hotter than other tree types (fallen/open $\bar{x}$ = 31.59 ± 0.29, fallen/closed $\bar{x}$ = 29.15 ± 0.30 and standing/closed $\bar{x}$ = 27.95 ± 0.25). Averaged across tree types, mean microhabitat T_{max} temperatures were arranged in the order top/north > side/south > ambient > frass (contrast $F_{1,6}$ = 112.88, p < 0.001). The highest mean T_{max} for all tree type by microhabitat combinations was the top side of fallen/open canopy trees ($\bar{x}$ =

37.03 ± 0.54) (Fig. 3a). Mean T_{min} was significantly affected by microhabitat ($F_{2,6}$ = 110.97, p < 0.001) but not tree type ($F_{2,6}$ = 0.96, p = 0.4363). The contrast top/north = side/south = frass > ambient accounted for the most variation in mean T_{min} ($F_{1,6}$ = 22.53, p < 0.0001).

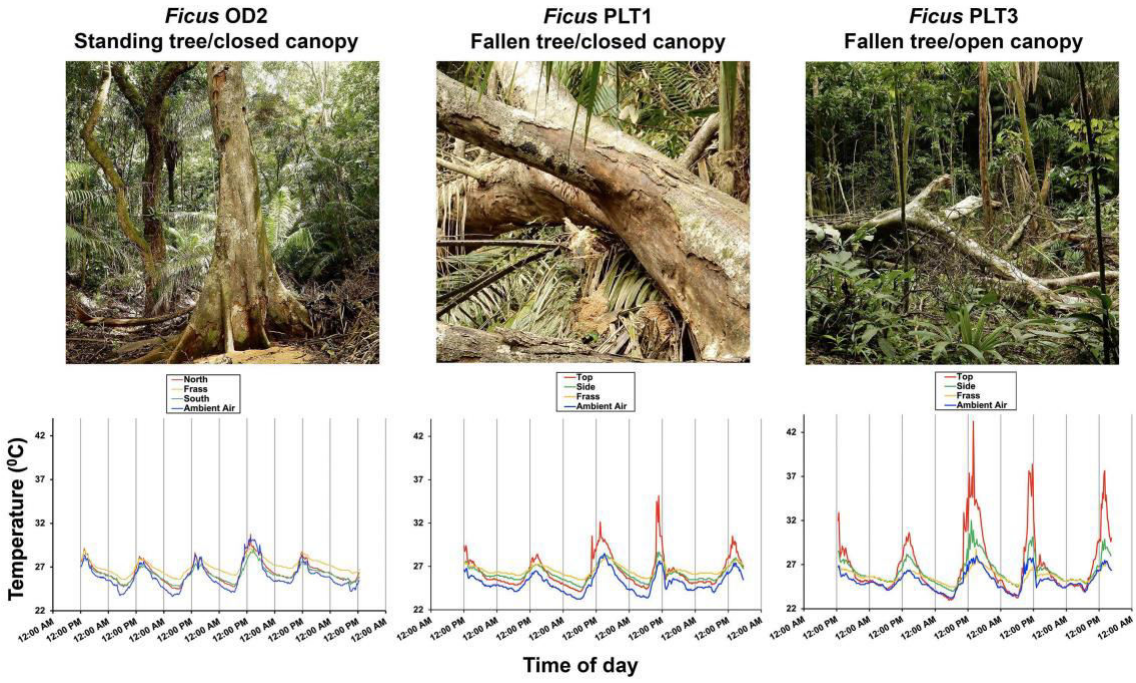

Figure 2. Diurnal temperature fluctuations shown below photographs of representative decaying *Ficus* tree types and microhabitats utilized by the harlequin beetle riding pseudoscorpion, *Cordylochernes scorpioides* in central Panamá. Despite extensive harlequin beetle damage, the OD2 tree was still alive, with leaves providing canopy shade.

DTR results were nearly identical to those for T_{max} (Fig. 3b), with highly significant main effects of tree type ($F_{2,6}$ = 47.93, p = 0.0002), microhabitat ($F_{3,6}$ =

50.15; $p = 0.0001$) and tree type by microhabitat interaction ($F_{6,6} = 19.95$, $p = 0.0010$). The contrast, top/north > side/south > ambient > frass, accounted for most variation in mean DTR ($F_{1,6} = 133.17$, $p < 0.0001$).

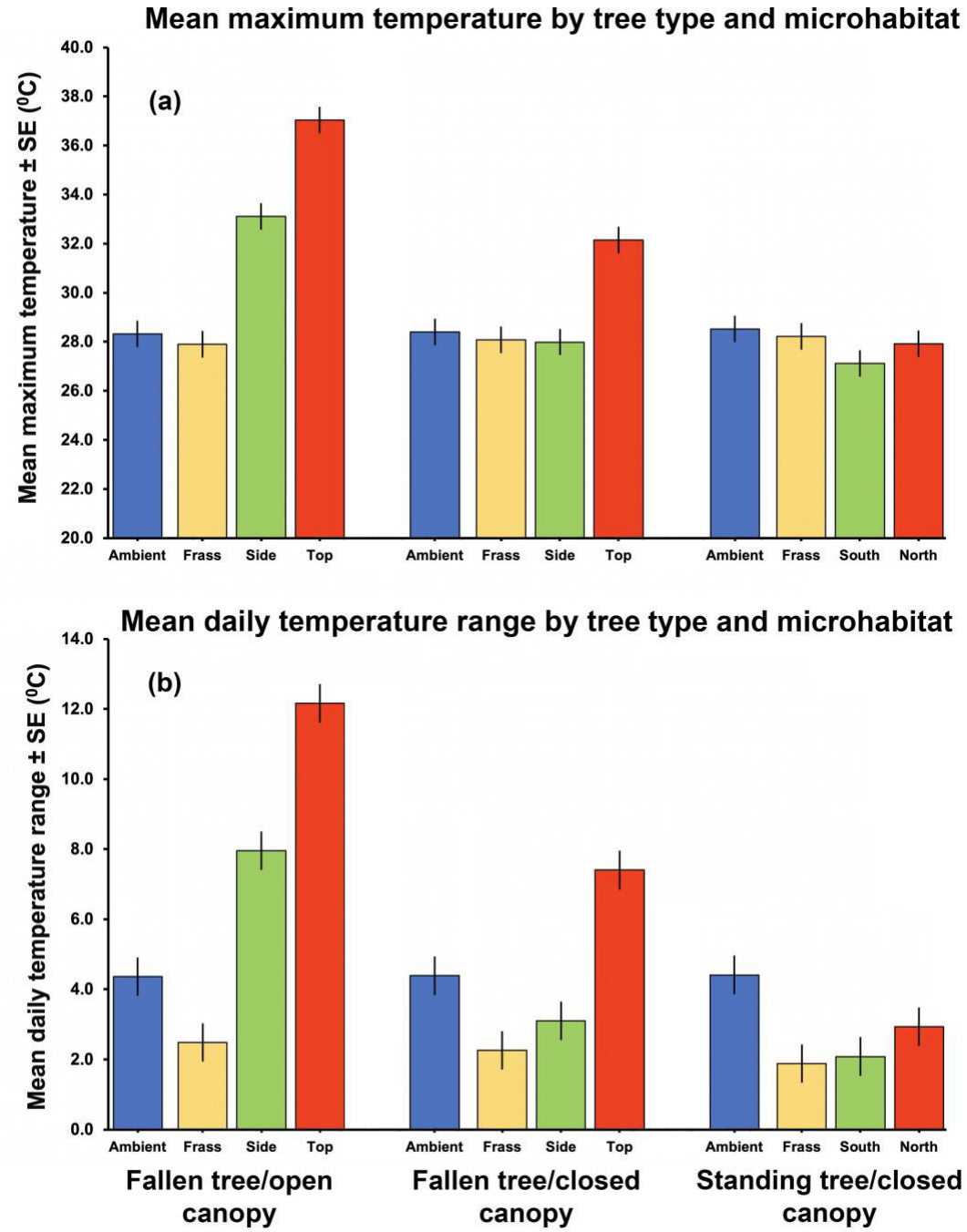

Figure 3. Effect of tree type/microhabitat combination on (a) mean daily maximum temperature and (b) mean daily temperature range for seven fallen and standing *Ficus* trees.

The T_{mean} analysis yielded a highly significant main effect of tree type ($F_{2,6}$ = 325.12, $p < 0.0001$) and microhabitat ($F_{3,6}$ = 1,485.88; $p < 0.0001$) on T_{mean}, as well as a significant interaction between tree type and microhabitat ($F_{6,6}$ = 618.40, $p < 0.0001$). Most variance in T_{mean} between tree types was explained by the relationship fallen/open > fallen/closed > standing/closed (fallen/open $\bar{x}$ = 26.74 ± 0.02, fallen/closed $\bar{x}$ = 26.33 ± 0.02, standing/closed $\bar{x}$ = 26.15 ± 0.01, contrast: $F_{1,6}$ = 645.04, $p < 0.0001$). Averaged across tree types, all microhabitats were significantly hotter (1-1.5°C) than mean ambient air temperature (contrast $F_{1,6}$ = 4,132.55, $p < 0.0001$). Much of the variance in T_{mean} across microhabitats was also explained by the relationship top/north > frass > side/south > ambient (contrast $F_{1,3}$ = 3,646.92, $p < 0.0001$). The top side of fallen/open canopy trees exhibited the highest T_{mean} ($\bar{x}$ = 27.85 ± 0.03) (**Fig**. 4A).

A total of 297 *C. scorpioides* were collected from the seven *Ficus* trees. Tree type significantly affected number of pseudoscorpions collected ($F_{2,4}$ = 11.92, p = 0.0206) (Fig. 4B). Standing/closed canopy trees, with their low mean temperatures and minimal diurnal temperature fluctuations, supported the highest *C. scorpioides* abundance ($\bar{x}$ = 59.00 ± 7.58), followed by fallen/closed ($\bar{x}$ = 47.50 ± 9.29) and fallen/open canopy trees ($\bar{x}$ = 12.50 ± 9.29).

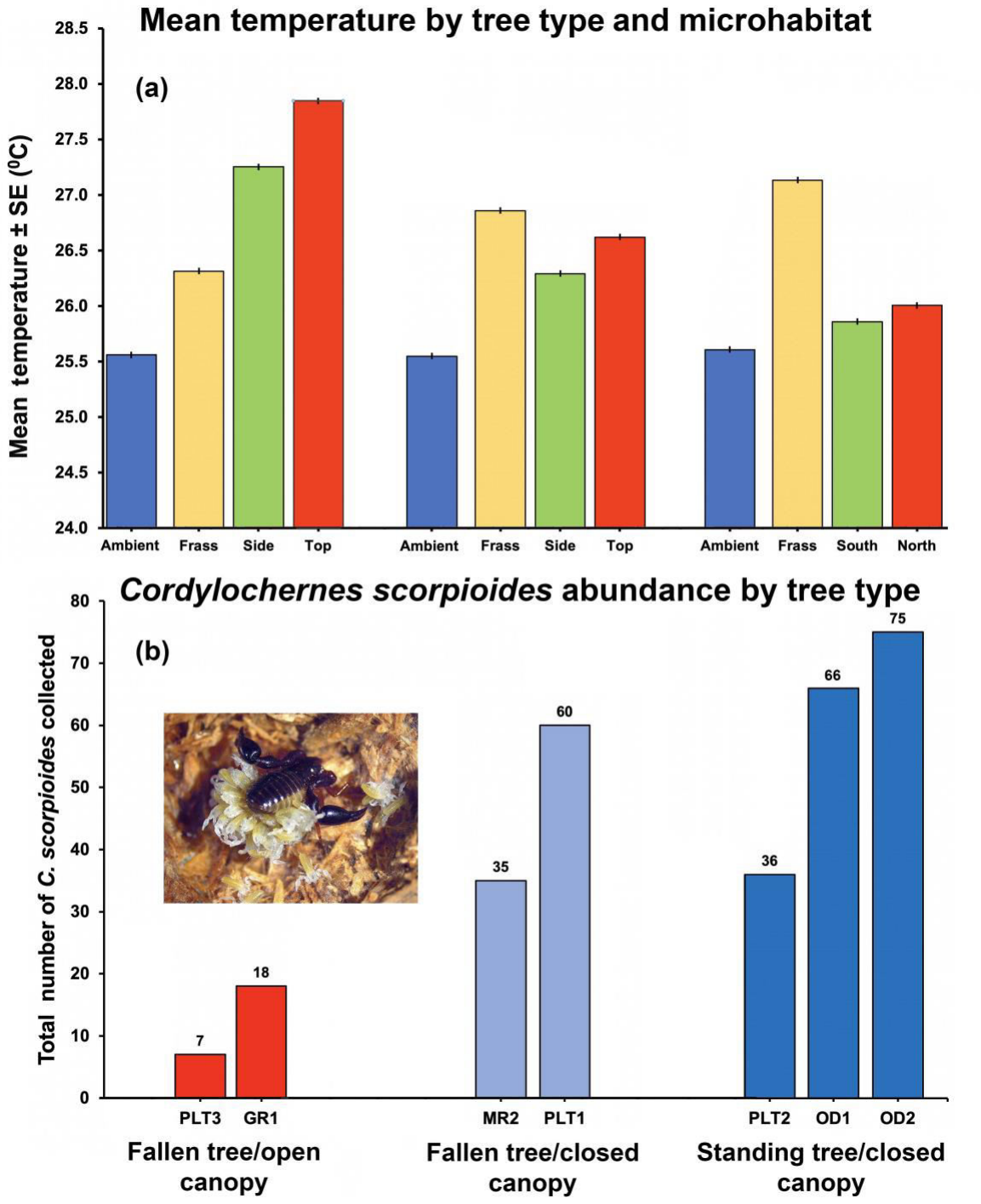

Figure 4. (a) Effect of tree type/microhabitat combination on mean temperature and (b) effect of tree type on *C. scorpioides* abundance for the seven fallen and

standing *Ficus* trees assayed in this study. The insert shows a gravid *C. scorpioides* female giving birth to a brood of protonymphs.

4 | DISCUSSION

This study demonstrated significant microclimate variation in habitats utilized by *C. scorpioides.* Mean temperature, maximum temperature and diurnal temperature ranges were greatest on the top sides of fallen/open canopy trees where, on the hottest days, T_{max} and DTR exceeded 40^0C and 20^0C, respectively, values that surpassed those for ambient air temperature by 10^0C to 15^0C. Across all tree types, mean ambient air temperatures were consistently 1^0C to 1.5^0C cooler than any of the pseudoscorpion's microhabitats. Microhabitat temperature buffering was only evident in frass which retained relatively modest temperatures despite variability in maximum daily temperature and diurnal temperature range. Frass also exhibited a 2˚C reduction in daily temperature range compared to that of ambient air. However, previous research indicates that even constant diurnal temperatures only reduce but do not eliminate the fitness costs of elevated temperature for *C. scorpioides* (Zeh et al. 2012, 2014).

Beyond investigation into behavioral adaptations to climate warming, the harlequin beetle-riding pseudoscorpion has proved to be a model organism for experimental investigation of the phenotypic, genetic and epigenetic effects of climate change on a tropical ectotherm. In simulated climate warming studies, a

3.5ºC increase altered levels of small, noncoding RNA expression in *C. scorpioides* (Su-Keene, et al., 2018), significantly reduced survival, development time and adult size but had its most detrimental effects on sperm production and male and female reproductive success (Zeh et al., 2012; Zeh et al., 2014). Control temperature regimes in these previous studies were based on long-term shaded air temperatures recorded by a weather station in the pseudoscorpion's native habitat (Zeh et al., 2012; Zeh et al., 2014). However, mapping of microclimates is increasingly recognized as essential for understanding and predicting species' responses to climate change (Bramer et al., 2018; Buckley, et al., 2018; Zellweger, et al., 2018). The results of this study indicate that mean temperatures experienced by *C. scorpioides* in decaying *Ficus* trees may exceed mean ambient air temperature by as much as 2.5ºC. Here, we found a significant effect of tree type on pseudoscorpion abundance, with cooler standing/closed canopy trees supporting the largest *C. scorpioides* populations, and very few individuals in hotter fallen/open canopy trees. However, it seems unlikely that microhabitat selection could mitigate the long-term effects of global warming on this tropical ectotherm.

Following the brief period when newly dead and dying trees attract harlequin beetles for mating and oviposition, *C. scorpioides* populations remain marooned in dead trees for two or three generations until beetle larvae complete development, eclose as adults and the pseudoscorpions climb on board to disperse en masse (Zeh, et al., 1992). The wood-boring activity of *A. longimanus*

larvae generates a microhabitat of detached bark and accumulated frass that serves as a breeding site for a myriad of saproxylic invertebrates (Ulyshen, 2018) that colonize the dead tree. Consequently, the effects of natural temperature variation on *C. scorpioides* abundance reported here suggest that the temperature increases projected for the tropics over the coming decades (IPCC 2014) may well greatly elevate extinction risk not only for the harlequin beetle-riding pseudoscorpion but for the entire diverse but poorly studied community of saproxylic invertebrates that inhabit decaying *Ficus* trees and play a vital role in nutrient recycling in tropical rainforests.

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From parents to offspring: intergenerational transmission of climate warming effects on a tropical ectotherm

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Abstract

Climate change is projected to increase by a minimum of 1.0°C-5.7°C by the end of the century. Many species will be affected by such rapid changes in global surface temperature. Of particular concern are tropical ectotherms, as the metabolic rate of ectothermic species increases exponentially with temperature and tropical species already live close to their thermal optima. While many experiments focus on the direct effects of climate warming on species, fewer investigate the intergenerational and transgenerational effects. Epigenetic modifications, such as DNA methylation, histone modification, and the production of regulatory small and large noncoding RNAs, are highly mutable under stressful environmental conditions and are heritable from parent to offspring. Such modifications may alter the gene expression of offspring after parental exposure to stressful temperatures. Here, we utilize the model organism *C. scorpioides* to determine how increased temperatures experienced by the parent generation affect phenology and reproductive traits in offspring, and whether affected traits are passed primarily by the mother or father. We take advantage of *C. scorpioides* mitochondrial variation naturally co-occurring in the native population to determine how increased temperature and mitochondrial variation interact, as many epigenetic processes are directly influenced by metabolites used in the production of ATP. While female reproductive traits are generally robust to intergenerational effects of simulated climate warming, male reproductive traits,

notably sperm number, were highly negatively affected. Offspring of high temperature females had reduced survivorship and reduced adult male size, and they also exhibited differences in reduced survivorship and sperm number depending on mitochondrial haplotype, suggesting mitochondrial variation indeed plays a role in organismal reaction to high temperature exposure. Surprisingly, one positive intergenerational effect was recorded, as offspring with high temperature fathers yielded higher counts of protonymphs after mating and were more likely to successfully reproduce.

1 | INTRODUCTION

Global climate change has been well documented for decades, but the extent to which it will affect weather patterns and erode biodiversity remains uncertain (Moss, et al., 2010). However, the most recent report by the United Nations' International Panel on Climate Change (IPCC) assesses the physical science basis of climate change and reaches the unequivocal conclusion that, even under the best-case scenario in which global warming is limited to an increase of 1.0°C to 1.8°C by the end of the century, global temperatures will continue to increase until at least 2050, despite mitigation efforts (IPCC, 2021). This report assessed climate response to five predictive scenarios that vary in levels of carbon dioxide (CO_2) and greenhouse gas (GHG) emissions, compared to pre-industrial levels during the period 1850–1900 (used as a baseline). Very low and low CO_2 and GHG emission scenarios involve a decline in emissions to net zero by the middle of the century and a period of net negative emissions. With intermediate CO_2 and GHG emissions, current levels of emissions are maintained until the middle of the century. In the case of high and very high CO_2 and GHG emissions, current levels double by 2100 and 2050, respectively. Under the intermediate and high GHG emission scenarios, an increase of at least 2°C by the middle of the century is very likely to occur. An increase of 2.1°C to 3.5°C is predicted for the intermediate scenario, and an increase of 3.3°C to 5.7°C for the high and very high GHG scenarios (IPCC 2021). To set these

predictions in a broader time context, the authors of the report turn to paleoclimate evidence (Haywood et.al., 2020) and point out that the Earth last sustained global surface temperatures reaching or in excess of 2.5°C above the 1850-1900 baseline temperatures during the Pliocene Epoch three million years ago.

For each unit increase in global surface temperature, disruptive climatic events increase in number and intensity, with heatwaves, heavy precipitation, and meteorological droughts occurring in unprecedented numbers (Donat, et al., 2016; IPCC, 2021; Mazdiyasni & AghaKouchak, 2015; Trenberth, 2011). Some mid-latitude regions are projected to experience the highest increase on the hottest days, approximately 1.5 to 2 times that of the global projected increase. The disproportionate effects that this increase will have on the tropics is likely to have a major impact on biodiversity (Sala, et al., 2000). Tropical regions are a hotbed for biodiversity (Brown, 2013; Fine, 2015), but the number of species has been declining due to anthropogenic activity (Hooper, et.al., 2005; Malcolm. et al., 2006; Novotny & Miller, 2014). Arthropods comprise the vast majority of species in the tropics (Erwin, 1982), and will be disproportionately affected by climate change and anthropogenic activity (Cardoso, et al., 2020). In ectotherms, temperature influences the rate of basic metabolic functions, and a single unit increase in temperature results in an exponential increase in metabolism (Gillooly, 2001; Klok & Harrison, 2013). Tropical arthropods have responded thus far to climate warming through phenology shifts (Forrest, 2016) and geographic

range shifts (Chen, et al., 2011) but, if species cannot shift fast enough to match the pace of warming, survival will depend on thermal sensitivity (Fitzgerald, et al., 2021). Since tropical arthropods experience low seasonal variability in temperature and already live close to the limit of their thermal tolerances (Addo-Bediako, Chown, & Gaston, 2000; Deutsch, et.al., 2008), biodiversity will likely be severely affected in the coming decades unless species can rapidly evolve to new conditions.

While evolution by genetic mutations is a slow process that takes many generations to bring about adaptation to a changed environment, epigenetic mechanisms can provide a rapid and heritable way for organisms to mount a response to external stressors (Bonduriansky, et al., 2012; Jablonka & Raz, 2009). Epigenetics, that is, the study of heritable gene expression changes without changes in DNA sequence, encompasses three main processes that influence the expression of genes: (1) DNA methylation; (2) histone protein modifications; and (3) post-transcriptional regulation of gene expression by short and long noncoding RNAs (Bird, 2007; Bonilla, et al., 2016; Bossdorf, et al., 2008). Given their high mutability (van der Graaf, et al., 2015) and sensitivity to environmental conditions (Burton & Metcalfe, 2014; Feil & Fraga, 2012), epigenetic processes may provide a way for endangered species to adapt to an anthropogenically-changed environment. However, responses may also be negative and induce maladaptive responses in offspring (Bonduriansky & Day, 2009; Donelan, et.al., 2020; McGuigan, et al., 2021; Yona, et al., 2015).

Disruption of epigenetic mechanisms has been linked to fertility dysfunction through a class of noncoding RNAs known as piwi-interacting RNAS (piRNAs) (Bartel, 2009; Ashe, et al., 2012). In mice, piRNAs are highly expressed in reproductive tissue and, particularly in sperm, protect against rogue transposable element activity (Carmell, et.al., 2007; Crichton, et al., 2014), while, in *Drosophila melanogaster* and *Caenorhabditis elegans*, PIWI proteins are required for germ-line stem cell division (Cox, et al., 1998; Ozata, et al., 2019). Once an epigenetic state has been changed, it then exhibits metastability, that is, it remains unchanged unless sufficiently disturbed (Feil & Fraga, 2012; Morgan, et al., 2005). Thus, even if global climate change is mitigated, organismal responses to such changes may persist for several generations after environmentally stable conditions have been re-established. Without mitigation, climate warming will likely result in increasingly unstable environmental conditions, with successive generations of populations or species exhibiting potentially maladaptive epigenetic states, thereby augmenting the probability of extinction.

Experiments investigating the impact of climate change on study organisms have typically focused on effects of environmental stress directly experienced by the individual, but the last twenty years have yielded insights into the potential for intergenerational and, less commonly, transgenerational epigenetic inheritance (Bossdorf, et al., 2008; Cavalli & Heard, 2019; Danchin, et al., 2011; Heard & Martienssen, 2014). Maternal or paternal exposure to environmental conditions can alter the phenotype of offspring through a range of

processes (Donelson, et al., 2018), with growing evidence demonstrating the heritability of these phenotypes (Cecere, 2021; Lempradl, 2020). Intergenerational epigenetic inheritance involves transmission of altered epigenetic states from parents to offspring, the simplest explanation for this being that both generations are directly exposed to the factor responsible for the changes (Perez & Lehner, 2019). In males, direct exposure to stressful environmental stimuli can alter the epigenetic state of both somatic and germline cells, resulting in intergenerational inheritance and the transmission of effects from fathers to their F_1 offspring. By contrast, in viviparous species, three generations are directly exposed to stressful environments in gestating females: the F_0 -generation females, their developing fetuses that will constitute the F_1 generation, and the primordial germ cells of the F_1 female fetuses that will contribute to the F_2 generation (Bonilla, et al., 2016; Heard & Martienssen, 2014). Thus, transgenerational epigenetic inheritance differs from intergenerational epigenetic inheritance in that it requires transmission to the next generation in the absence of the environmental trigger. Consequently, in live-bearing species, transgenerational effects are distinguishable from intergenerational effects in that they persist after the F_1 generation in males and after the F_2 generation in gestating females. Transgenerational effects are less well documented than intergenerational effects, but both phenomena have been observed. For example, Anway et al. (2005) exposed gestating female rats to an anti-androgenic compound during embryonic gonadal sex determination and induced

F1 offspring with decreased spermatogenic capacity, which lasted for three subsequent generations. Exposure to an estrogenic compound also produced offspring with decreased reproductive capacity, but those exposure effects lasted for two generations. A study by Guillaume et al. (2016) manipulated thermal environments of the marine broadcast spawning polychaete, *Galeolaria caespitosa,* to determine how parental effects impacted offspring performance and found that sires exposed to warmer environmental temperatures had reduced fertilization potential and offspring survival.

With its unique reproductive biology and intriguing natural history, the pseudoscorpion, *Cordylochernes scorpioides,* is a model organism for investigating the impact of climate warming on a tropical ectotherm. Ranging throughout rainforests from southern Mexico to northern Argentina, *C. scorpioides* is a small, flightless arthropod that solves the challenge of dispersing between its ephemeral habitats of decaying trees in the families, Moraceae and Apocynaceae, by climbing under the elytra of the giant harlequin beetle, *Acrocinus longimanus*, and using the beetle as its dispersal agent (Zeh & Zeh, 1992). This novel method of dispersal generates intense, size-based competition between males to monopolize beetle abdomens as sites for inseminating dispersing females (Zeh, et al., 1997). In this pseudoscorpion, males transfer sperm to females by means of a stalked spermatophore deposited on the substrate, making it possible to intercept mating in the laboratory and retrieve sperm in order to quantify sperm number and assess sperm quality. Another

feature of *C. scorpioides'* reproductive biology that greatly facilitates assessment of temperature effects is its "external womb" form of viviparity, where first-stage nymphs are born live. Developing embryos are nourished by the mother in a transparent brood sac overlying her genital aperture, enabling non-invasive monitoring of female reproductive state and embryonic development (Su-Keene, et al., 2018; Zeh J., et al., 2012; Zeh J., et al., 2014). This pseudoscorpion species also exhibits a high level of mitochondrial sequence divergence, with three mitochondrial haplogroups, A1, A2, and B2, co-occurring in populations in central Panamá. Identified through next-generation sequencing, the A1 and A2 haplogroup sequences diverge by 1.9% from each other across the mitochondrial genome, and by 8% from the B2 haplogroup (Padua, et al., 2014; Zeh et al., unpublished). These mitochondrial haplogroups have demonstrable effects on reproductive fitness. Although the B2 haplogroup occurs at a frequency of only 12% in populations in central Panamá, B2 haplogroup males produce sperm with a competitive advantage over other males (Padua, et al., 2014). Further investigation revealed significant differential expression of eleven mitochondrial oxidative phosphorylation (OXPHOS) genes between the A and B2 haplogroups, with B2 haplogroup males upregulating the *atp8* gene, which is partially responsible for cellular energy (Zeh, et al., 2019).

Mitochondria play a principal role in cellular metabolism and energy provision through oxidative phosphorylation. Consequently, the mitochondrial genome is a target of selection and is likely to influence evolutionary responses

to climate change (Ballard & Pichaud, 2014). The catalytic capacity of enzymes involved in the generation of ATP is highly sensitive to thermal variation (Christen, et al., 2018; Michaelsen, et al., 2021), and studies have linked mitochondrial dysfunction to heat stress in plants, mice, trout, and humans (Blier, et al., 2014; Rurek, 2014; White, et al., 2012; Wilkening, et al., 2018). Mitochondria have also been linked to epigenetic mechanisms, since large amounts of ATP and acetyl-CoA molecules phosphorylate and acetylate chromatin, thereby allowing nuclear DNA transcription (Wallace & Fan, 2010; Wiese & Bannister, 2020), while impaired mitochondria induce DNA methylation in the nuclear genome (Minocherhomji, et al., 2012; Smiraglia, et al., 2008). Taking advantage of naturally occurring *C. scorpioides* mitochondrial haplogroups in studies investigating the impact of climate warming on this species may shed light on mitochondrial effects and the potential influence of mitochondrial variation on epigenetic responses.

Previous research on climate warming consequences for *C. scorpioides* demonstrated that a 3.5°C increase above current temperatures in the pseudoscorpion's natural habitat reduced male fertility by half and rendered females functionally sterile (Zeh JA., et al., 2012; Zeh JA., et al., 2014). To determine the tipping point beyond which temperature increase exerts irreversible effects on *C. scorpioides*, a range of more moderate temperature increases were then evaluated. Whereas an increase of 3°C caused high offspring mortality (unpublished data), a 2°C increase resulted in relatively

modest decreases in sperm production and nymph survival (Hernandez et.al., in prep.). Exposure to an increase of 2.5°C during nymphal development reduced survival, adult body size, sperm production and female reproductive success (Bonham, 2021).

Although the effects of the 2.5°C increase were significant, they were not too extreme to prevent offspring production and preclude investigation of parent-to-offspring transmission of those effects (intergenerational inheritance). In the study reported here, a 2.5°C temperature increase was therefore used in a multifactorial experiment designed to determine whether the effects of parental exposure to elevated temperature are transmitted through mothers, fathers or through both parents. Developmental, morphological and reproductive traits were quantified in the offspring of males and females exposed during nymphal development to the increased temperature regime and compared to the same traits in the offspring of parents reared under the control temperature regime.

2 | METHODS

Cordylochernes scorpioides pseudoscorpions used in this research were derived from the F5 through F8 generations of a laboratory population established from 297 individuals collected in Central Panamá from seven decaying *Ficus* trees in July 2017 (for locations, see Chapter 1). The laboratory population was perpetuated each generation by outcrossing the isofemale lines randomly with respect to mitochondrial haplogroup, thereby establishing a relatively homogeneous nuclear genetic background across the A1, A2 and B2 mitochondrial haplogroups represented in this study.

As reported previously (Bonham, 2021), the effects on *C. scorpioides* of exposure to a 2.5°C increase were assessed by carrying out a split-brood experiment, in which virgin females were each mated to an unrelated male to generate 82 families (n = 23 A1 haplogroup; 27 A2 haplogroup, and 32 B2 haplogroup families). For each family, protonymphs (first-stage nymphs) were randomly allocated at birth, with 20 assigned to a control (C) and 20 to a high (H) temperature treatment incubator for rearing to adulthood. After birth, pseudoscorpions go through three nymphal (protonymph, deutonymph and tritonymph) molts prior to the adult stage, at which point the pedipalps and cephalothorax become fixed in size and gender is morphologically visible (Weygoldt, 1969). The C temperature regime simulated diurnally oscillating temperatures in this pseudoscorpion's natural habitat and was based on

microhabitat temperature data collected by iButton data loggers fixed to the seven *Ficus* trees from which the founders of the *C. scorpioides* laboratory population were collected (for details, see Chapter 1). For the H temperature regime, the C oscillating temperatures were increased by 2.5ºC, with the control temperature regime averaging 28.38ºC and the high temperature regime averaging 30.88ºC. After reaching the adult stage, C and H males and females were assayed to investigate potential temperature, mitochondrial haplogroup, and temperature by haplogroup interaction effects on developmental, morphological, and reproductive traits, as well as on protein-coding expression in testicular tissue (Bonham 2021).

2.1 | Establishment of Female Outcross (FOUT) and Male Outcross (MOUT) Lines

In the study presented here, C and H adults from the 82 split-brood families described above were used in a reciprocal outcross experiment designed to investigate patterns of intergenerational inheritance of temperature-induced effects in *C. scorpioides*. To determine whether the effects of exposure to a 2.5°C increase are transmitted through males, females, or through both sexes, female outcross (FOUT) families were established by mating C and H females (n = 51 and 84, respectively) each to a C male. Similarly, male outcross (MOUT) families were established by mating C and H males (n = 98 and 140,

respectively) to C females. Temperature had a significant effect on offspring production resulting from the FOUT matings, with H dams less successful at birthing a brood than their C counterparts ($\bar{x}_H$ = 0.581 ± 0.051, $\bar{x}_C$ = 0.799 ± 0.067; χ^2 = 6.51, P = 0.0107). They gave birth to significantly fewer nymphs ($\bar{x}_H$ ± SE = 27.8 ± 3.0, $\bar{x}_C$ = 47.5 ± 3.9, $F_{1,127}$ = 8.72, P = 0.0037), and nymphs born to H females exhibited lower survival to adulthood than C nymphs ($\bar{x}_H$ = 0.830 ± 0.017, $\bar{x}_C$ = 0.900 ± 0.019, $F_{1,72}$ = 7.00, P = 0.0100). Elevated temperature had an even greater impact on the outcome of the MOUT matings, with a highly significant difference between H and C males in their ability to sire a brood ($\bar{x}_H$ = 0.42 ± 0.041, $\bar{x}_C$ = 0.681 ± 0.049, χ^2 = 15.30, P < 0.0001). MOUT offspring production was also affected by dam haplotype, with A2 haplogroup dams significantly less likely to produce a brood than either A1 or B2 dams ($\bar{x}_{A2}$ = 43.22% ± 5.28% , $\bar{x}_{A1}$ = 57.96% ± 5.39%, $\bar{x}_{B2}$ = 62.23% ± 5.5%, χ^2 = 7.22, P = 0.0270).

The reciprocal outcross matings generated a total of 79 FOUT families for use in this study, 37 produced by C females and 42 produced by H females, and 111 MOUT families, 59 sired by C males and 52 by H males. After birth, 22 protonymphs from each of the FOUT and MOUT families were reared to adulthood under the C temperature regime in individual vials to ensure virginity. Nymphs were fed once per week, initially with *Drosophila melanogaster* larvae and switching to small *Tribolium confusum* larvae shortly before the deutonymph-to-tritonymph molt. The development time of each individual was monitored, and

the survival rate of each family from birth to adulthood was assessed. After molting to the adult stage, all individuals in the FOUT and MOUT families were examined to determine gender and a subset of the males and females in each family were assayed for morphometric traits and male and female reproductive traits.

2.2 | Development Time and Survival

Developmental time was defined in this study as the number of days from birth to adulthood, and previous research on the effects of exposure to a 2.5°C increase established that reaching adulthood requires a minimum of 31 days (Bonham, 2021). Consequently, vials were visually inspected daily for adult emergence between 28 and 65 days after birth, and time to adulthood was recorded for each surviving FOUT and MOUT individual. Vials not yielding an adult by the end of the inspection period were thoroughly searched. Nymphs not found were recorded as dead, and the proportion of surviving offspring in each family was calculated.

2.3 | Morphometric Traits

To investigate the possible intergenerational inheritance of temperature-induced effects on morphology, at least three females and three males from each FOUT

and MOUT family were photographed and three measurements were taken of the sclerotized pedipalps and cephalothorax: (1) chela hand depth; (2) pedipalpal femur depth, and (3) cephalothorax length. These are the three most informative and reliably measured morphological traits that are fixed in size following the final molt from tritonymph to adult (Weygoldt, 1969; Zeh et.*al*. 2012). Individuals were restrained under a glass slide with the right pedipalp outstretched on 2 mm graph paper for size calibration, and photographed at approximately 30X magnification, using an ALTRA20 digital camera attached to an Olympus SZ6145TR stereomicroscope (Olympus Corporation of the Americas, Center Valley, PA, USA). Images were imported into ImageJ 1.52q (National Institutes of Health, Bethesda, MD, USA) and each trait was measured using the “straight line” tool.

In previous research (Zeh et al., 2012), principal component analysis demonstrated that the trait most tightly correlated with a composite measure of size (PC1) in this pseudoscorpion is hand depth in males (r = 0.97) and cephalothorax length in females (r = 0.95), prompting inclusion of these traits as size covariates in analyses of male and female reproductive traits, respectively.

2.4 | Male Reproductive Traits

***Mating Behavior.* –** Mating in *C. scorpioides* involves a sequence of well-defined behaviors (Zeh & Zeh, 2011) in which the male initiates mating by grasping the female by both her chelae (T1). If the female acquiesces, the male becomes

stationary in order to initiate spermatophore production (T2), which involves construction of a stalk, a droplet of fluid and a sperm packet at the apex of the stalk. After spermatophore deposition is complete and the sperm packet is affixed to the stalk (T3), the male reverses and attempts to guide the female over the sperm packet. For successful sperm transfer to occur, the female must flex her abdomen as she passes over the spermatophore, so that the sperm packet adheres to her genital aperture. To assess intergenerational transmission of temperature effects on mating behavior, adult virgin males from the FOUT and MOUT families were each placed in a 52-mm-diameter mating arena with a virgin, non-experimental female. Each mating was observed under a Leica EC4 stereomicroscope with video objective 1.0X (Leica Microsystems Inc., Buffalo Grove, IL) and times for T1, T2 and T3 were recorded.

Sperm Number and Viability. – As described elsewhere (Bonilla, et al., 2011), matings staged in the laboratory can be interrupted immediately after the male has deposited a spermatophore on the substrate and the sperm packet picked up using a dissecting needle. In this study, sperm packets were collected from 30 FOUT males and 39 MOUT males for quantification of sperm number and viability. After collection, packets were broken open in 9 µL of phosphate-buffered saline (140 mM NaCl; 2.7 mM KCl; 8.1 mM Na2HPO4; 8.1 mM KH2PO4; pH = 7.4) to release the sperm. To distinguish between live and dead sperm, sperm samples were stained with 1 µL of a 1:50 dilution of 1 mM SYBR

14 and 1 µL of 2.4 mM propidium iodide, following the manufacturer's instructions (Invitrogen Live/Dead Sperm Viability Kit). SYBR14 causes green fluorescence in live sperm, while propidium iodide causes red fluorescence in inviable sperm. The 11-µL samples were pipetted onto a hemocytometer and viewed at magnifications ranging from 40X to 400X under an Olympus BX51 fluorescence microscope. Live and dead sperm were counted, and the total number of sperm in the packet was estimated by multiplying the number counted in a 0.9-µL volume of the sample by a factor of 12.2 (11 µL/0.9 µL). The proportion of live sperm to total number of sperm provided an estimate of sperm viability.

2.5 | Female Reproductive Traits

To assess female reproductive traits in the female and male outcross families, virgin daughters of the FOUT dams and MOUT sires were each given the opportunity to mate with a non-experimental male. The reproductive status of each female was then monitored to determine whether she became gravid, spontaneously aborted her brood of embryos, or gave birth to protonymphs.

***Fecundity.* –** Fecundity was evaluated by counting the number of early-stage embryos in the brood sacs of females that became gravid. On the fifth day after mating, gravid females were immobilized supine (ventral side up) under a glass slide and their brood sacs photographed at high (45X) magnification, using an

Olympus SZ6145TR stereomicroscope equipped with an ALTRA20 digital camera (Olympus Corporation of the Americas, Center Valley, PA, USA). Images were imported into ImageJ 1.52q (National Institutes of Health, Bethesda, MD, USA) and the number of embryos contained in each brood sac was counted. Females that did not become gravid received a score of zero embryos produced.

***Reproductive Success.* –** After their brood sacs were photographed, gravid females were monitored until they either spontaneously aborted the brood or gave birth to protonymphs. Female reproductive success was quantified by counting the number of protonymphs born to each female. Females that failed to give birth to nymphs because they did not become gravid or spontaneously aborted their brood of embryos received a score of zero protonymphs produced.

2.6 | Statistical Analyses

All analyses were carried out using SAS statistical software, v9.4 (SAS Institute Inc, 2016). Effects of either sire (MOUT) or dam (FOUT) temperature, mitochondrial haplogroup and full factorial interaction effects on developmental time, survival, morphometric traits, male reproductive traits and female reproductive traits were analyzed. In data sets in which replications included related individuals, family identity was included in the analysis as a random effect to avoid pseudoreplication. Because sex exerts large and well characterized

effects on development time and morphology (Zeh et.*al.* 2012), two sets of analyses were carried out for these traits. First, sex was included as a fixed factor to quantify gender effects. Analyses were then carried out separately by sex to provide a more focused assessment of temperature, haplogroup and temperature by haplogroup interaction effects within each sex. Morphometric traits, development time, and mating times (T1, T2, T3) are normally distributed and were analyzed using a Generalized Linear Mixed Model (GLMM), implemented in SAS as PROC GLIMMIX. The GLMM for these models assumed a Gaussian distribution and incorporated a Laplace maximum likelihood approximation, and the SAS containment method for determining degrees of freedom (Ferron et al., 2009; SAS Institute, 2011). Sex ratio, survival, and sperm viability were recorded as proportions, and thus analyzed by fitting the PROC GLIMMIX procedure with a logit link function to include a binomial response predictor. Male and female reproductive traits, i.e., embryo, protonymph and sperm numbers, are non-normally distributed count data and were analyzed using a generalized Poisson distribution model for over-dispersed count data (SAS Institute Inc, 2016: 3919-2926). For analyses of broods born to experimental females and broods sired by experimental males, a score of 1 was assigned to broods with nymphs born and a score of 0 was assigned to aborted broods or when the female failed to become gravid. Consequently, the categorical analysis procedure PROC CATMOD was used to analyze these data.

Two-tailed tests were used for all hypothesis testing and a p-value less than 0.05 was considered significant for all tests.

3 | RESULTS

Temperature and haplogroup main effects for all traits (means, standard errors (SEs) and statistical significance) for the FOUT and MOUT experiments are summarized in Tables 1 and 2, respectively. Comparable data for the directly-treated generation (from Bonham 2021) are also included as a baseline reference. Table 3 provides a summary of temperature by haplogroup interaction effects for the directly-treated study and the FOUT and MOUT experiments. For clarity, only statistically significant effects are included in Table 3.

3.1 | Developmental Time and Survival

FOUT developmental time effects. – FOUT developmental times were recorded for 756 females and 754 males from 79 full-sibling families. Sex exerted a highly significant effect on developmental time ($F_{1,146} = 39.65$, $P < 0.0001$), with females developing on average 4.36 days faster than males ($\overline{x} = 39.83 \pm 0.4903$ days for females; $\overline{x} = 44.20 \pm 0.4900$ days for males). For analyses performed separately by sex, main and interaction effects of dam temperature and mitochondrial haplogroup were not significant in either sex (Tables 1-3; $P > 0.05$).

MOUT developmental time effects. – MOUT developmental times were recorded for 1,121 females and 979 males from 111 full-sibling families. The effect of sex on developmental time was highly significant ($F_{1,210}$ = 40.57, P < 0.0001), with females requiring on average 40.13 ± 0.3805 days to reach adulthood and males averaging 43.58 ± 0.3865 days. For analyses carried out separately by sex, there was a significant positive intergenerational effect of high temperature on developmental rate in females ($F_{1,105}$, = 5.47, P = 0.0213), but not in males ($F_{1,105}$ = 2.04, P = 0.1557). Female offspring sired by high temperature MOUT males (HM female offspring) reached adulthood 1.72 days faster than female offspring sired by control temperature MOUT males (CM female offspring; see Table 1). HM male offspring did achieve adulthood 1.14 days more rapidly than CM males but this difference was not significant ($F_{1,105}$ = 2.04, P = 0.1557). There were no significant sire haplogroup or sire temperature by sire haplogroup interaction effects.

Trait	Direct High Temp Mean ± SE	Direct Control Temp Mean ± SE	Direct Temp Effect P	FOUT High Temp Mean ± SE	FOUT Control Temp Mean ± SE	FOUT Temp Effect P	MOUT High Temp Mean ± SE	MOUT Control Temp Mean ± SE	MOUT Temp Effect P
Female Developmental Time (days)	36.56 ± 0.42	39.37 ± 0.42	0.0001	40.39 ± 0.59	39.28 ± 0.63	NS	39.27 ± 0.54	40.99 ± 0.5	0.0213
Male Developmental Time (days)	38.79 ± 0.48	43.66 ± 0.47	0.0001	44.26 ± 0.74	44.15 ± 0.79	NS	43.01 ± 0.58	44.15 ± 0.55	NS
Birth to Adult Survivorship (%)	0.78 ± 0.02	0.86 ± 0.02	0.0001	0.83 ± 0.02	0.9 ± 0.02	0.0065	0.88 ± 0.02	0.88 ± 0.02	NS
Female Cephalothorax Length (mm)	1.27 ± 0.01	1.34 ± 0.01	0.0001	1.31 ± 0.01	1.31 ± 0.01	NS	1.33 ± 0.01	1.34 ± 0.01	NS
Male Cephalothorax Length (mm)	1.24 ± 0.01	1.34 ± 0.01	0.0001	1.29 ± 0.01	1.34 ± 0.01	0.0012	1.34 ± 0.01	1.36 ± 0.01	NS
Female Chela Hand Depth (mm)	0.75 ± 0	0.8 ± 0	0.0001	0.8 ± 0.01	0.8 ± 0.01	NS	0.8 ± 0.01	0.82 ± 0.01	NS
Male Chela Hand Depth (mm)	0.84 ± 0.01	0.99 ± 0.01	0.0001	0.93 ± 0.01	0.99 ± 0.01	0.0040	0.99 ± 0.01	1.03 ± 0.02	NS
Female Femur Depth (mm)	0.58 ± 0	0.61 ± 0	0.0001	0.6 ± 0	0.6 ± 0	NS	0.6 ± 0	0.62 ± 0.01	0.0423
Male Femur Depth (mm)	0.66 ± 0.01	0.74 ± 0.01	0.0001	0.7 ± 0.01	0.74 ± 0.01	0.0028	0.73 ± 0.01	0.76 ± 0.01	0.0376
Female Reproduction: Embryos Produced	57.29 ± 3.73	67.34 ± 4.79	NS	69.26 ± 4.75	66.55 ± 5.8	NS	82.2 ± 2.81	85.07 ± 4.19	NS
Female Reproduction: % Broods Carried to Term	58.05% ± 5.13%	79.89% ± 6.73%	0.0107	81.68% ± 6.49%	77.62% ± 8.17%	NS	85.21% ± 6.54%	69.92% ± 7.46%	NS
Female Reproduction: Protonymphs Born	27.8 ± 3	47.47 ± 3.94	0.0037	44.74 ± 4.47	44.62 ± 5.64	NS	49.91 ± 4.49	40.97 ± 5.12	0.0267
Male Mating Speed: Time to Initiation (T1 in seconds)	107.6 ± 17.15	65.97 ± 15.89	NS	87.59 ± 21.68	105.91 ± 21.82	NS	69.52 ± 11.89	108.9 ± 13.2	0.0339
Male Mating Speed: Time to Acquiescence (T2-T1 in seconds)	209 ± 24.27	120.68 ± 22.08	0.0216	154.33 ± 17.53	168.24 ± 17.65	NS	160.84 ± 15.4	146.07 ± 17.1	NS
Male Mating Speed: Spermatophore Production Time (T3-T2 in seconds)	318.51 ± 12.22	299.25 ± 11.11	NS	296.86 ± 9.55	330.46 ± 9.61	0.0221	309.14 ± 7.47	295.37 ± 8.29	NS
Sperm Number	401.28 ± 77.15	736.21 ± 69.98	0.0001	380.61 ± 85	853.02 ± 85.58	0.0004	587.02 ± 56.48	721.13 ± 62.71	0.0005
Sperm Viability	90.83% ± 1.82%	93.54% ± 1.47%	NS	88.07% ± 2.22%	89.18% ± 2.08%	NS	91.69% ± 1.15%	90.61% ± 1.22%	NS

Table 1. Summary of temperature treatment main effects in the simulated climate warming study of *Cordylochernes scorpioides*. Means, standard errors (SEs) and temperature treatment statistical significance for the directly-treated generation (from Bonham 2021) are included as a baseline. For clarity, non-significant results are reported as " NS" but actual *P* values are provided in the Results section.

FOUT birth-to-adult survival effects. – There was a significant negative intergenerational effect of high temperature on offspring survival, with offspring born to high temperature FOUT dams (HF offspring) surviving significantly less well than offspring born to control FOUT dams (CF offspring) ($F_{1,27}$ = 8.68, P = 0.0065; Table 1; Fig. 1). While the main effect of offspring mitochondrial haplogroup on survival was not significant ($F_{2,27}$ = 1.31, P = 0.2863), there was a significant interaction between dam temperature treatment and offspring haplogroup *(*$F_{2,27}$ = 3.74, P = 0.0368; Table 3). The B2 haplogroup was most severely negatively affected by the dam's temperature treatment (Fig. 2). Sex ratio, as measured by the proportion of male offspring, was not significantly affected by dam temperature, haplogroup or the interaction between dam temperature and haplogroup (P > 0.05).

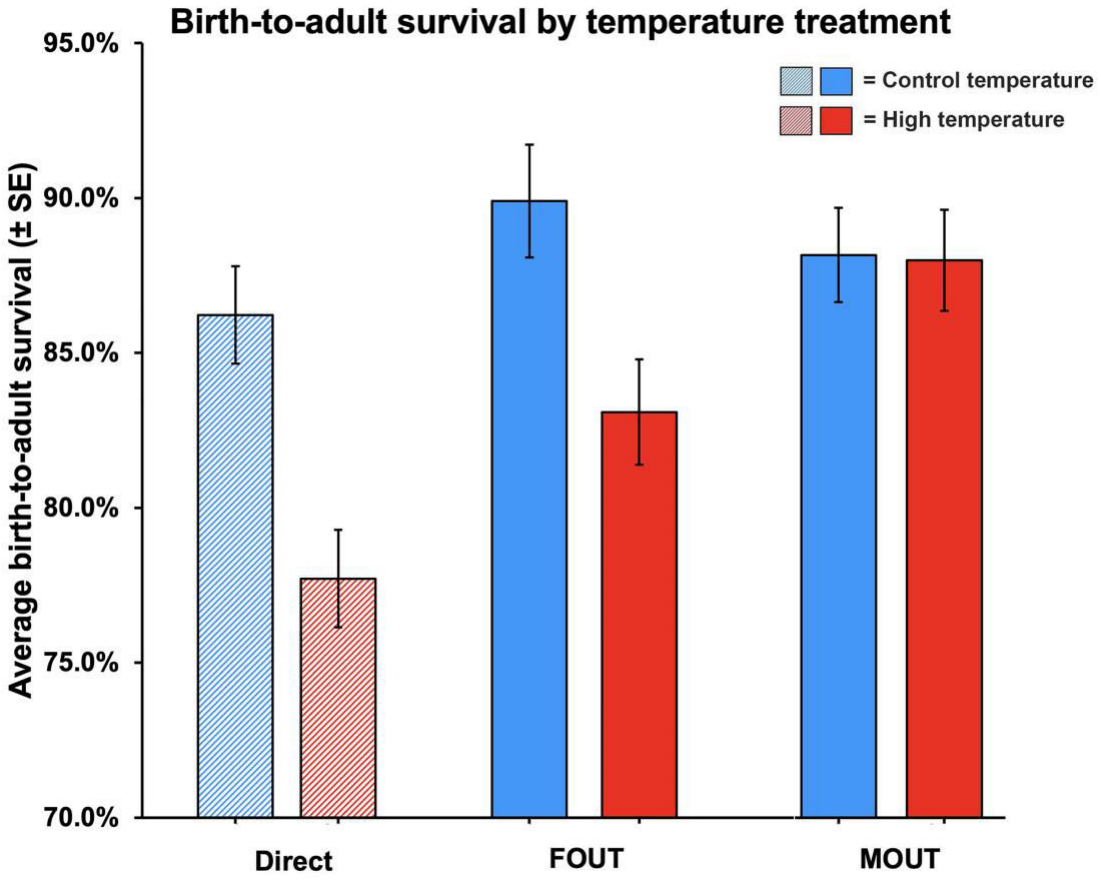

Figure 1. High-temperature treatment of female (FOUT) but not male (MOUT) parents significantly decreases birth-to-adult survival in *Cordylochernes scorpioides* offspring. The negative intergenerational effect of high temperature on FOUT survivorship is nearly as great as the effect in individuals directly experiencing the high-temperature treatment, as reported in Bonham (2021). Data are plotted as means ± SEs.

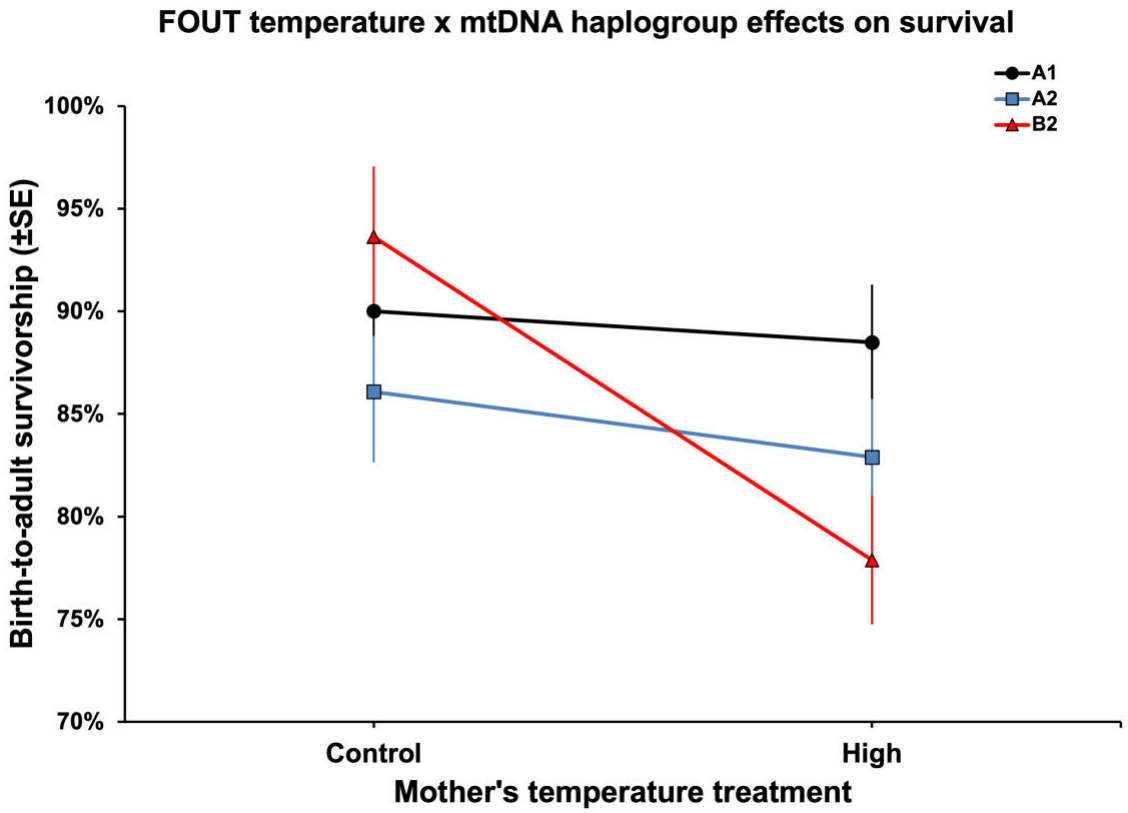

Figure 2. Negative effect of mother's high-temperature treatment on offspring survivorship is mediated by mitochondrial haplogroup. High temperature exerts a disproportionately negative effect on offspring of B2-haplogroup females. Data are plotted as means ± SEs.

***MOUT birth-to-adult survival effects*. –** There were no significant intergenerational effects of sire temperature treatment (Fig. 1) or mitochondrial haplogroup on offspring survival (sire temperature: $F_{1,31} = 0.01$, $P = 0.9394$; sire haplogroup: $F_{2,31} = 0.61$, $P = 0.5492$). The sire temperature by sire haplogroup effect was also not significant ($F_{2,31} = 1.41$, $P = 0.2586$). The proportion of male

offspring was not significantly affected by sire temperature, haplogroup or the interaction between sire temperature and haplogroup ($P > 0.05$).

3.2 | Morphometric Traits

FOUT morphometric effects. – FOUT morphological data were recorded from 237 female and 282 male offspring from 78 full-sibling families. Not surprisingly, sex had no significant effect on the sexually monomorphic trait, cephalothorax length ($F_{1,144} = 0.03$, $P = 0.8545$). However, there was a significant, negative intergenerational effect of high temperature on the carapace length of male offspring. Averaged across the two sexes, cephalothorax length was 1.3% smaller for HF offspring than for CF offspring ($\bar{x} = 1.30 \pm 0.006475$ for HF offspring versus $\bar{x} = 1.32 \pm 0.006644$ for CF offspring; $F_{1,144} = 6.96$, $P = 0.0093$). There was also a significant interaction between dam temperature treatment and offspring sex ($F_{1,144} = 7.13$, P = 0.0084), with HF males suffering a more significant size reduction than females (Fig. 3). Compared to CF males, HF males were 3.71% smaller for cephalothorax length.

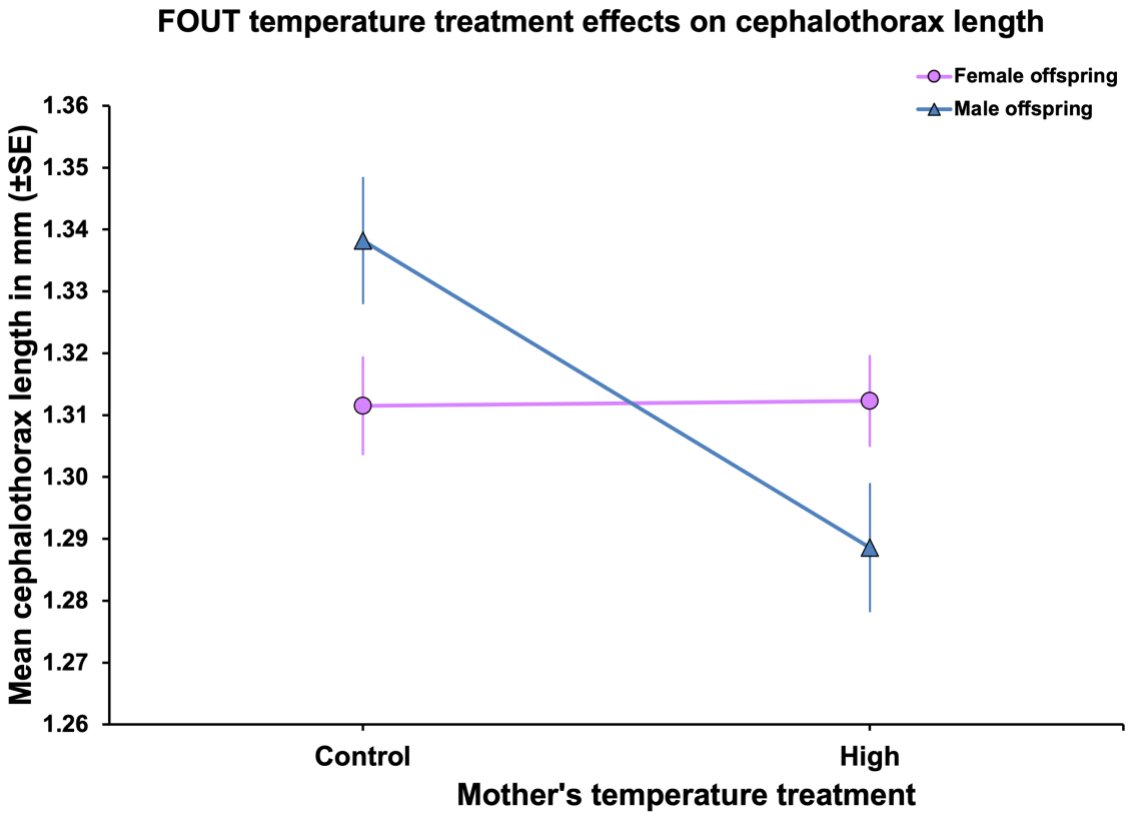

Figure 3. Negative effect of mother's high-temperature treatment on offspring body size (cephalothorax length) is mediated by sex. Only male offspring experience a reduction in body size as a result of the mother's high-temperature treatment. Data are plotted as means ± SEs.

There were significant effects on chela hand depth (HD) of sex ($F_{1,144}$ = 191.64, $P < 0.001$), dam temperature ($F_{1,144}$ = 6.28, P = 0.0133), and the interaction of sex and dam temperature ($F_{2,144}$ = 8.24, P = 0.0047). Males exhibited a larger overall hand depth than females ($\bar{x}$ = 0.9585 ± 0.007874 vs $\bar{x}$ = 0.8019 ± 0.008116), and CF offspring had a larger hand depth than HF offspring ($\bar{x}$ = 0.8944 ± 0.008124 vs $\bar{x}$ = 0.8660 ± 0.007874). The significant effect of dam

temperature on offspring hand depth was exclusively due to reduction in male size, with HF males averaging a 6.15% reduction in hand depth relative to CF males (Fig. 4).

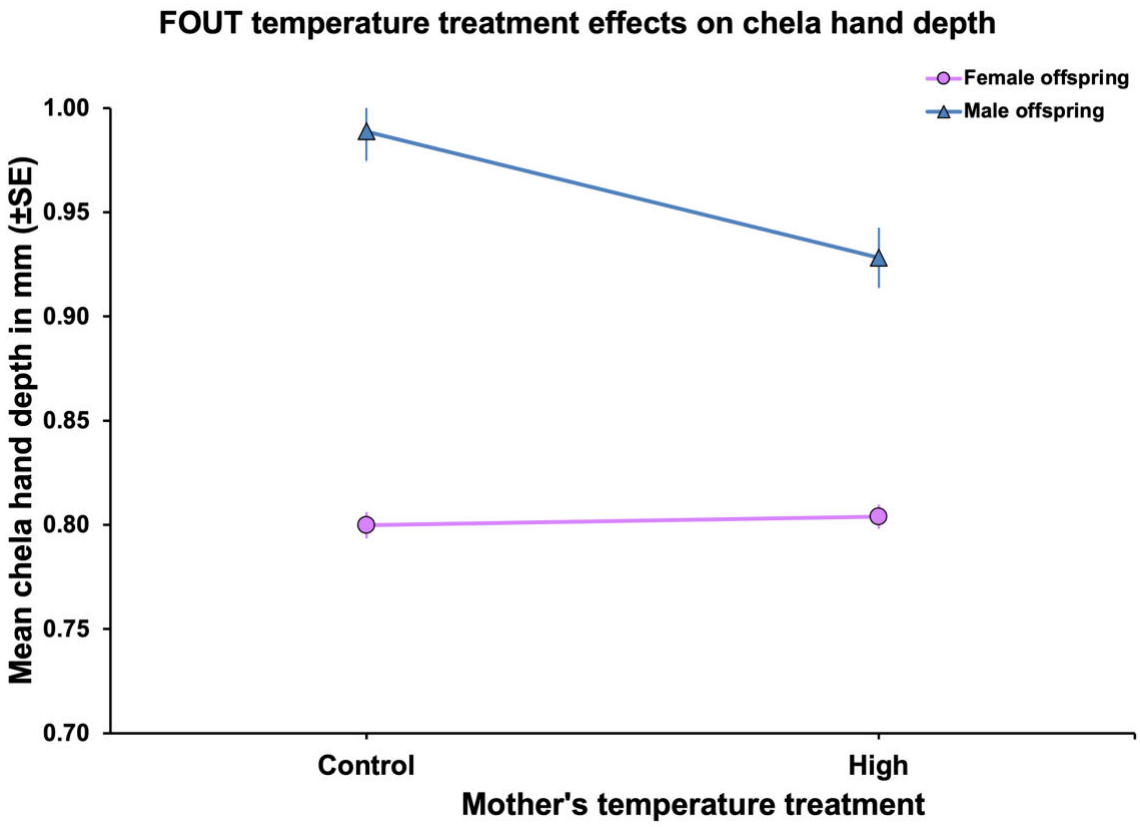

Figure 4. Negative effect of mother's high-temperature treatment on the sexually- dimorphic trait, chela hand depth, is mediated by sex. Only male offspring experience a reduction in hand depth as a result of the mother's high-temperature treatment. Data are plotted as means ± SEs.

Similarly, femur depth (FD) was also significantly affected by sex ($F_{1,144}$ = 233.20, P < 0.0001), dam temperature ($F_{1,144}$ = 8.49, P = 0.0041), and the

interaction between sex and dam temperature ($F_{2,144}$ = 6.30, P = 0.0132). Femur depth was significantly larger in males ($\bar{x}$ = 0.7202 ± 0.005429) than in females ($\bar{x}$ = 0.6012 ± 0.005588). Compared to HF offspring, CF offspring exhibited a slightly larger femur depth ($\bar{x}$ = 0.6720 ± 0.005597 vs $\bar{x}$ = 0.6493 ± 0.005421). As in the case of chela hand depth, the significant effect of dam temperature on offspring femur depth was exclusively due to reduction in male size, with HF males averaging a 5.71% reduction in femur depth relative to CF males. (Fig. 5).

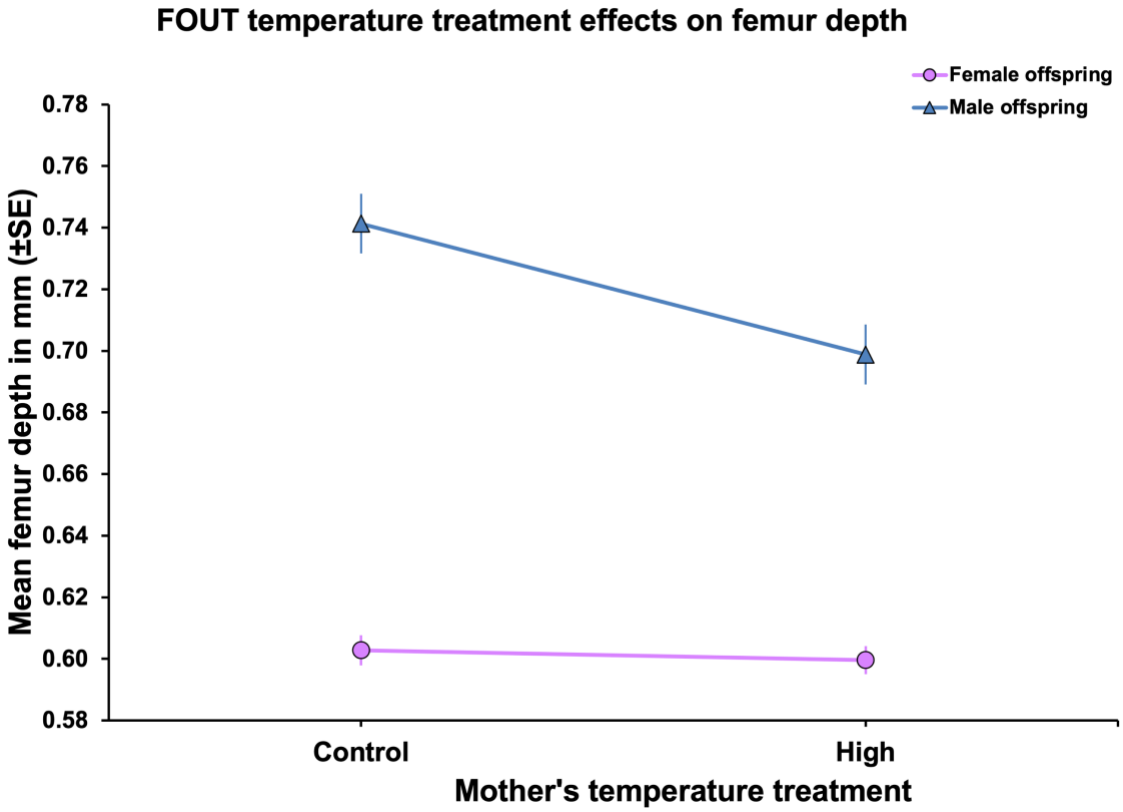

Figure 5. Negative effect of mother's high-temperature treatment on the sexually- dimorphic trait, femur depth, is mediated by sex. Only male offspring

experience a reduction in femur depth as a result of the mother's high-temperature treatment. Data are plotted as means ± SEs.

The main and interaction effects of dam haplogroup were not significant for any of the measured traits, i.e., cephalothorax length, hand depth and femur depth (Tables 2-3).

MOUT morphometric effects. – MOUT morphological data were recorded from 369 females and 426 males from 111 full-sibling families. While the MOUT experiment was designed to assess possible sire temperature and sire haplogroup effects, exploratory analyses revealed significant dam haplogroup and dam haplogroup by sire haplogroup interaction effects in male offspring. Therefore, both sire haplogroup and dam haplogroup were included in the model results described below. There were no significant main effects of sire haplogroup in any of the analyses (Table 2).

As expected, sex did not significantly affect the sexually monomorphic trait, cephalothorax length, with males and females roughly equal in length ($\overline{x}_{female}$ = 1.3352 ± 0.007239; $\overline{x}_{male}$ = 1.3521 ± 0.007235; $F_{1,186}$ = 2.74, P = 0.0996). Sex did exert highly significant effects on both chela hand depth ($\overline{x}_{female}$ = 0.8103 ± 0.008422; $\overline{x}_{male}$ = 1.0122 ± 0.008421; $F_{1,186}$ = 287.20, P < 0.0001) and femur depth ($\overline{x}_{female}$ = 0.6104 ± 0.005657; $\overline{x}_{male}$ = 0.7488 ± 0.005658; $F_{1,186}$ = 299.09, P < 0.0001). Males were approximately 25% and 23% larger than females for hand depth and femur depth, respectively.

For analyses performed separately by sex, the intergenerational effect of sire temperature on cephalothorax length was not significant for either female ($F_{1,93} = 1.54$, $P = 0.2174$) or male offspring ($F_{1,93} = 1.08$, $P = 0.3018$; Table 1). However, for male offspring, both dam haplogroup ($F_{2,315} = 5.00$, $P = 0.0073$), and the interactions between sire temperature and dam haplogroup ($F_{1,315} = 3.62$, $P = 0.0278$) and between sire haplogroup and dam haplogroup ($F_{4,315} = 4.41$, $P = 0.0018$) were significant. The B2 haplogroup inherited by male offspring from their mother was associated with small size, particularly when the father's haplogroup was A1 or A2 (Fig. 6).

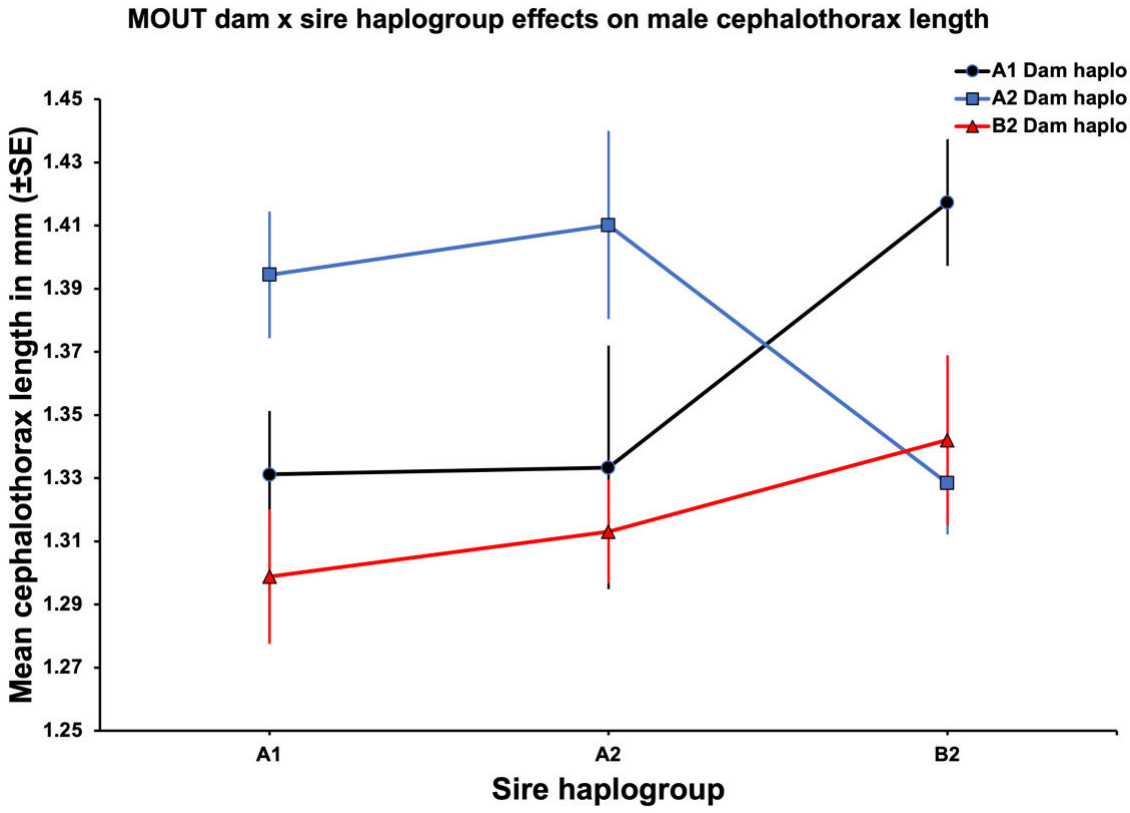

Figure 6. Dam-by-sire mitochondrial haplogroup interactions influencing male

body size (cephalothorax length) in male outcross (MOUT) lines. The B2 haplogroup inherited by male offspring from their mother was associated with small size, particularly when the father's haplogroup was A1 or A2.

The intergenerational effect of sire temperature on chela hand depth was positive but only marginally significant for both female ($F_{1,93}$ = 3.79, P = 0.0546) and male offspring ($F_{1,315}$ = 3.38, P = 0.0691), with offspring sired by high-temperature males being 2% and 4% smaller than control-sire female and male offspring, respectively (Table 1). No sire haplogroup, dam haplogroup or interactions effects were significant for female hand depth but both dam haplogroup ($F_{2,315}$ = 5.81, P = 0.0033) and sire haplogroup by dam haplogroup interaction effects ($F_{4,315}$ = 6.13, P < 0.0001) were highly significant in male offspring. As in the case of cephalothorax length, the B2 haplogroup carried by male offspring was associated with small hand depth, particularly when the sire's haplogroup was A1 or A2 (Fig. 7).

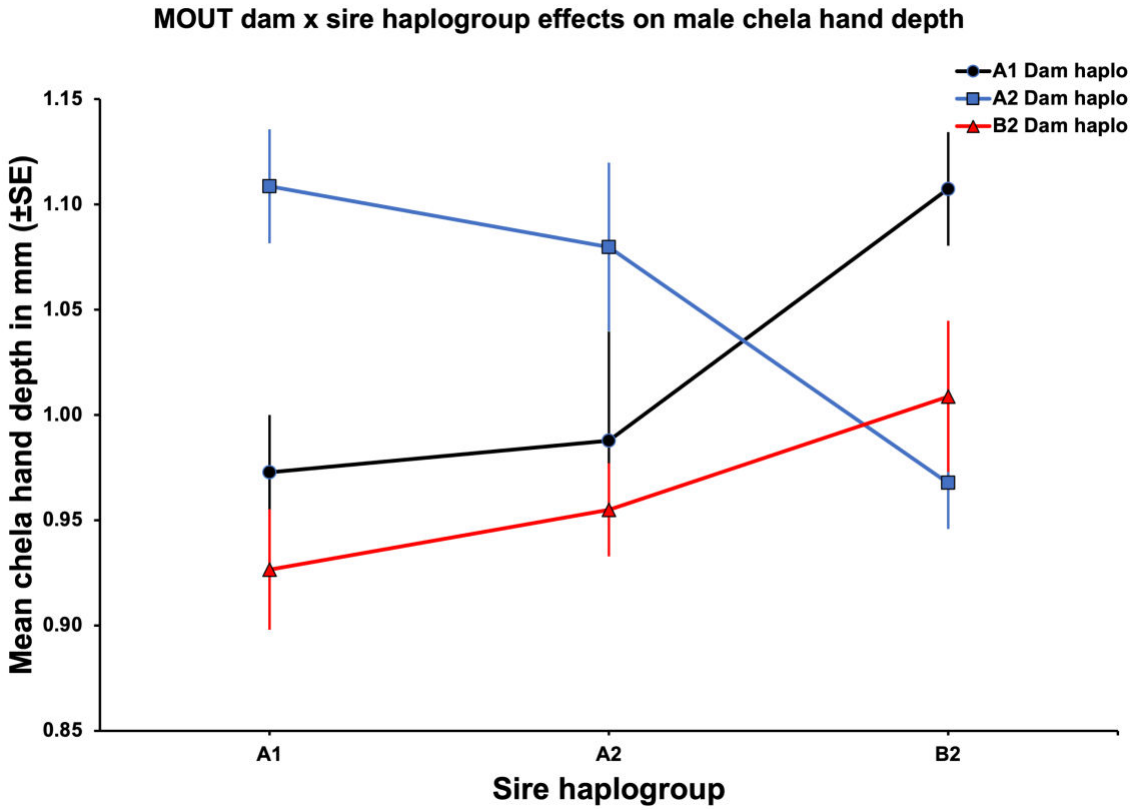

Figure 7. Dam-by-sire mitochondrial haplogroup interactions influencing male chela hand depth in male outcross (MOUT) lines. The B2 haplogroup inherited by male offspring from their mother was associated with small hand depth, particularly when the father's haplogroup was A1 or A2.

The results for femur depth parallel those for hand depth although the intergenerational effect of sire temperature is stronger and fully statistically significant in both female ($F_{1,93}$ = 4.24, P = 0.0423) and male offspring ($F_{1,93}$ = 4.45, P = 0.0376). Again, no sire haplogroup, dam haplogroup or interactions effects were significant for female hand depth but both dam haplogroup ($F_{2,315}$ = 6.73, P = 0.0014) and sire haplogroup by dam haplogroup interaction effects

($F_{4,315}$ = 6.33, P < 0.0001) were highly significant in male offspring. As with cephalothorax length and chela hand depth, the B2 haplogroup was associated with small femur depth, particularly in males fathered by A1 or A2 sires (Fig. 8).

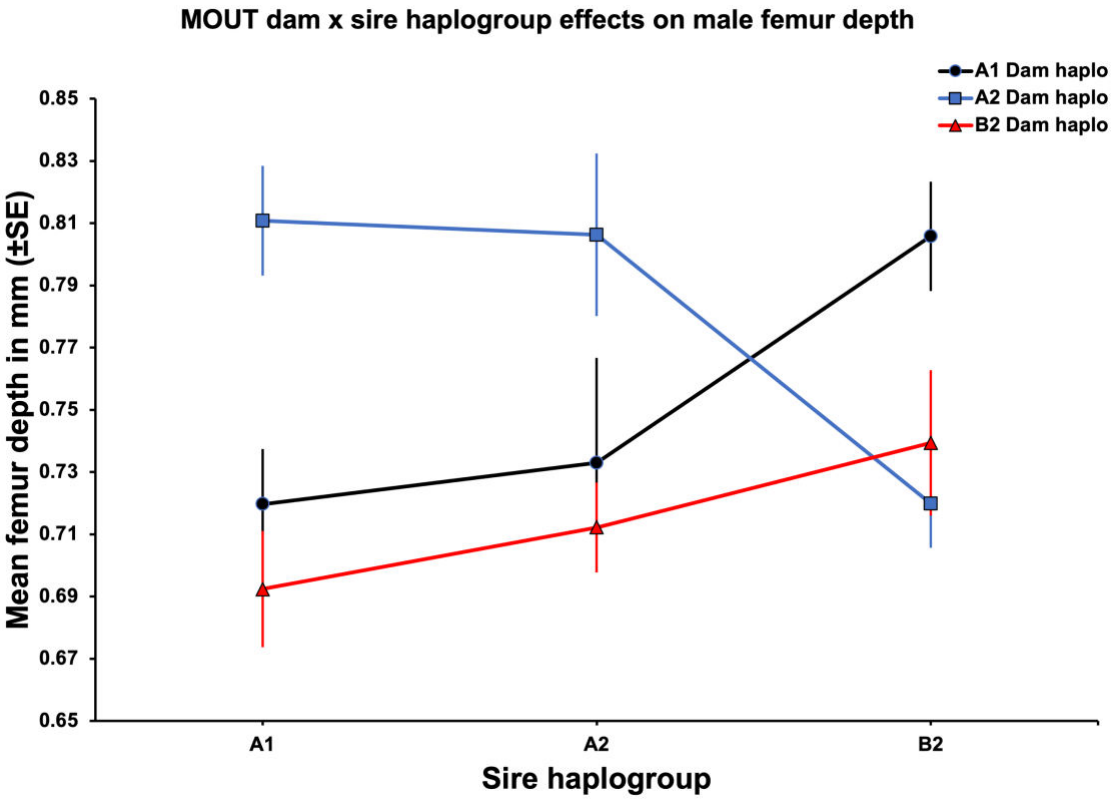

Figure 8. Dam-by-sire mitochondrial haplogroup interactions influencing femur depth in male outcross (MOUT) lines. The B2 haplogroup inherited by male offspring from their mother was associated with small femur depth, particularly when the father's haplogroup was A1 or A2.

3.3 | Male Reproductive Traits

FOUT male reproductive trait effects. – Matings were observed and sperm packets collected for a total of 36 males. Sire temperature did not significantly affect time to initiate mating (T1) ($P > 0.05$), although HM males were approximately 17% faster than CM males (Table 1). The effects of temperature on time to female acquiescence (T2-T1) was also not significant, but HM males were again faster than CM males (Table 1). Temperature did exert a significant intergenerational effect on sperm packet deposition time (T3-T2) ($F_{1,29} = 5.85$, $P = 0.0221$) with HM males approximately 10% faster than CM males (Table 1). Haplogroup effects on T1, T2-T1, and T3 were all statistically insignificant ($P > 0.05$; Table 2).

Trait	Direct A1 Mean ± SE	Direct A2 Mean ± SE	Direct B2 Mean ± SE	Direct Haplo Main Efffect P	FOUT A1 Mean ± SE	FOUT A2 Mean ± SE	FOUT B2 Mean ± SE	FOUT Haplo Main Efffect P	MOUT A1 Mean ± SE	MOUT A2 Mean ± SE	MOUT B2 Mean ± SE	MOUT Haplo Main Efffect P
Female Developmental Time (days)	37.72 ± 0.54	38.74 ± 0.51	37.43 ± 0.49	NS	39.01 ± 0.74	40.57 ± 0.68	39.92 ± 0.81	NS	39.72 ± 0.68	40.73 ± 0.63	39.94 ± 0.61	NS
Male Developmental Time (days)	41.45 ± 0.61	41.41 ± 0.57	40.8 ± 0.55	NS	43.67 ± 0.94	44.3 ± 0.85	44.62 ± 1.01	NS	43.27 ± 0.74	44.22 ± 0.68	43.25 ± 0.65	NS
Birth to Adult Survivorship (%)	0.85 ± 0.02	0.83 ± 0.02	0.79 ± 0.02	NS	0.89 ± 0.02	0.84 ± 0.02	0.86 ± 0.02	NS	0.89 ± 0.02	0.86 ± 0.02	0.89 ± 0.02	NS
Female Cephalothorax Length (mm)	1.31 ± 0.01	1.31 ± 0.01	1.3 ± 0.01	NS	1.32 ± 0.01	1.31 ± 0.01	1.31 ± 0.01	NS	1.33 ± 0.01	1.32 ± 0.01	1.34 ± 0.01	NS
Male Cephalothorax Length (mm)	1.29 ± 0.01	1.29 ± 0.01	1.28 ± 0.01	NS	1.33 ± 0.01	1.31 ± 0.01	1.3 ± 0.01	NS	1.32 ± 0.01	1.34 ± 0.01	1.35 ± 0.01	NS
Female Chela Hand Depth (mm)	0.78 ± 0.01	0.77 ± 0.01	0.77 ± 0.01	NS	0.81 ± 0.01	0.79 ± 0.01	0.8 ± 0.01	NS	0.8 ± 0.01	0.81 ± 0.01	0.81 ± 0.01	NS
Male Chela Hand Depth (mm)	0.93 ± 0.01	0.91 ± 0.01	0.91 ± 0.01	NS	0.98 ± 0.02	0.95 ± 0.02	0.95 ± 0.02	NS	0.97 ± 0.02	0.99 ± 0.02	1.01 ± 0.02	NS
Female Femur Depth (mm)	0.6 ± 0	0.59 ± 0	0.59 ± 0	NS	0.61 ± 0.01	0.59 ± 0.01	0.61 ± 0.01	NS	0.6 ± 0.01	0.61 ± 0.01	0.61 ± 0.01	NS
Male Femur Depth (mm)	0.71 ± 0.01	0.7 ± 0.01	0.7 ± 0.01	NS	0.73 ± 0.01	0.72 ± 0.01	0.71 ± 0.01	NS	0.72 ± 0.01	0.74 ± 0.01	0.75 ± 0.01	NS
Female Reproduction: Embryos Produced	64.03 ± 5.13	65.41 ± 4.75	57.51 ± 5.16	NS	69.06 ± 6.41	71.67 ± 6.3	62.97 ± 6.65	NS	86.03 ± 5	88.79 ± 4.08	76.08 ± 3.8	NS
Female Reproduction: % Broods Carried to Term	65.86%2 ± 7.15%	79.51%2 ± 6.66%	61.54%2 ± 7.13%	NS	77.79%2 ± 9.06%	78.80%2 ± 8.43%	82.34%2 ± 9.51%	NS	75.49%2 ± 8.76%	80.95%2 ± 9.06%	76.25%2 ± 7.99%	NS
Female Reproduction: Protonymphs Born	36.5 ± 4.19	43.96 ± 3.9	32.44 ± 4.17	NS	48.56 ± 6.24	41.46 ± 5.81	44.02 ± 6.56	NS	45.23 ± 6.01	48.39 ± 6.22	42.7 ± 5.48	NS
Male Mating Speed: Time to Initiation (T1 in seconds)	101.2 ± 18.09	85.28 ± 15.59	73.87 ± 17.14	NS	104.32 ± 26.17	75.1 ± 24.87	110.84 ± 27.44	NS	118.6 ± 16.7	67.16 ± 15.91	81.87 ± 14.67	NS
Male Mating Speed: Time to Acquiescence (T2-T1 in seconds)	138.76 ± 25.15	157.35 ± 21.68	198.4 ± 24.37	NS	149.7 ± 21.17	159.73 ± 20.12	174.44 ± 22.2	NS	157.59 ± 21.62	164.9 ± 20.61	137.86 ± 19	NS
Male Mating Speed: Spermatophore Production Time (T3-T2 in seconds)	326.81 ± 12.66	288.66 ± 10.91	311.18 ± 12.27	NS	323.92 ± 11.53	300.21 ± 10.96	316.83 ± 12.09	NS	305.45 ± 10.49	296.52 ± 10	304.79 ± 9.22	NS
Sperm Number	601.49 ± 80.92	559.4 ± 69.73	545.35 ± 74.78	0.0002	567.56 ± 102.64	680.26 ± 97.54	602.62 ± 107.61	0.0015	599.63 ± 79.32	799.85 ± 75.59	562.73 ± 69.7	0.0346
Sperm Viability	92.89% ± 1.73%	88.59% ± 1.64%	95.07% ± 1.77%	0.0460	91.83% ± 2.80%	87.68% ± 2.29%	86.36% ± 2.66%	NS	90.29% ± 1.58%	92.98% ± 1.46%	90.17% ± 1.42%	NS

Table 2. Summary of mitochondrial haplogroup treatment main effects in the simulated climate warming study of *Cordylochernes scorpioides*. Means, standard errors (SEs) and haplogroup treatment statistical significance for the directly-treated generation (from Bonham 2021) are included as a baseline. For clarity, non-significant results are reported as " NS" but actual *P* values are provided in the Results section. For the FOUT experiment, haplogroup is the dam haplogroup. For the MOUT experiment, haplogroup is the sire haplogroup.

The intergenerational effect of sire temperature on number of sperm produced was highly significant ($F_{1,29}$ = 16.33, P = 0.0004). Averaged across the three haplogroups, CM males produced 853 ± 86 total sperm compared to 381 ± 85 sperm for HM males, for an overall 55.38% decrease in number of sperm produced (Table 1; Fig. 9). Dam haplogroup significantly affected sperm number ($F_{2,29}$ = 8.21, P = 0.0015; Table 2) and the interaction between dam temperature treatment and male haplogroup was also highly significant ($F_{2,29}$ = 5.48, P = 0.0042), with B2-haplogroup males most negatively affected by the dam's high temperature treatment (Fig. 10).

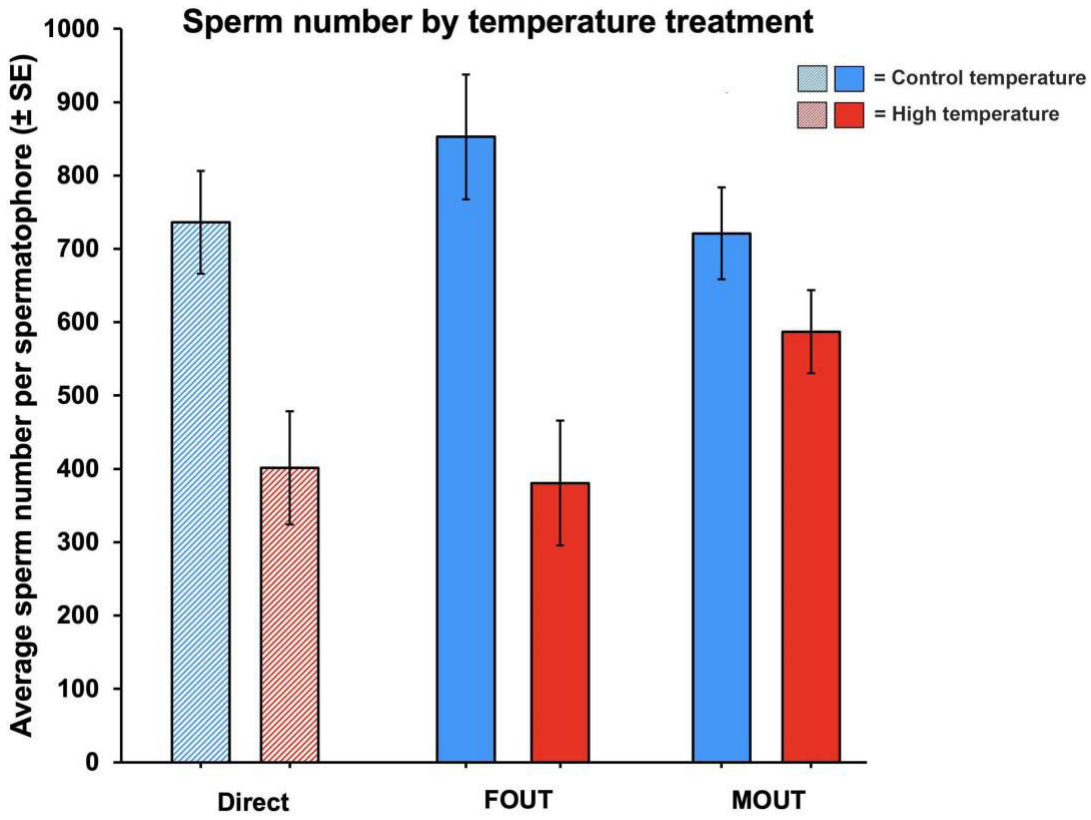

Figure 9. High-temperature treatment of both female (FOUT) and male (MOUT)

parents significantly decreases sperm production in *Cordylochernes scorpioides* male offspring. The intergenerational negative effect of high temperature on FOUT sperm production is as great as the effect in males directly experiencing the high-temperature treatment, as reported in Bonham (2021). Data are plotted as means ± SEs.

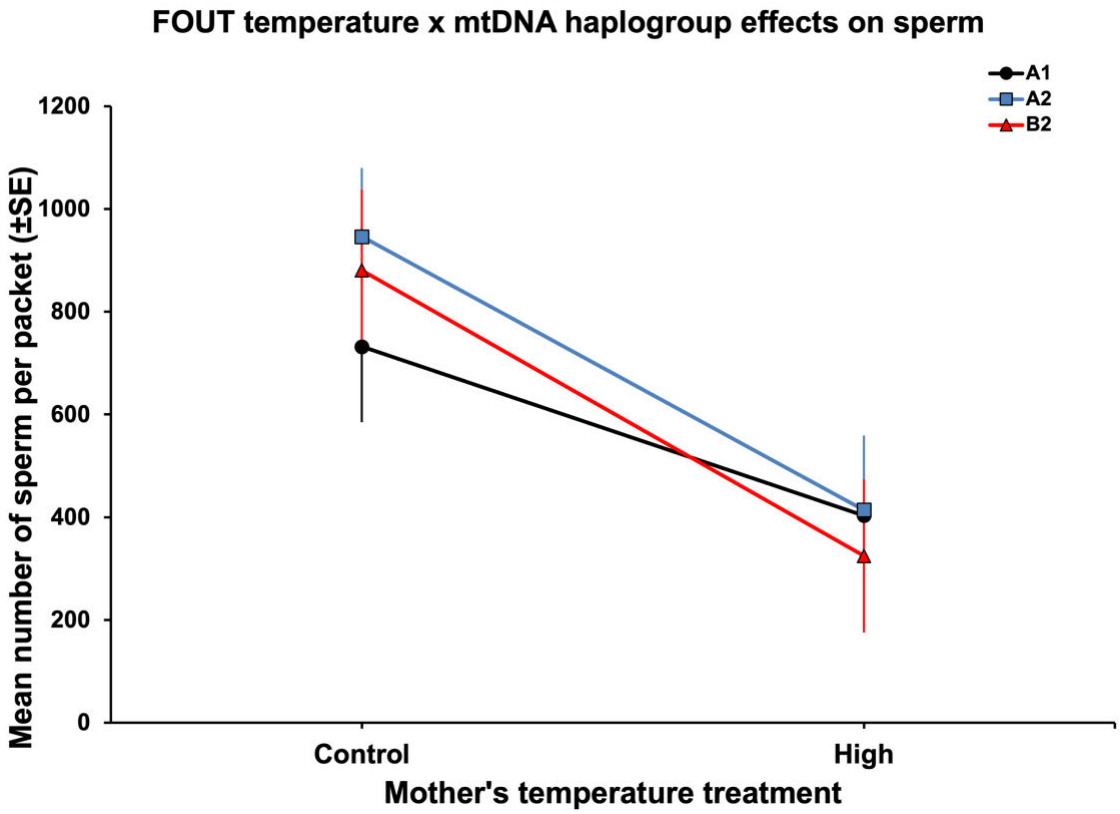

Figure 10. Negative effect of mother's high-temperature treatment on offspring sperm production is influenced by mitochondrial haplogroup in the female outcross (FOUT) line. Exposure of the dam to high temperature has a disproportionately negative effect on males born to B2-haplogroup females. Data are plotted as means ± SEs.

Sperm viability, i.e., the proportion of viable sperm per packet, exhibited little variation and was not significantly affected by dam temperature treatment ($F_{1,25}$ = 0.15, P = 0.693), dam haplogroup ($F_{1,25}$ = 0.66, P = 0.5272) (Tables 1-2) or the interaction between temperature and haplogroup ($F_{1,25}$ = 2.46, P = 0.1054).

MOUT male reproductive trait effects. – Matings were observed and sperm packet collected for a total of 39 males. Sire temperature exerted a significant intergenerational effect on mating initiation time ($F_{1,32}$ = 4.91, P = 0.0339) with HM males initiating mating at a rate 36% faster than CM males (Table 1). Neither sire temperature, sire haplogroup, nor the sire temperature by haplogroup interaction had significant effects on female acquiescence time (P > 0.05; Tables 1-3). Similarly, there were no significant main or interaction effects for deposition time (P > 0.05; Tables 1-3), contrary to the results of the FOUT experiment. However, sire temperature did significantly affect the total number of sperm produced ($F_{1,32}$ = 14.91, P = 0.0005), as CM males produced 23% more sperm than HM males (Table 1; Fig. 9). Sire haplogroup also significantly affected the total number of sperm produced ($F_{2,32}$ = 3.72, P = 0.0346; Table 2) but the interaction between sire temperature and haplogroup was not significant (($F_{2,32}$ = 0.71, P = 0.7037; Fig. 11).

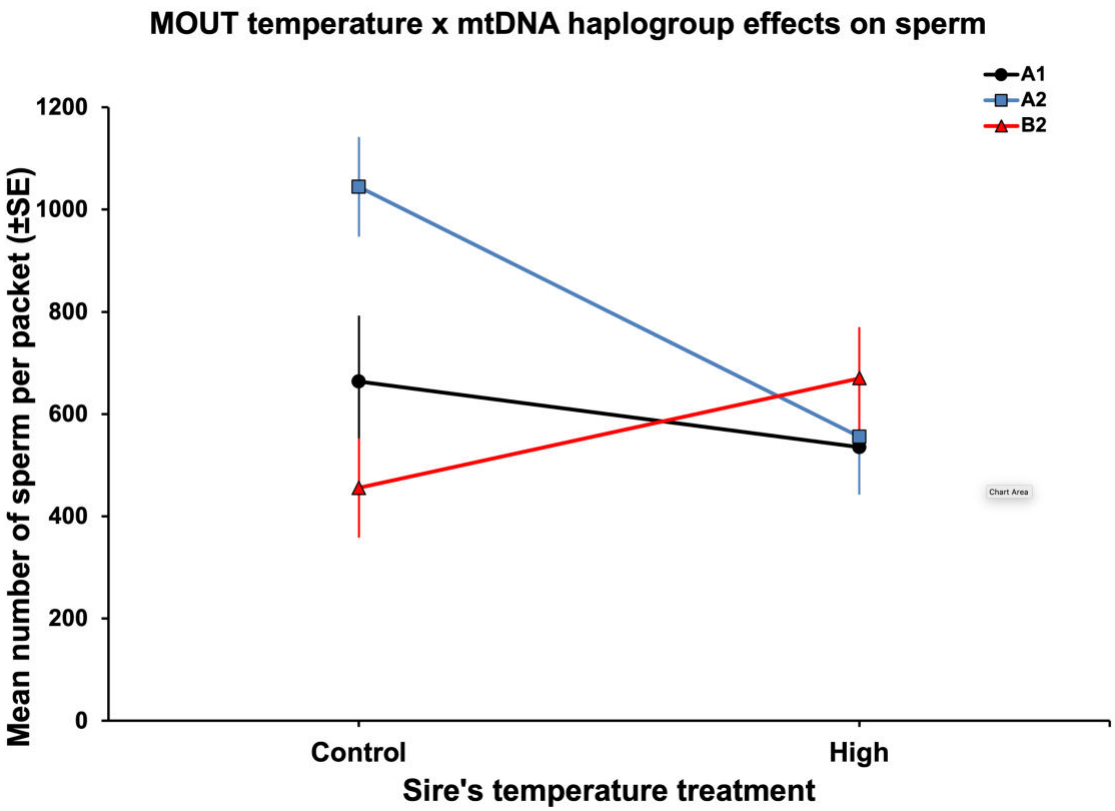

Figure 11. Negative effect of father's high-temperature treatment on offspring sperm production is influenced by mitochondrial haplogroup in the male outcross (MOUT) line. Males sired by A1- and A2-haplogroup males are disproportionately negatively affected by the high temperature compared to sons of B2-haplogroup males. Data are plotted as means ± SEs.

As was the case in the FOUT experiment, sperm viability was not significantly affected by temperature treatment ($F_{1,30}$ = 0.31, P = 0.5808), haplogroup ($F_{1,30}$ = 0.80, P = 0.4592) (Tables 1-2) or the interaction between temperature and haplogroup ($F_{2,30}$ = 1.37, P = 0.2096).

3.4 | Female Reproductive Traits

FOUT reproductive trait effects. – A total of 61 F_1 females were mated, and of those, 49 gave birth to a brood. There were no significant effects of female temperature, haplogroup or the temperature by haplogroup interaction on any aspect of female reproductive output, including the percentage of broods carried to term (temperature: $\chi^2 = 0.02$, $P = 0.8826$; haplogroup: $\chi^2 = 0.23$, $P = 0.8894$; temperature x haplogroup: $\chi^2 = 0.69$, $P = 0.7070$), the number of embryos produced (temperature: $F_{1,50} = 0.65$, $P = 0.4246$; haplogroup: $F_{2,50} = 1.07$, $P = 0.3519$; temperature x haplogroup: $F_{2,50} = 0.48$, $P = 0.6208$) and the number of nymphs born (temperature: $F_{1,54} = 0.31$, $P = 0.5820$; haplogroup: $F_{2,54} = 0.13$, $P = 0.8789$; temperature x haplogroup: $F_{2,54} = 0.93$, $P = 0.3998$; see Tables 1-3).

MOUT female reproductive traits effects. – Out of 69 mated MOUT females, 54 successfully carried broods to term. In a pattern similar to the FOUT experiment, there were no significant effects of sire temperature, sire haplogroup or the temperature by haplogroup interaction on either the percentage of broods carried to term (temperature: $\chi^2 = 2.09$, $P = 0.1480$; haplogroup: $\chi^2 = 0.14$, $P = 0.9313$; temperature x haplogroup: $\chi^2 = 1.28$, $P = 0.5275$) or the number of embryos produced (temperature: $F_{1,38} = 0.49$, $P = 0.4896$; haplogroup: $F_{2,38} = 0.41$, $P = 0.6661$; temperature x haplogroup: $F_{2,38} = 1.71$, $P = 0.1949$). However,

there was a significant, positive effect of sire temperature on the number of nymphs born ($F_{1,62}$ = 5.15, P = 0.0267), with HM females giving birth to 22% more nymphs than CM females (Table 1). The number of protonymphs born is the product of the number of embryos produced, an intergenerational component, and the survival of embryos to birth, a transgenerational component. Since embryo number was unaffected by sire temperature, it can be inferred that the significant, positive effect of sire temperature on number of protonymphs born represents a transgenerational effect of increased embryonic survival. Finally, neither sire haplogroup ($F_{2,62}$ = 0.62, P = 0.5401) nor the temperature by haplogroup interaction ($F_{2,62}$ = 1.27, P = 0.2809) significantly influenced number of nymphs born (Tables 1-3).

Trait	Direct Birth to Adult Survivorship (%) Mean SE	FOUT Birth to Adult Survivorship (%) Mean SE	Direct Male Mating Speed: Time to Aquiescence Mean ± SE	Direct Sperm Number Mean ± SE	FOUT Sperm Number Mean ± SE
A1 Control Mean	87.71% ± 2.94%	90.01% ± 3.28%	126.91 ± 35.36	733.08 ± 113.23	732.05 ± 146.78
A1 High Mean	81.97% ± 2.94%	88.49% ± 2.81%	150.6 ± 38.66	469.89 ± 124.41	403.08 ± 143.16
A2 Control Mean	84.23% ± 2.71%	86.08% ± 2.72%	135.65 ± 31.28	802.41 ± 99.67	946.32 ± 133.97
A2 High Mean	80.84% ± 2.71%	82.89% ± 2.83%	179.06 ± 37.01	316.39 ± 119.12	414.21 ± 144.65
B2 Control Mean	86.71% ± 2.52%	93.63% ± 3.44%	99.48 ± 36.9	673.14 ± 114.92	880.69 ± 156.9
B2 High Mean	70.31% ± 2.52%	77.88% ± 3.14%	297.32 ± 36.18	417.56 ± 110.89	324.56 ± 148.73
Interaction P-Value	0.0294	0.0368	0.0259	0.0013	0.0096

Table 3. Summary of significant temperature-by-haplogroup interactions in the directly-treated generation (Bonham 2021) and the FOUT and MOUT experiments. For the FOUT experiment, haplogroup is the dam haplogroup. For the MOUT experiment, haplogroup is the sire haplogroup.

4 | DISCUSSION

To place these results in context, in Figure 12, elevated temperature effects on the range of *C. scorpioides* morphological, life history and reproductive traits are compared for the directly-treated F_0 generation (Bonham, 2021) and the FOUT and MOUT experiments carried out in this study. Directly-treated *C. scorpioides* were significantly negatively impacted for all traits except development time. High-temperature pseudoscorpions were less sexually dimorphic, exhibited lower birth-to-adult survival and were smaller. Females gave birth to fewer nymphs and males were slower to mate and produced fewer sperm than control-temperature individuals. The only potential fitness benefit was significantly reduced development time, but this occurred at the cost of smaller adult size, particularly in males. Nonetheless, the magnitude of negative effects in the F_0 generation varied extensively by trait, with by far the greatest impact on reproductive traits closely linked to fitness in both females and males (Fig. 12). Similar results in an earlier study of *C. scorpioides* prompted the authors to conclude that "… reproduction may be the Achilles' heel of tropical ectotherms, as climate warming subjects them to an increasingly adverse thermal environment." (Zeh et al., 2012). In the case of male reproduction, the findings reported here extend this pattern to the intergenerational level, with greatly reduced sperm counts in both FOUT and MOUT offspring. Interestingly, female reproductive traits linked to fitness, including numbers of embryos produced, broods carried to term, and

protonymphs born, were not so affected and appear to be resistant to the intergenerational effects of high temperature.

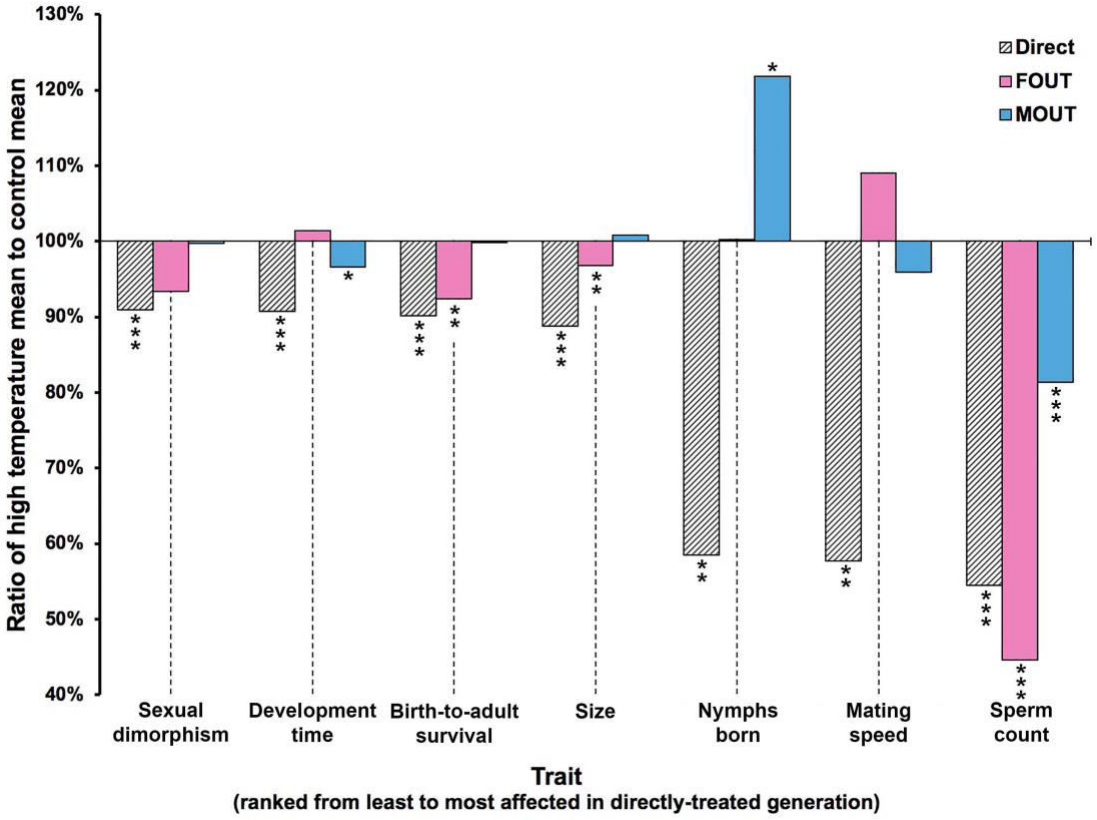

Figure 12. Summary comparison of the direct and intergenerational maternal (FOUT) and paternal (MOUT) phenotypic consequences of simulated climate warming in *Cordylochernes scorpioides.* Bars are scaled relative to control temperature means with equivalence at 100%, and averaged across the two sexes for development time, survival and size. Data for the directly-treated generation are from Bonham (2021). Significance levels are indicated by asterisks below or above the bars: * ≤ 0.05; ** ≤ 0.01; *** ≤ 0.0001.

As expected from the magnitude of direct high-temperature effects, intergenerational effects on non-reproductive traits were either less apparent or absent. In addition, the likelihood and magnitude of significant intergenerational effects varied by inheritance mode, with more and greater effects transmitted through females than through males. In particular, the negative effect of high temperature was perpetuated in the lower survivorship of FOUT offspring, and significantly reduced size of males born to high-temperature females. Only 83% of nymphs born to FOUT high-temperature females survived to the adult stage, compared to 90% of offspring born to control females. No such difference in survival was detected in offspring sired by high and control MOUT males (Fig. 1). An intriguing pattern of intergenerational inheritance to emerge from this study was the transmission of reduced body size to their offspring by females directly exposed to the elevated temperature. The sons but not the daughters of high FOUT females were significantly smaller than their control counterparts (Fig. 3). Through a process of elimination, the results of the MOUT and FOUT assays of morphometric traits reported here generate a hypothesis to explain how negative effects on body size of birth-to-adult exposure to increased temperature are transmitted from mothers to offspring in *C. scorpioides*.

In the F_0-generation individuals directly exposed to elevated temperature, cephalothorax length, chela hand depth and femur depth were all significantly smaller in both males and females than in control individuals. This temperature-induced reduction in size was likely the consequence of significantly reduced

male and female developmental times in the F_0 generation. Indeed, high-temperature-induced size reduction associated with more rapid development is a pervasive pattern in invertebrates and these two life-history traits are causally related (Angilletta, et al., 2004; Gardner, et al., 2011; Sheridan & Bickford, 2011). However, this explanation cannot be invoked in the case of the F_1 generation, in which the effect of elevated temperature on developmental time was uncoupled from its effect on size. The daughters of high MOUT males developed significantly faster than the daughters of control males but did not differ in size, except in the case of femur depth. Paradoxically, the sons of high FOUT females exhibited significant reduction in cephalothorax length, chela hand depth and femur depth but no reduction in developmental time compared to the sons of control females.

Temperature effects on mitochondria or other components of the egg cytoplasm could account for transmission exclusively through females but should affect both their male and female offspring. Cytoplasmic maternal effects can therefore be rejected as an explanation for the pattern of female transmission detected in this study, since size reduction was apparent in the sons but not the daughters of high FOUT females. With other potential causes rejected, the most likely explanation for the pattern of intergenerational inheritance of size reduction in morphometric traits documented in this study lies in the XX/XO system of sex determination in *C. scorpioides* (Šťáhlavský et al. 2009). Since males have only a single X chromosome, they are more vulnerable than females to the negative

consequences of disruption of X-linked gene expression, as occurs in many X-linked human diseases, such as hemophilia A (Graw et al., 2005), red-green color blindness (Neitz & Neitz, 2000) and Duchenne muscular dystrophy (Duan et al., 2021). These diseases are largely restricted to males and result from mutations in the DNA sequence of genes on the X chromosome. However, the cause of reduced size in the sons of high females in this study was likely epigenetic rather than genetic. According to this hypothesis, exposure to elevated temperature during development induced changes in epigenetic regulation of expression of X-linked genes influencing growth in the somatic and germline cells of exposed individuals. Epigenetic mechanisms provide a more plausible explanation for these short-term maladaptive responses to environmental change, as epigenetic changes occur more rapidly than genetic evolution (Skinner, 2015). The sons of high FOUT females exhibited reduced size as a result of intergenerational inheritance of temperature-induced epigenetic changes to their maternally-inherited, single X chromosome. With their two X chromosomes, the daughters of high FOUT females exhibited no reduction in size due to the rescuing effect of the X chromosome they inherited from their control father. In support of this interpretation, preliminary RNA sequencing studies have revealed significant differential X-linked gene expression in FOUT but not MOUT embryos (Chapter 3).

The intergenerational effects transmitted through the male lines are more difficult to interpret. On the one hand, sperm number in F_1 offspring was

significantly negatively affected by exposure of sires to high temperature, although the level of sperm reduction was less marked than in FOUT lines (Fig. 9). On the other hand, all other significant intergenerational effects were positive and included reduced female developmental time , faster mating initiation by males, and, most significantly, increased female reproductive success. Indeed, F_1 MOUT females sired by high temperature males gave birth to 22% more protonymphs than females sired by control males (Table 1). Perhaps the most likely explanation for this fitness enhancement is selection, in which only the most genetically- or epigenetically-fit F_0 MOUT males were able to sire offspring. This hypothesis of selection-generated fitness benefits is consistent with lower percentage of broods carried to term by females mated to MOUT sires (42.2%) than by FOUT dams mated to control males (58.05%) (Table 1).

Strict maternal inheritance of mitochondria is hypothesized to act as a constraint on the adaptive evolution of mitochondria for male function, particularly in gametes (Frank & Hurst, 1996; Zeh & Zeh, 2005; Padua, et.al., 2014; Vaught & Dowling, 2018). Consistent with this theory was the finding that sperm number was the only trait to exhibit significant haplogroup effects in the F_1 generation (Table 2). The significant main intergenerational effect of haplogroup was apparent in both FOUT and MOUT lines, with the A2 haplogroup males producing the greatest number of sperm in both cases. In terms of potential evolutionary responses to climate change, of greater relevance are interactions between temperature treatment and haplogroup, and significant interactions were

detected for two traits in the FOUT line. For both birth-to-adult survival (Fig. 2) and number of sperm produced (Fig. 10), the rare B2 haplogroup was most negatively affected by high-temperature treatment of the mother. This result is perhaps not surprising, as evidence suggests that the geographic origin of the B2 haplogroup is in western Panamá at higher elevations subjected to cooler temperature regimes (Zeh et al., 2003). This result is of particular interest because of the sister's (or mother's) curse phenomenon acting on the B2 haplogroup, which has a roughly three-fold advantage in sperm competition with the A1 haplogroup (Padua et al., 2014; Zeh et al., 2019). An RNA sequencing study has strongly linked this sperm competitive advantage to differential mitochondrial gene expression, particularly of the *atp8* gene. Nonetheless, because males do not transmit mitochondria to offspring, selection cannot act directly to increase the frequency of the B2 haplogroup.

One interesting albeit perplexing result was the detection of significant dam haplogroup and sire temperature by dam haplogroup effects on male but not female cephalothorax length, hand depth and femur depth (Figs. 6-8) in the MOUT but not in the FOUT lines. The male offspring of high MOUT sires exhibited small size of all three traits, if they inherited B2 haplogroup mitochondria from their mothers, particularly when their father's haplogroup was A1 or A2. These findings suggest that, in *C. scorpioides*, exposure of the male parent to high temperature affects crosstalk between nuclear and mitochondrial genes in their offspring. Experimental studies on mice and *Drosophila*

melanogaster have demonstrated that mitonuclear communication has major consequences for the phenotype (Innocenti et al., 2011; Roubertoux et al., 2003; Vaught et al., 2020), and growing evidence indicates that this crosstalk between the mitochondrial genome and the nuclear genome is largely mediated by epigenetic mechanisms (Wiese & Bannister, 2020).

The results of this study present a more complex picture than would be suggested by the overwhelmingly negative consequences for the directly treated F_0 generation. While many traits were robust to intergenerational effects of simulated climate warming, there was a lingering negative effect on FOUT male body size and both MOUT and FOUT sperm production, but mildly increased nymph numbers in MOUT females and mildly reduced developmental time for MOUT females. The patterns of intergenerational inheritance detected in this study are not unusual, as paternally and maternally inherited traits have been demonstrated to have conflicting outcomes in offspring (Burke, et al., 2020; Curley, et al., 2011). For example, a study by Ducatez et al. (2012) found that in the butterfly *P. brassicae*, males exposed to stressful environment were characterized by parental effects from the egg stage until adult emergence, whereas females exposed to stress produced effects late in progeny life history. Earlier evidence suggests that parental effects produce an increase in parent fitness, regardless of offspring fitness (Marshall & Uller, 2007). Data from our experiment also supports this hypothesis, since, as reported earlier, MOUT females in the F_1 generation produced more nymphs on average compared to

control females, increasing their own reproductive success. Newer evidence also suggests parent-of-origin effects are also likely to be mediated by whether the effect produces a differential fitness benefit between the two sexes in the same environment (Burke, et al., 2020). In their natural habitat, *C. scorpioides* clamber onto the elytra of harlequin beetles for dispersal to new fallen trees, and dispersal of the population induces temporally oscillating sexual selection on body size of males, with larger males monopolizing space, and thus mating opportunities, with females during dispersal (Zeh, et al., 1997). Here, reduced size in FOUT sons but not in FOUT daughters may produce differential fitness effects between males and females in the F_1 generation contributing to conflicting patterns of intergenerational inheritance caused by mutations in the males' defective X chromosome.

The most striking conflicting pattern of inheritance in this study is the difference in male and female reproductive success. One component of male reproductive success, i.e., sperm number, was the most negatively impacted trait for both MOUT and FOUT males, but the number of protonymphs born, a component of female reproductive success, in MOUT females was the most improved trait. It is possible that the increase in protonymph number is due to rapid positive selection in fitter MOUT males. Evolutionary rescue occurs when adaptive evolutionary changes restore population growth to prevent extinction. Theory and laboratory experiments suggest that the probability of evolutionary rescue depends on several intrinsic and extrinsic factors, including standing

genetic variation and mutation rate, population size, density dependence dynamics, and amount of gene flow between the source and sink populations (Carlson, et al., 2014; Hoffmann & Sgro, 2011; Seebacher, et al., 2015). For expeditious evolutionary rescue to occur, the optimum state per each of several factors must be reached, therefore most studies place a higher emphasis on behavioral and plastic changes as a more obtainable immediate strategy for combating extinction due to climate change (Beever, et al., 2017; Parmesan, 2006; Wong & Candolin, 2015). Behaviorally, environmental heterogeneity potentially allows ectotherms to compensate for lack of thermal plasticity by moving between microhabitats and modulating heat exposure (Pincebourde & Suppo, 2016). In natural populations in central Panamá, *C. scorpioides* inhabits decaying *Ficus* trees, and tree type (standing or fallen) and extent of canopy cover (closed or open) produces fine-scale microclimate variation, with trees in open canopy conditions experiencing greater diurnal variability in temperature and averaging approximately 2.5ºC hotter than trees in closed canopy conditions (Chapter 1). Microclimatic heterogeneity may also contribute to standing phenotypic variation in natural populations of *C. scorpioides* as individuals carry a legacy of microenvironments experienced in previous generations.

Plastic epigenetic changes are much more sensitive to environmental perturbations than is the genome itself (Furrow, et al., 2011; Skinner & Nilsson, 2021), and therefore provide a more direct way of inducing phenotypic change under rapid environmental change (Bonduriansky & Day, 2009; Colicchio &

Herman, 2020; Harrisson, et al., 2014). High levels of microclimate heterogeneity are likely a factor that induces intergenerational effects, as theory suggests that high levels of epigenetic modifications are favored in frequently changing environments to allow organisms to repeatedly reach the optimal phenotype (Herman, et al., 2014), and plastic responses in offspring are adaptive when parent environment predicts offspring environment (Ezard, et al., 2014; Yin, et al., 2019). Epigenetic alterations have also been demonstrated to further genetic evolution, as nearly all genetic mutations can be directly influenced by epigenetic processes (Ghalambor, et al., 2015; Klironomos et al., 2013; Scoville & Pfrender, 2010; Skinner, 2015; Stajic & Jansen, 2021; Zeh, et al., 2009), allowing organisms to respond rapidly to environmental change through epigenetic alterations that then beget adaptive genetic mutations.

Moreover, epigenetic mechanisms have been associated with silencing transposable element expression (Yi & Goodisman, 2021). Transposable elements are DNA sequences able to copy or excise themselves and “move” to new genes in the genome, resulting in gene disruption. Physical stress induced by climate change or ecological disruptions, may temporarily disable the system of epigenetic mechanisms suppressing transposable element activity, resulting in restructuring of the genome, as conserved genes are disrupted or as genes change to allow species to reach new peaks on the fitness landscape (Casacuberta & Gonzalez, 2013; Werren, 2011; Zeh et al., 2009). Previous RNA-seq analysis on *C. scorpioides* testicular tissue (Bonham, 2021) revealed that 11

differentially expressed genes derived from transposable elements were upregulated in males reared at high temperatures, suggesting breakdown in epigenetic control over transposable elements for high temperature exposed individuals. The relationship between intergenerational effects and heat exposure is required to better understand how environmental volatility and epigenetic adaptation may have to spur beneficial genetic mutations or produce an extinction spiral of pathological responses to climate change, not just for *C. scorpioides*, but for the countless species impacted by rising temperatures over the coming decades.

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Activation of transposable elements in a warming world

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Abstract

Anthropogenic greenhouse gas emissions are currently projected to cause an increase in average global temperatures of 2.4-3.5ºC by the end of the century. Tropical regions are a hotbed for biodiversity, comprised primarily of arthropods. Tropical ectotherms are at a disproportionate risk of detrimental effects from high temperature exposure because they are adapted to narrow thermotolerances and their metabolic rate increases exponentially with linear increases in temperature. Long-term response to climate change must occur through evolutionarily changes, however short-term responses can confer immediate advantages. Epigenetics, known as the study of gene expression changes without changes in DNA sequence, can provide a way for tropical arthropods to exhibit plastic changes in response to rapidly increasing temperatures. Epigenetic modifications consist of DNA methylation, histone modifications, and small non-coding RNAs, and these modifications have shown to be heritable across generations. Transposable elements are short genomic sequences capable of relocating throughout the genome and disrupting essential gene activity. Epigenetic modifications repress transposable element activity, but exposure to stressful environments can disrupt epigenetic regulatory mechanisms designed to suppress transposable element mobilization. PIWI-interacting small non-coding RNAs have been implicated in germline suppression of transposable elements to prevent mobilization during embryogenesis. Here, I

utilized the model tropical arthropod *Cordylochernes scorpioides* to quantify differential expression of testicular protein coding and transposable element expression, and testicular and spermatic PIWI-interacting non-coding RNA expression in the germline of individuals exposed to high temperatures during development to determine how epigenetic regulatory mechanisms and transposable element expression can contribute to the intergenerational inheritance of phenotypes in offspring. Testicular protein coding and transposable element expression were up-regulated and testicular and spermatic PIWI-interacting RNAs were down-regulated in *C. scorpioides* exposed to high temperatures, indicating that epigenetic dysregulation of transposable element suppression may play a role in the inheritance of adaptive or maladaptive offspring phenotypes.

1 | INTRODUCTION

As the climate continues to warm, its effects on biodiversity remains uncertain. Global anthropogenic greenhouse gas (GHG) emissions were higher during the previous decade (2010-2019) than at any previous time during human history, and it is highly likely that existing infrastructure for GHG emissions will cause an increase of at least 1.5ºC measured from the beginning to the end of this century (IPCC, 2022). Current policies and energy-production infrastructure suggest that, without further mitigation, warming has the potential to increase by a total of 2.4-3.5ºC. Tropical regions are a hotbed for biodiversity, containing 91% of terrestrial birds, as well as over 75% of terrestrial mammals, freshwater fish, and reptiles (Barlow, et al., 2018). Of particular concern are tropical ectotherms, which comprise the vast majority of tropical species, with 1,359,129 species described (IUCN 2019), while the true number of species estimated at least several million (Stork, 2018). Since tropical ectotherms are already adapted to narrow thermal limits (Addo-Bediako, et al., 2000; Deutsch, 2008) and metabolic rate increases exponentially with increases in ambient temperature (Dillon, et al., 2010; Gillooly, 2001; Klok & Harrison, 2013), they are at a disproportionate disadvantage in combating the effects of climate change. Indeed, long-term analysis of tropical arthropod populations has found a massive 10- to 60-fold decrease in arthropod diversity since the 1970s, correlated with a 2ºC increase in temperature between 1978 and 2015 (Lister & Garcia, 2018).

Tropical species have responded to climate warming through behavioral modifications such as phenology shifts (Forrest, 2016), elevational range shifts (Chen et al., 2016), and taking advantage of microclimatic heterogeneity (Chapter 1). However, these strategies have limitations for the mitigation of climate change. Long-term adaptation to increased thermal tolerances must occur through adaptive evolutionary changes to restore population growth and prevent extinction, a process termed evolutionary rescue (Carlson, et al., 2014). Evolutionary rescue is the most effective long term strategy for species to mitigate the deleterious effects of climate change, but the probability of evolutionary rescue occurring depends on several intrinsic and extrinsic factors, such as standing genetic variation, mutation rate, population size, density dependence dynamics, and the amount of gene flow between populations (Carlson, et al., 2014; Hoffmann & Sgro, 2011; Seebacher, et al., 2015). Therefore, short-term plastic changes that immediately affect the phenotype confer the maximum advantage for responses to climate warming. Plasticity in arthropods is well documented, with diapause (Saunders, 2014), body pigmentation (Sibilia, et al., 2018), behavior (Abram, et al., 2017), and sex determination (Blackmon, et al., 2017) all linked to temperature. However, epigenetics, the study of gene expression changes without changes in DNA sequence, has only recently started receiving attention in arthropods as a key component of phenotypic plasticity (Villagra & Frías-Lasserre, 2020), even

though epigenetic mechanisms have been found in a variety of arthropod species (Burggren, 2016; Glastad, et al., 2019). Epigenetic mechanisms regulate levels of gene expression, and are required for cell differentiation during embryonic development (Skinner & Nilsson, 2021) and the suppression transposable element activity (Slotkin & Martienssen, 2007).

Transposable elements (TEs), also known as transposons or mobile genetic elements, are relatively short DNA sequences capable of moving throughout the genome (Bourque, et al., 2018). Since TEs are highly mutagenic, host genomes use regulatory epigenetic molecular mechanisms (EMMs) to prevent active transposons from transcribing proteins (reviewed in Slotkin & Martienssen, 2007). Transposable elements are categorized into two classes: DNA transposons and retrotransposons (Bourque, et al., 2018). DNA transposons are mobilized by a DNA intermediate and utilize “cut-and-paste” mechanism, whereas retrotransposons are mobilized by an RNA intermediate using a “copy-and-paste” mechanism, and thus retrotransposons are typically found in greater copies in eukaryotic genomes (Kim, et al., 2012). Retrotransposons can be further divided into long terminal repeat (LTR) retrotransposons, such as endogenous retroviruses, and non-LTR transposons, such as long interspersed elements (LINEs) and short interspersed elements (SINEs) (Kohlrausch, et al., 2022). The proportion of the genome occupied by transposons varies by species, with over 80% of the maize genome consisting of transposable elements (Schnable & et al., 2009); around 50% in mice (Todd, et al., 2019); 40% of the

human genome (Kohlrausch, et al., 2022), and less than 6% of the genome in the Antarctic midge (Petersen, et al., 2019). The majority of transposons reach fixation primarily through genetic drift, often eroded by single base-pair mutations that lead to their inactivation and “domestication” into the genome where, in many cases, transposon sequences undergo exaptation and develop functional roles within the host (Bourque, et al., 2018; Naville, et al., 2016). For example, *env* genes derived from retrotransposons have been found to function in the formation of placenta in live-bearing species by facilitating cell-cell fusion (Dupressoir, et al., 2012). However, prior to domestication, transposon activity has the potential to cause major disruptions in the genetic sequence. At least 120 transposon insertion events have been associated with human disease (Hancks & Kazazian, 2017) due to interruption of essential protein-coding genes.

There are three main EMMs that influence TE silencing and the expression of genes: (1) DNA methylation; (2) histone protein modifications; and (3) post-transcriptional regulation of gene expression by short and long noncoding RNAs (Bird, 2007; Bossdorf, et al., 2008). DNA methylation is the most well-studied EMM, and consists of gene silencing by adding methyl groups at cytosine residues (Skinner & Nilsson, 2021). Histone modifications operate by affecting chromatin structure to impact the accessibility of genes for transcription, with acetylation and phosphorylation the most well-studied (Bartova, et al., 2008; Rothbart & Strahl, 2014; Taylor & Young, 2021). Noncoding RNAs (ncRNAs) consist of small- and long-ncRNAs that regulate gene expression independent of

DNA sequence by binding to messenger RNA (mRNA) transcripts to cause degradation (Huang, et al., 2014; Forrest & Khalil, 2017; Wei, et al., 2018). Epigenetic mechanisms are also capable of interacting with each other, as, for example, long ncRNAs are known to facilitate DNA methylation processes (Urquiaga, et al., 2021), and DNA methylation can modify histone modifications (Lobo, et al., 2019). EMMs are highly sensitive to environmental conditions experienced by the individual and they exhibit metastability, that is, they remain unchanged unless sufficiently disturbed (Feil & Fraga, 2012; Morgan, et al., 2005). Thus, the epigenome provides a direct mechanism for the environment to impact gene expression in the individual independent of DNA sequence. Factors such as nutrition (Vaiserman & Lushchak, 2022), temperature (Xu, et al., 2020), stress (Cao-Lei, et al., 2021), trauma (Thumfart, et al., 2022), exposure to toxins (Liu, et al., 2022), and microbiome and microbial diversity (Woo & Alenghat, 2017; Ye, et al., 2017, but see Chapter 4) have been documented as altering EMMs, thereby altering phenotype. EMM dysfunction has been implicated in many human diseases, including cancer, metabolic disorders, autism, autoimmune disease, asthma, and type-2 diabetes (Samata, et al., 2017; Shamsi, et al., 2017) as well as in mice and rat models (Rosenfeld, 2010), and nonhuman primates (Dettmer & Suomi, 2014). EMMs altered early in development can even contribute to phenotypes that influence acquisition of disease later in life (Nilsson, et al., 2018).

Furthermore, direct exposure to stressful environments induces alterations not only in somatic cells in the individual, but also in germline cells, creating intergenerational and/or transgenerational modes of inheritance (Ben Maamar, et al., 2022; Lempradl, 2020; Margueron & Reinberg, 2010). In intergenerational inheritance, exposure of the parent also affects germline cells for the next generation of offspring, since both generations are directly exposed to the factor responsible for the changes, occurring in, namely, mature and premature sperm that contribute to the F_1 generation in males and oocytes in females (Skinner & Nilsson, 2021). In gestating females, three generations are simultaneously exposed to stressful conditions: the F_0 mother, F_1 developing fetuses, and F_2 primordial germline cells within the gestating fetus. Transgenerational inheritance differs from intergenerational inheritance in that epigenetic changes persist after the directly exposed generation, occurring in the F_2 generation in males and F_3 generation in gestating females. Both types of epigenetic inheritance have been documented in the literature, with intergenerational inheritance found in model organisms such as *D. melanogaster* (Öst & al, 2014), mice (Carone & al, 2010; Huypens, et al., 2016), and *C. elegans* (Miersch & Döring, 2012; Tauffenberger & Parker, 2014) and transgenerational inheritance documented in several species such as mice (Gapp, et al., 2014), fish (Kelley, et al., 2021), and birds (Skinner, et al., 2014).

Since intragenerational epigenetic modifications may be passed down through the germline, exposure to environmental stress may result in disease

etiologies in the next generation (Nilsson, et al., 2018). To avoid ambiguity in differentiating between maternal epigenetic markers of the oocyte and epigenetic markers acquired early in embryological development, research efforts have focused largely on paternal epigenetic inheritance through spermatozoa. Sperm deliver a complex mix of RNAs to the oocyte, including mRNA transcripts, TE transcripts, and a variety of snRNAs such as microRNAs (miRNAs), small interfering RNAs (siRNAs) and piwi-interacting RNAs (piRNAs) (Godia, et al., 2022). These RNAs have marked effects on several components of male fitness such as on reproduction, with the absence of the full suite of snRNAs in sperm correlated with a 46% reduction in the probability of achieving live birth (Jodar, et al., 2015); on offspring development, with the post-transcriptional regulation and degradation of mRNAs by miRNAs and siRNAs essential for healthy embryonic cell division (Jodar, et al., 2013); and on silencing transposon expression by piRNAs. A study by Krawetz et al. (2011) found that small RNA transcripts most prevalent in human sperm are nonfunctional mRNA fragments derived from repetitive elements, representing 65% of transcripts, followed by piRNAs, representing nearly 17% of transcripts. A more recent review by Kohlrausch et. al., (2022) reported that the vast majority of EMMs are reset in humans during primordial germ cell development, with *de novo* methylation reestablished in embryonic development. Reprogramming of methylation states in early prenatal development poses a serious risk of transposon activation during blastocyst implantation, leading to deleterious genomic instability. Therefore, alternative

mechanisms to methylation and histone modifications to maintain transposon control during reproduction necessitate an abundance of piRNAs in spermatozoa.

Both EMMs and TEs have been associated with increased dysregulation as a result of physiological stress. Alternative epigenetic states have been documented as a consequence of physiological stress from UV exposure, temperature, radiation, wounding, and pathogen infection, and physiological stress is also capable of directly inducing activation of some transposons, as reviewed in Capy, et al. (2000). Most significantly, TEs can be reactivated through alteration of EMMs designed to control and reduce their expression, making physiological stress response an important component of species fitness, with a reduction in epigenetic control over TEs responsible for disruption of genome integrity (Slotkin & Martienssen, 2007; Chen, et al., 2016). It is not yet clear whether tropical arthropods exposed to heat stress through climate change will exhibit adaptive or maladaptive states, and how those states may be transmitted to offspring through intergenerational or transgenerational inheritance. In the present study, I quantified the differential expression of protein-coding genes, transposable element transcripts, and piRNAs in testicular and spermatic tissue to determine whether nymphal exposure to high temperature is indeed capable of causing disruption of epigenetic control mechanisms over transposable element expression and how simulated climate warming through exposure to high temperature during nymphal development can

affect the potential for adaptive or maladaptive intergenerational inheritance in the model tropical arthropod, *Cordylochernes scorpioides*.

Previous climate warming studies of *C. scorpioides* indicate that reproduction is likely the “Achilles heel” of this species (Zeh, et al., 2012). Nymphal exposure to an increase of 3.5ºC above ambient temperatures in this pseudoscorpion’s natural habitat reduced male fertility by half and left females functionally sterile, unable to carry a brood to term (Zeh, et al., 2014). Climate warming studies consisting of several smaller temperature increase increments were then carried out to determine at which point detrimental reproductive responses in *C. scorpioides* become unsustainable for species propagation. An increase of 3ºC caused high offspring mortality and the experiment had to be discontinued (Zeh, et al., unpublished), whereas an increase in 2ºC resulted in relatively modest decreases in sperm production and nymph survival (Hernandez, et al., in prep).

Exposure to an increase of 2.5ºC resulted in widely negative results consisting of reductions in survival, adult body size, sperm production and female reproductive success (Bonham, 2021), though effects were not too extreme as to prevent offspring production. An intergenerational effects study was also carried out to determine how exposure to high temperature affects offspring phenotype (Chapter 2). In a reciprocal outcross experiment, males exposed to high temperature during nymphal development were mated to both control temperature and high temperature females, and, similarly, females exposed to high temperature during nymphal development were mated to both control and

high temperature males. Morphology, development time, and female and male reproductive traits in offspring were assayed to determine which traits were inherited through the male and female lineage. Reduced sperm counts occurred in the sons of both high-temperature mothers and fathers, and this trait also exhibited significant haplogroup effects in the F_1 generation. Mitochondrial variation in this species was also implicated in differential responses to climate change. In offspring with high-temperature mothers, negatively affected traits passed from the directly treated generation were reduced survivorship and reduced body size of only male offspring. Interestingly, high-temperature fathers transmitted some positive intergenerational effects to their offspring: sons of high temperature males were more likely to successfully sire broods and they fathered significantly more offspring than the sons of control males. Conflicting results between offspring of high temperature dams and sires suggest further investigation is required into epigenetic regulatory mechanisms that guide intragenerational and intergenerational plasticity in this species.

1.1 | The Study Organism

Cordylochernes scorpioides is a small, flightless, neotropical arthropod that inhabits decaying trees in the families, Moraceae and Apocynaceae, and gains access to these ephemeral and patchily-distributed habitats by “hitchhiking” under the elytra of its dispersal agent, the giant harlequin beetle, *Acrocinus*

longimanus (Zeh & Zeh, 1992; Zeh J., 1997). This pseudoscorpion has a unique reproductive biology that enables accurate and non-invasive study of reproductive traits. During mating, males and females engage in a stereotypical display in which the male grasps the female and remains stationary until a spermatophore is produced and deposited on the substrate. If the female acquiesces, she allows the male to guide her over the spermatophore and flexes her abdomen so that the sperm packet adheres to her genital aperture and sperm are evacuated into her reproductive tract. Mating can be interrupted following spermatophore deposition to retrieve sperm packets for assessment of RNA abundance or to quantify sperm number and viability. Females of this species are polyandrous, produce mixed paternity broods sired by up to four males (Zeh, 1997), and exhibit a unique form of viviparity, in which embryos are nourished in an external, transparent brood sac overlying the female's genital aperture.

This species also exhibits high levels of mitochondrial variation, with three mitochondrial haplogroups, A1, A2, and B2, co-occurring in central Panamanian populations where study organisms were obtained, at frequencies of, respectively, 67%, 21% and 12%. Next-generation sequencing indicates that A1 and A2 haplogroups diverge by 1.9% and A1 and B2 sequences diverge by 8% across the mitochondrial genome (Padua, et al., 2014; Zeh, et al., unpublished). These haplogroups have a functional impact on reproductive success: in two-male sperm competition experiments, B2 males sire three times as many

offspring as A1 males (Zeh et al. 2019). RNA sequencing suggests this advantage is due to differential expression of mitochondrial oxidative phosphorylation genes between haplogroups, with eleven genes found to be differentially expressed between A and B2 males, including upregulation of *atp8* by over 20-fold (Zeh, et al., 2019).

Maternally inherited mitochondria produce the vast majority of adenosine triphosphate (ATP) used for cellular energy (Meiliana, et al., 2021). Consequently, the mitochondrial genome is a target of selection and is likely to influence evolutionary responses to climate change (Ballard & Pichaud, 2014). Mitochondria have also been linked to epigenetic mechanisms, since large amounts of ATP and acetyl-CoA phosphorylate and acetylate chromatin, thereby allowing nuclear DNA transcription (Wallace & Fan, 2010), while impaired mitochondria induce DNA methylation in the nuclear genome (Smiraglia, et al., 2012). Mitochondria detect and respond to a variety of physical stressors, and alter their function to meet cellular needs, thus they are a significant mediator of environmental cues and plasticity. Within the framework of climate change, thermal variation has significant influence over the catalytic capacity of enzymes involved in the generation of ATP (Christen, et al., 2018; Michaelsen, et al., 2021), with several studies linking mitochondrial dysfunction to heat stress in plants, mice, trout, and humans (Blier, et al., 2014; Rurek, 2014; White, et al., 2012; Wilkening, et al., 2018). Mitochondrial intermediates influence epigenetic processes used in manufacturing cellular energy, and enzymes involved in the

removal of these marks use mitochondrial metabolites to carry out their function (Schvartzman, et al., 2018). For example, S-adenosylmethionine (SAM) is a key methyl donor to prevent gene expression through methylation, with its synthesis involving conversion of methionine into SAM by combining with the adenosyl moiety of ATP (Ouyang, et al., 2020), indirectly linking ATP production with key epigenetic processes. Substantial mitochondrial variation between *C. scorpioides* haplogroups may help elucidate the role of mitochondria in intergenerational epigenetic response to simulated climate warming.

1.2 | The *Cordylochernes scorpioides* Genome

The genome contains a high proportion (~70%) of transposable elements and repetitive sequences as well as extensive gene duplications (Zeh et al., unpublished). Sequencing the genome was completed using a de novo assembly pipeline involving PacBio Sequell II sequencing followed by FALCON (Chin, et al., 2016) contig assembly, and Chicago and Dovetail™ Hi-C based scaffolding. High molecular weight DNA (average molecular weight >100 KB) was extracted from an inbred *C. scorpoides* line to create PacBio SMRTbell libraries and sequenced at 40X coverage using the Sequel II system. Chicago™ Libraries utilized a combination of *in vitro* chromatin fixation, digestion and crosslink reversal to create a library type unique to Dovetail Genomics, known as Dovetail™ Hi-C. These libraries utilize the restriction enzyme *Dpn*II for chromatin

digestion prior to proximity ligation, which has been shown to improve ordering, orientation and contiguity even in highly accurate assemblies.

Pacbio Sequel II yielded 6.3M reads generated 106 billion base-pairs (bp), with a mean read length of 15,735 bp, an N50 of 27,860 bp, and a maximum read length of 103,850 bp. The FALCON assembly, based on 5.6M error-corrected reads, yielded 9,807 contigs, and an N50 and maximum contig length of 960,000 bp and 18,800,000 bp, respectively. Chicago and Dovetail™ Hi-C scaffolding improved scaffold N50 100-fold, from 960 Kb to 99.6 Mb. The final assembly included 5,279 scaffolds, with an assembly length of 2.94 Gb. The longest scaffold is ~224 Mb, the approximate size of the largest human chromosome. Nearly 90% of the assembly (2.6 Gb) is in 24 scaffolds, corresponding to the haploid number of *C. scorpioides* chromosomes. The 25th largest scaffold is only 2% of the size of Scaffold 24 and the various smaller scaffolds may represent contaminant DNA. Moreover, the scaffold length distribution precisely matches the distribution of chromosome lengths in the *C. scorpioides* karyotype. The third largest scaffold, predicted to be the X chromosome based on size in the karyotype, bears a hypervariable locus confirmed through DNA typing to be hemizygous, as the *C. scorpioides* has an XO, male heterogametic sex determination system.

Protein-coding sequences were annotated by by comparison with coding sequences from *Mesobuthus martensii*, *Ixodes scapularis*, *Centruroides sculpturatus*, *Stegodyphus mimosarum* and *Limulus polyphemus* from the NCBI

database for training in AUGUSTUS software (version 2.5.5; Stanke, et al., 2006) through three rounds of prediction optimization and an *ab initio* model in SNAP (version 2006-07-28; Johnson, et al., 2008). *C. scorpioides* mRNA sequencing libraries were prepared from early- and late-stage embryos by the Nevada Genomics Center at the University of Nevada Reno to improve prediction quality. Reads were sequenced using an Illumina NextSeq 500 sequencer and an Illumina Mid-Output flow cell, resulting in approximately 133 million paired-end reads. These reads were mapped onto the genome using the STAR aligner software (version 2.7; Dobin, et al., 2013) and intron hints generated using the bam2hints tools with AUGUSTUS. AUGUSTUS, SNAP, and MAKER software(Cantarel, et al., 2008) were used to predict genes in the repeat-masked reference genome, using Swiss-Prot peptide sequences from the UniProt database as a guide in the Maker pipeline. Only genes that were predicated by both AUGUSTUS and SNAP were used in the final gene sets. Genes were further characterized for function by performing a BLAST search of the peptide sequences against the UniProt database. Transposable and repetitive elements were identified de novo and classified using RepeatModeler software (version 2.0.1; Smit, 2008), which itself utilizes RECON (version 1.08; Levitsky, 2004) and RepeatScout (version 1.0.6; Price, et al., 2005). The custom repeat library obtained from RepeatModeler was used to discover, identify and mask the repeats in the assembly file using RepeatMasker (version 4.1.0). For piRNA analysis, small-RNA libraries for piRNA prediction were prepared by the Nevada

Genomics Center, and approximately 20 million, 21-34 bp RNA reads from combined sperm and testes samples were mapped to the *C. scorpioides* genome. piRNA clusters were annotated using proTRAC software (probabilistic Tracking and Analysis of Clusters) (version 2.4.2; Rosenkranz & Zischler, 2012), which, like miRCat2, requires sRNA sequences mapped to the genome from high throughput sequencing libraries. proTRAC identifies piRNA clusters based on (1) significant deviations from a uniform distribution regarding the density of mapped reads; (2) strand asymmetry; (3) frequency of putative piRNA loci with T at position 1 (1T) or an A at position 10 (10A); (4) the number of putative piRNA loci within the typical piRNA length range, and (5) the number of loci corresponding to infrequently mapped reads. Small RNA libraries were prepared for prediction by the Nevada Genomics Center, and approximately 20 million small bp RNA reads from sperm and testes samples were mapped to the *C. scorpioides* genome.

Annotation revealed 27,445 protein-coding genes, encompassing 51,621,360 base pairs. The total genome consists of 2.6 Gb, thus protein-coding genes only represent 1.97% of the genome. All major gene types involved in epigenetic regulation were identified, including Argonaute and piwi loci, histone deacetylase loci, DNA methyltransferase loci, a Dicer locus, and polycomb protein. Transposon-derived genes constituted a surprising fraction of protein-coding genes (7.23%) and consists mainly of retrovirus-related Pol polyprotein loci. Annotation of the genome also includes five copies of a PEG3 homolog, which

are proteins required in mammalian fetal development, though paternally expressed, exhibiting parent-of-origin effects, thus suggesting the possibility that the large number of transposon-derived domesticated genes is related to the evolution of live birth in *C. scorpioides* (Zeh et. al., unpublished). RepeatModeler annotation identified more than 6.2M copies of repetitive elements, comprising approximately 70% of the genome, an exceptionally high number compared with the majority of published arthropod genomes. The majority of repetitive elements are unknown sequences, with other classes of transposable elements consisting mainly of LINEs, LTR retrotransposons, and DNA transposons (Fig. 1). Given the paucity of published data on arachnid genomes, it is unsurprising that 27.69% of genes encode proteins of unknown function.

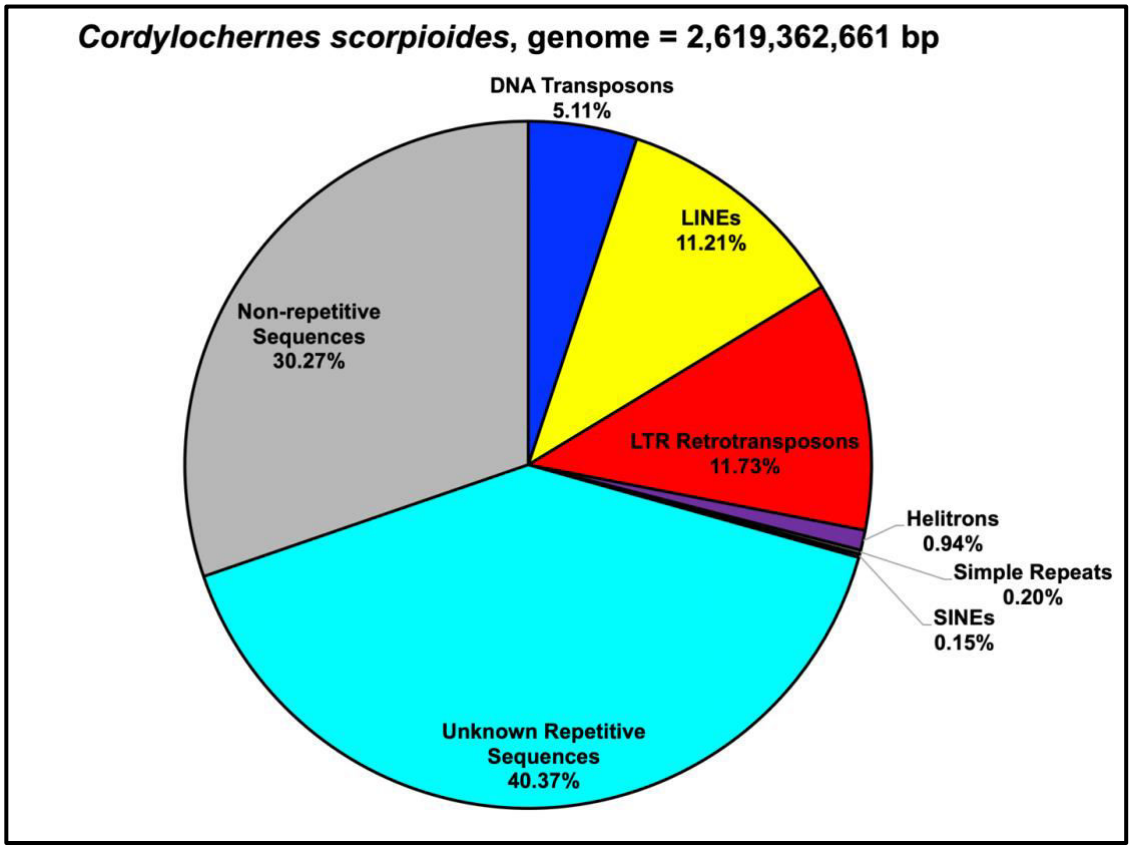

Figure 1. Relative abundances of repetitive regions in the *C. scorpioides* genome.

Testicular and sperm tissue were selected for RNA-seq, as nearly all protein-coding genes are readily and abundantly expressed in the testes and the sperm epigenome is highly specialized and vulnerable to environmental conditions (Bonilla, 2016). Indeed, RepeatModeler annotation confirms that transposons are transcribed into mRNAs. In testicular tissue, nearly 6.67% of all sequenced reads matched repetitive element sequences, with LTR/Gypsy sequences as the most

abundantly expressed transposon in the testes and approximately 20,000 transposon loci expressed at a level of 5 or more transcripts per million (TPM).

2 | METHODS

2.1 | Experimental Pseudoscorpions

Pseudoscorpions used for this study were descended from 297 individuals collected from seven decaying *Ficus* trees in Central Panamá in July 2017 (for locations, see Chapter 1). A laboratory population was perpetuated by outcrossing isofemale lines randomly with respect to mitochondrial haplogroup for multiple generations to establish a relatively uniform nuclear genome across haplogroups. Individuals for this experiment were generated as described elsewhere (see Chapter 2), as part of a larger study aimed at investigating the effects of a 2.5ºC increase on developmental, morphological and reproductive traits in *C. scorpioides*. To assess *C. scorpioides* exposure to high temperature, virgin females from the laboratory population were randomly mated to an unrelated male to generate A1, A2 and B2 haplogroup families. Pseudoscorpions go through three nymphal (protonymph, deutonymph and tritonymph) molts prior to the adult stage, at which point the pedipalps and cephalothorax become fixed in size and gender is morphologically visible (Weygoldt, 1969). A split-brood design was used, in which 40 protonymphs from each family were randomly

assigned at birth, with 20 each going to either a control (C) incubator or high (H) temperature incubator. The control temperature diurnally-oscillating regime had an average daily temperature of 28.38ºC, based on ambient temperature data collected by iButton loggers attached to the seven decaying trees from which the founding members of the *C. scorpioides* laboratory population were collected, (see Chapter 1). The high temperature regime involved an increase of 2.5ºC above the control regime, with average daily temperature of 30.38ºC. Nymphs were reared in individual vials and fed a weekly diet of *Drosophila melanogaster* larvae during the first two nymphal stages, and then switched to *Tribolium confusum* larvae for the tritonymph and adult stages, as described elsewhere (Zeh et. al., 2005). Development time to adulthood at the high temperature typically takes a minimum of 31 days, so daily visual inspection of vials for adult emergence commenced 28 days after birth. After developing to the adult stage, individuals were sexed and adult males chosen for testes dissection and sperm collection.

2.2 | RNA-Seq of Testicular and Sperm Tissue

Adult males chosen for testes expression analysis were frozen in liquid nitrogen, dissected under 20-40X magnification, and the testes were surgically removed, as described in Weygoldt (1969). Similarly, sperm was collected from each male by staging matings between experimental males and non-experimental females

in 28mm-diameter mating arenas and observing under a Leica EC4 stereomicroscope with video objective 0.63x (Leica Microsystems, Inc.). Immediately after spermatophore deposition, matings were interrupted and sperm packets collected using a dissecting needle. RNA from both tissues was extracted and purified using miRNeasy Micro Kit (Qiagen), and samples were stored at -80ºC prior to sequencing.

Fourteen libraries were prepared for testes protein-coding analysis of gene expression of several temperature treatments (represented by either “C” or “H”) and haplotype pairings (represented by either “A1,” “A2,” or “B2”), with two A1C, two A1H, two A2C, three A2H, two B2C, and three B2H replications used. Dissected testes from two young full-sibling adult males were pooled to obtain enough RNA per library. mRNA-Seq libraries were prepared using poly-A enrichment. Four libraries of three males each were prepared for sperm piRNA analysis with replications of one A1H, one A1C, one B2H, and one B2C. All RNA quantity and quality were assessed by the Nevada Genomic Center at the University of Nevada Reno, using Agilent Bioanalyzer RNA Nano (Agilent Technologies) or High Sensitivity DNA chips. Quantification was performed using Qubit dsDNA or RNA specific reagents. RNA quantity per sample was determined to be from 1.27 µg to 3.56 µg and RNA Integrity Numbers (RI) ranged from 7.90 to 9.60.

Library preparation and sequencing were also performed at the Nevada Genomic Center. Libraries were created according to manufacturer instructions using a Qiagen QIASeq Stranded mRNA Select kit and sequenced in multiplex using an Illumina NextSeq 500 and an Illumina Mid-Output flow cell (Illumina). Paired-end reads were obtained, with the sequencer configured to collect 76 bp forward and reverse reads for 76 cycles each, and eight cycles for barcode indices. Pre-base-call intensity data, logs, and metrics were sent to the Illumina BaseSpace service and processed by built-in BaseSpace FASTQ Generation module to call nucleotide bases, demultiplex samples by barcode, mask adapter sequences, and generate FASTQ format sequence files. Files were downloaded for further processing and filtered to remove bases below Q10 in quality score using bbduk from the Joint Genome Institute's BBTools Suite (Bushnell B. – sourceforge.net/projects/bbmap/). Number of reads per sample ranged from 42,681,956 to 58,023,048, with approximately 93% of reads greater than base quality ≥ Q20.

Nuclear protein-coding gene expression levels were determined by mapping individual sequencing libraries to the coding sequence (CDS) annotation of the entire, 24-chromosome *C. scorpioides* genome using BBMap (Bushnell B. – sourceforge.net/projects/bbmap/) plugin, a highly accurate and splice-aware global aligner for DNA/RNA sequencing reads, in Geneious Prime 2021, set to normal sensitivity and random mapping to multiple best matches. Since protein-coding genes only comprise 1.97% of total genes in the C. scorpioides genome,

all genes were included in analysis. The same procedures were used for transposon and piRNA expression, except that matches of mRNA transcripts were made to target genome sequences not CDS annotations, as both TEs and piRNAs lack introns. Given the large number of repetitive sequence loci identified by RepeatModeler (> 6 million) in the *C. scorpioides* genome, only the first chromosome (8.54% of total genome sequence) was used for transposable expression analysis. For protein-coding genes, TPM (see Wagner, et al., 2012) for each CDS and sample were calculated using Geneious Prime to generate count data. For TEs and piRNAs, counts per million (CPM) were used. Ambiguously mapped reads were considered partial matches and only loci that were expressed in at least one of the 14 samples were retained for differential expression analysis.

Differential expression analysis between treatment and haplogroups was performed using the Bioconductor software package edgeR, version 3.32.1 (Robinson, et al., 2010). Given the limited sample sizes and the similarities in gene code between A1 and A2 haplogroups, these haplogroups were pooled into a single “A” category. Temperature effects on differential expression were assessed by specifying haplogroup as a blocking factor and temperature as a treatment factor. Similarly, haplogroup effects on differential expression were assessed by specifying temperature as a blocking factor and haplogroup as a treatment factor. edgeR utilizes an overdispersed Poisson model to account for

biological and technical variability, and empirical Bayes methods to moderate overdispersion across transcripts (Robinson, et al., 2010). The Bonferroni–Holm method (Holm, 1979) was used to adjust *P*-values for multiple comparisons. Significant differential expression of mRNA and piRNA was determined using a false discovery rate (FDR) of below 0.05 per gene (Aubert, et al., 2004; Benjamini & Hochberg, 1995). For mRNA transcripts, gene function was determined using by performing a BLAST search of the peptide sequences against the UniProt database. piRNA function was assessed using RNAcentral database, but all differentially expressed piRNAs identified were not found to map to identified sequences. For protein-coding and TE expression, the edgeR filtering command “keep = filterByExpr(y, design)” was used to eliminate lowly and inconsistently expressed loci. For piRNA analyses, because of the small number of loci, filtering effects were minimal, and all expressed loci were included in the analyses.

3 | RESULTS

3.1 | *C. scorpioides* Differential Expression

Testes Protein-Coding Results

The number of protein-coding genes expressed in testicular tissue represents a large proportion of total genes, with 25,271 out of 27,445 nuclear genes expressed between samples. EdgeR analysis revealed that 535 mRNA transcripts were significantly differentially expressed between control and high temperature samples, with 63 loci being downregulated and 469 loci upregulated (FDR < 0.05) (Fig. 2 and Fig 3).

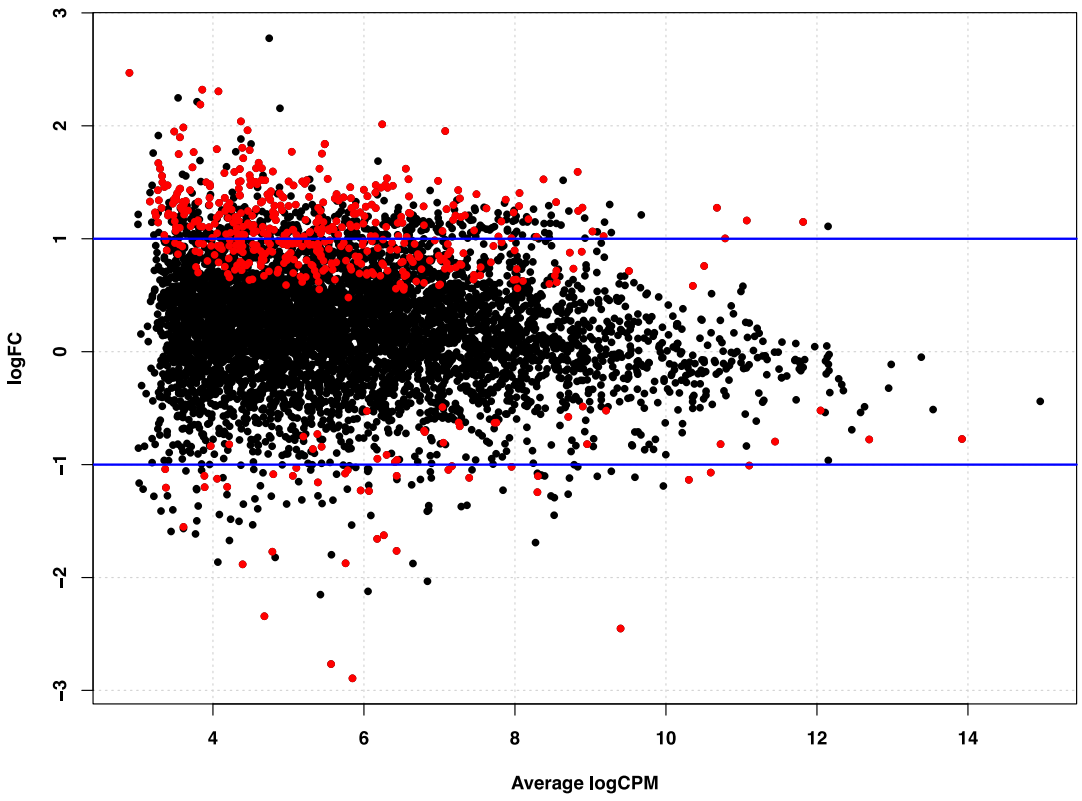

Figure 2. Smear plot of differentially expressed transcripts in testicular protein-coding genes between control and high temperature males. Genes colored red

are significantly differentially expressed. Blue lines indicate genes with greater than one-fold differential expression.

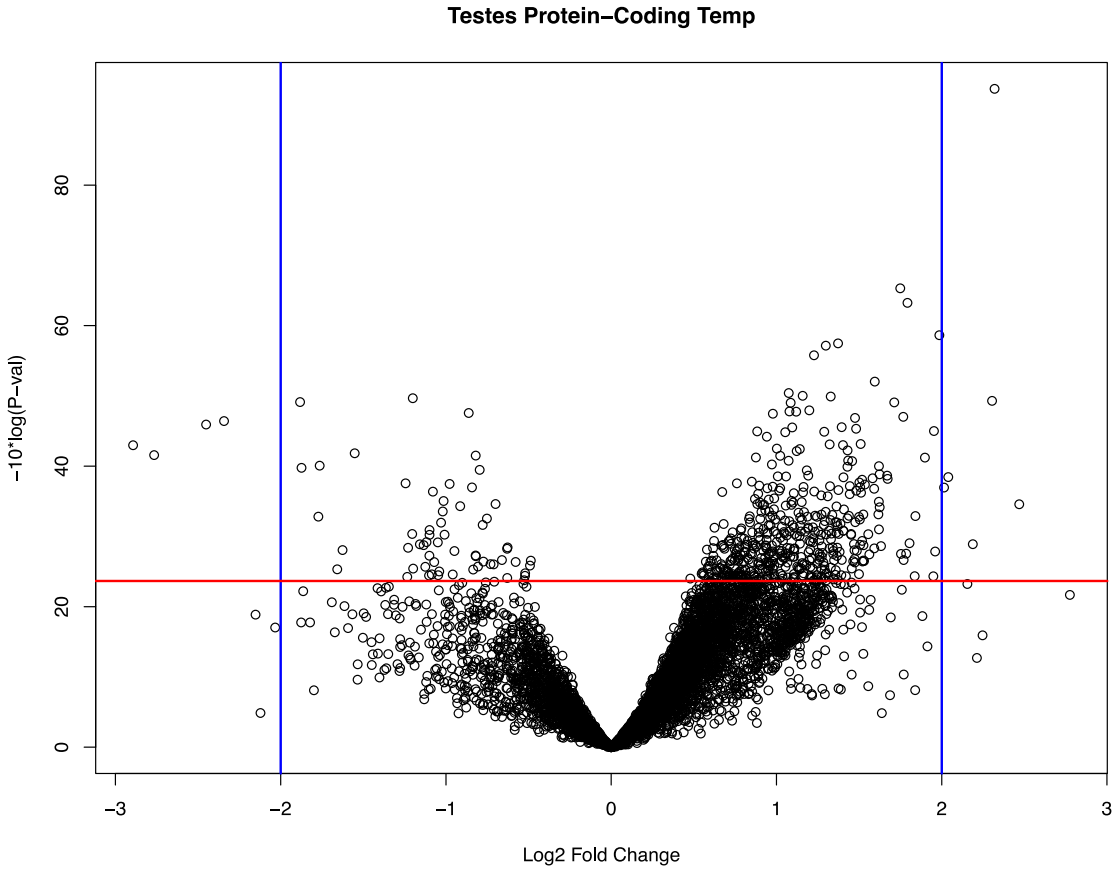

Figure 3. Volcano plot of differentially expressed transcripts in testicular protein-coding genes between control and high temperature males. Genes above the red line are significantly differentially expressed. Blue lines indicate genes with greater than two-fold differential expression.

Differentially upregulated transcripts include those that code for several heat shock proteins (eg. HSP-90AA1, HSP90b1, and HSP70); a number of transcripts for genes involved in the regulation of epigenetic mechanisms, such as SETMAR, known for inducing methylation of histone proteins, and RDR1, an RNA polymerase protein required for production of antiviral small RNAs; numerous transcripts involved transposon activity, such as *pol* proteins derived from transposons; several zinc finger transcripts, involved in transposon repression; and 2 transcripts for encoding PIWI proteins. Temperature treatment expression patterns suggest that genes are expressed similarly among control temperature males and among high temperature males (Fig. 4).

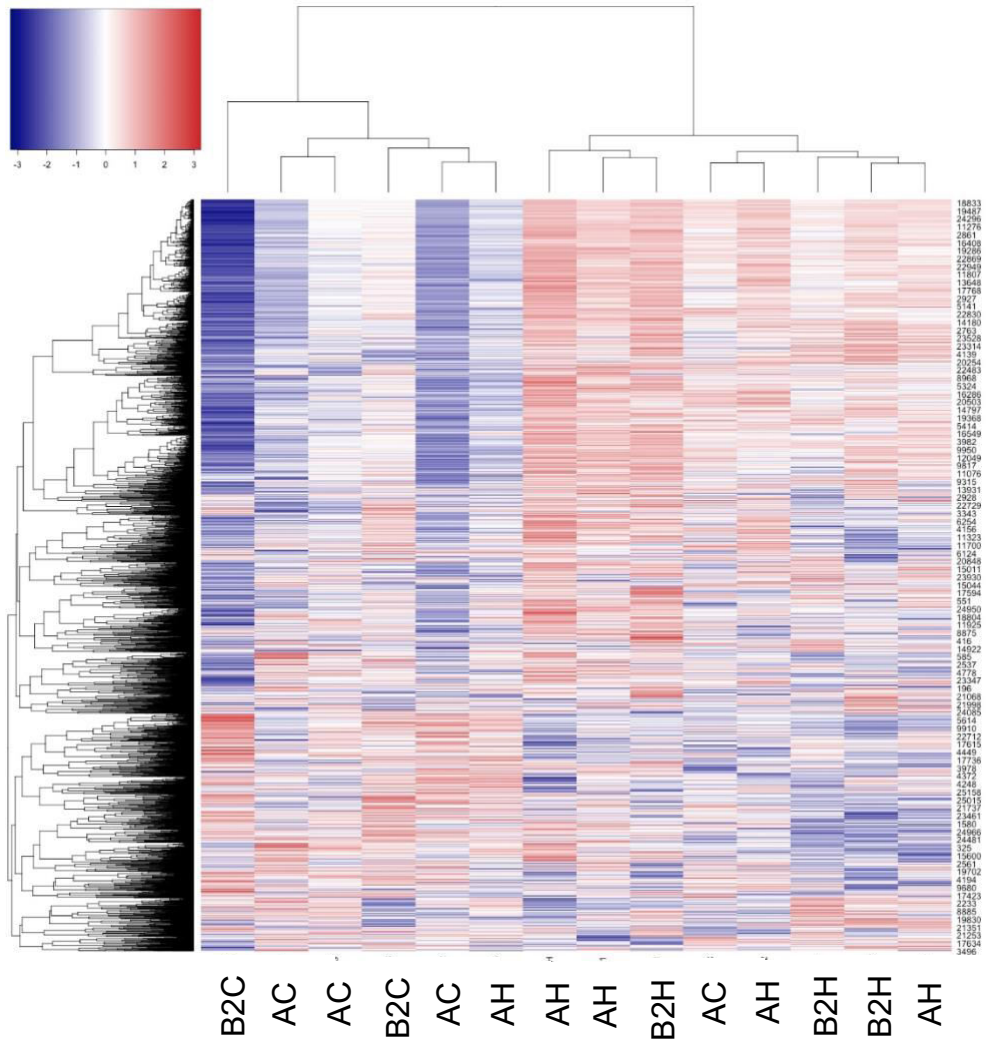

Figure 4. Heat map of 535 differentially expressed genes between control temperature and high temperature replications for testicular protein-coding genes. The right hand column consists of numbers representing the 535 differentially expressed genes. Replications consist of either “AC” or “B2C,” representing samples obtained from males treated with the control temperature regime from pooled A1/A2 haplogroups and the B2 haplogroup; or “AH” and “B2H”, representing samples obtained from males treated with the high

temperature regime from pooled A1/A2 haplogroups and the B2 haplogroup. Most control temperature-exposed *C. scorpioides* express genes similarly between replications, and high temperature-exposed *C. scorpioides* also express genes similarly between replications.

C. scorpioides mitochondrial haplogroup analysis revealed three significantly differentially expressed genes between A and B2 haplogroups (Fig. 5 and Fig. 6), with two genes significantly downregulated and one significantly upregulated. The upregulated protein is related to protein phosphatase, implicated in removal of phosphate groups of completed proteins; one downregulated protein, vitellogenin-3 is implicated in reproduction and oogenesis, and the other downregulated protein is of unknown function.

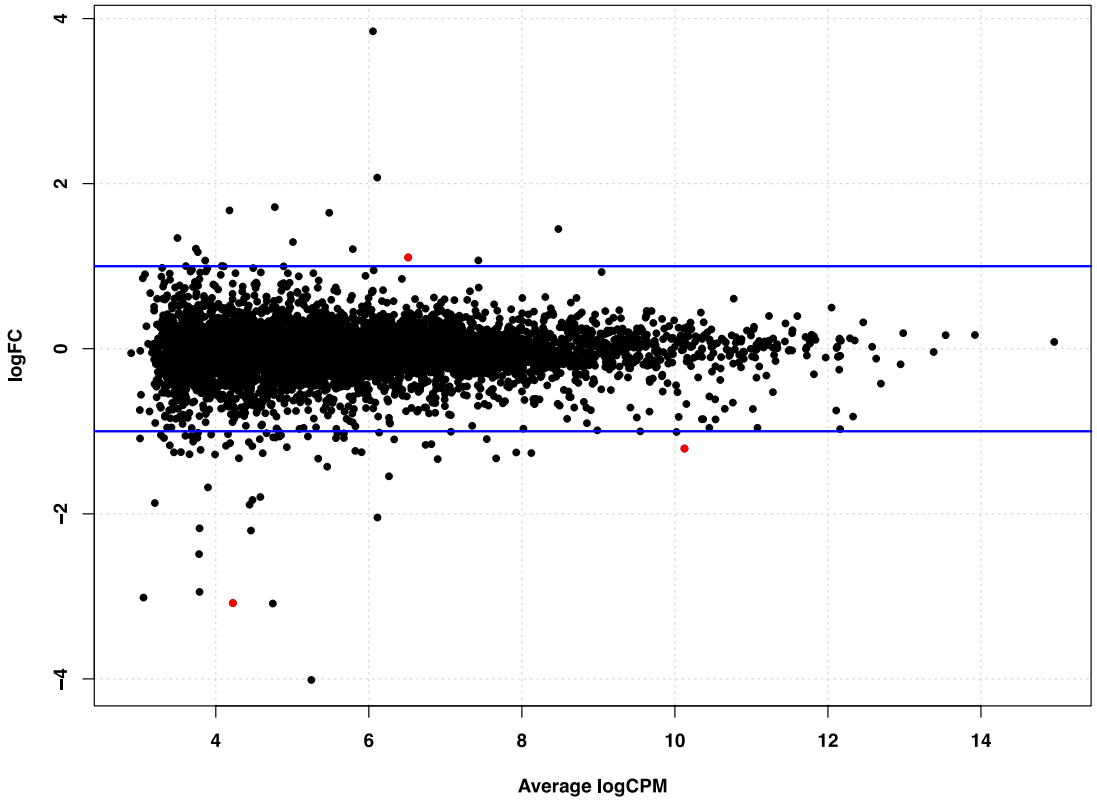

Figure 5. Smear plot of differentially expressed transcripts in testicular protein-coding genes between A1/A2 and B2 haplogroup males. Red genes are significantly differentially expressed. Blue lines indicate genes with greater than one-fold differential expression.

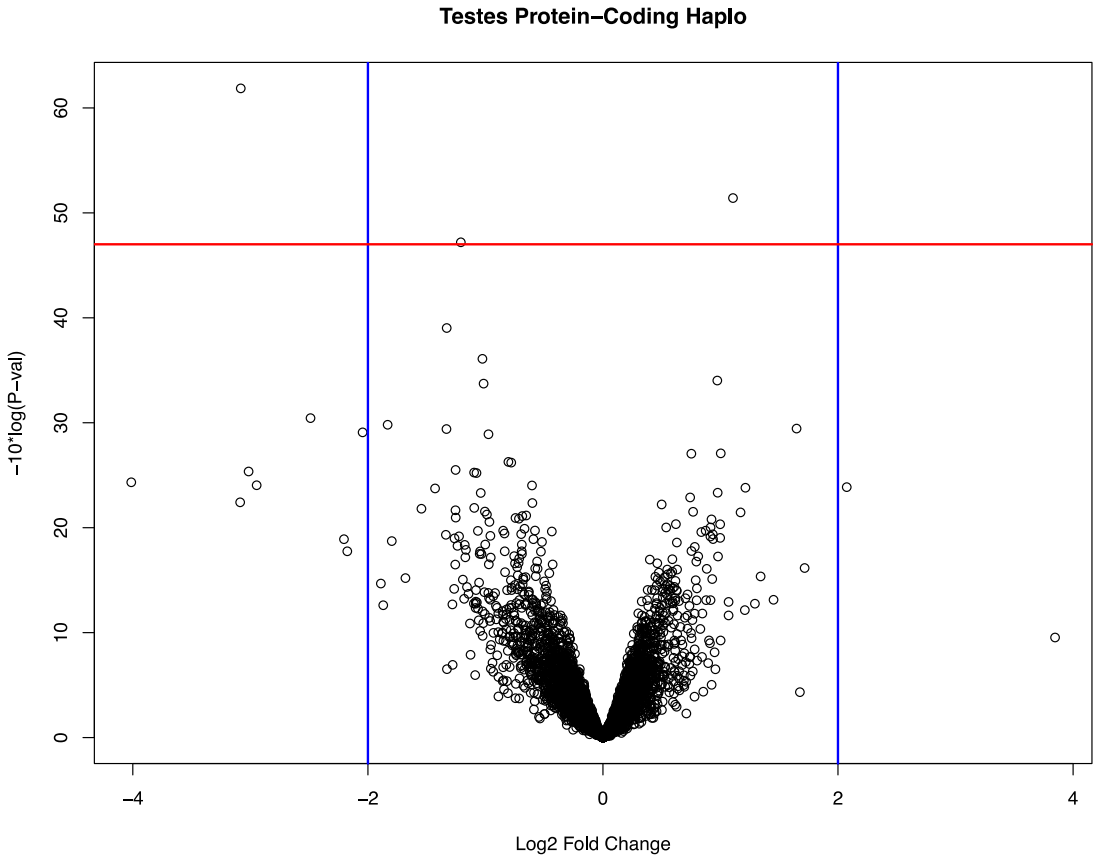

Figure 6. Volcano plot of differentially expressed transcripts in testicular protein-coding genes between A1/A2 and B2 haplogroup males. Genes above the red line are significantly differentially expressed. Blue lines indicate genes with greater than two-fold differential expression.

Testes Transposon Results

RepeatModeler identified 864,272 distinct repetitive elements on Chromosome 1 of the *C. scorpioides* genome. Of these, 207,474 loci were expressed in at least

one testes sample, but most loci were characterized by extremely low expression such that only 44,658 loci exhibited 10 or more counts per million summed across the 14 replicates. Further filtering by EdgeR reduced this number to 11,809 loci. Of these, 93 loci were significantly differentially expressed between control and high temperature samples, with 14 being downregulated and 79 being upregulated (Fig. 7 and Fig. 8). Of the upregulated transposable elements, the majority (26) were classified as LTR retrotransposons, 18 as DNA transposons, 18 as LINE elements, and 19 as of unknown class. A dozen of various other classes were also identified. Downregulated transposons consisted of mainly unknown classes of transposons.

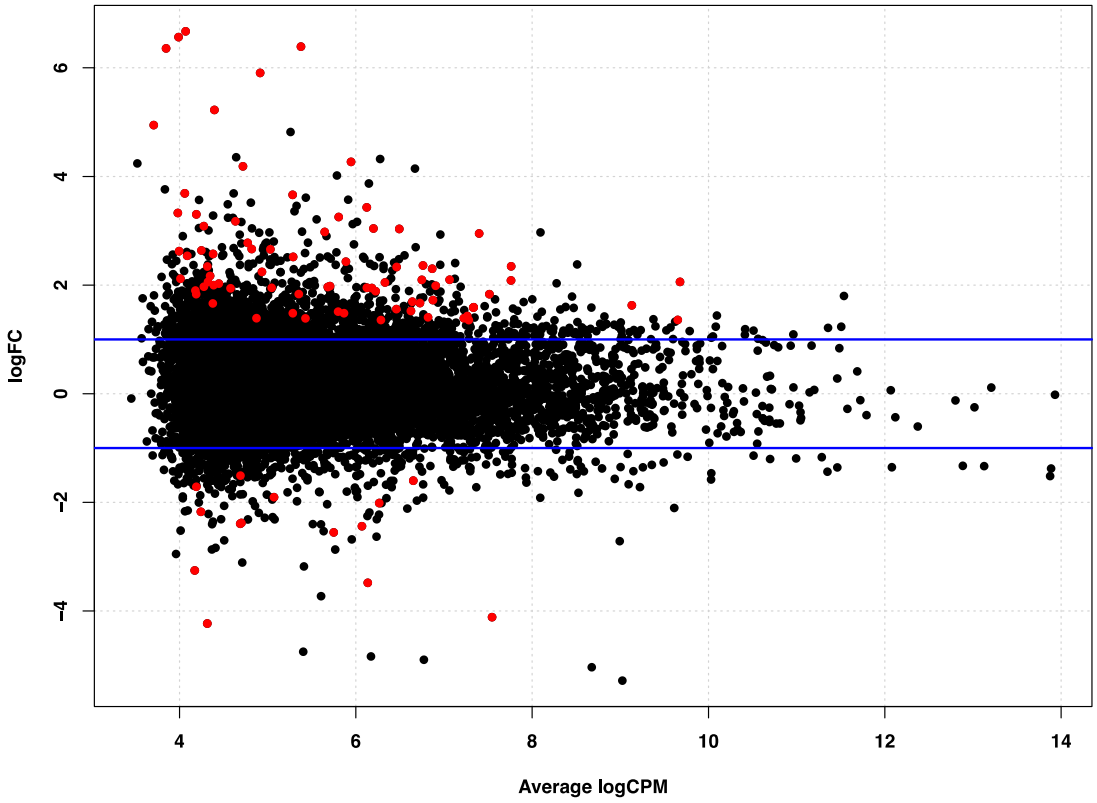

Figure 7. Smear plot of differentially expressed transcripts in testicular transposon transcripts between control and high temperature males. Red genes are significantly differentially expressed. Blue lines indicate genes with greater than one-fold differential expression.

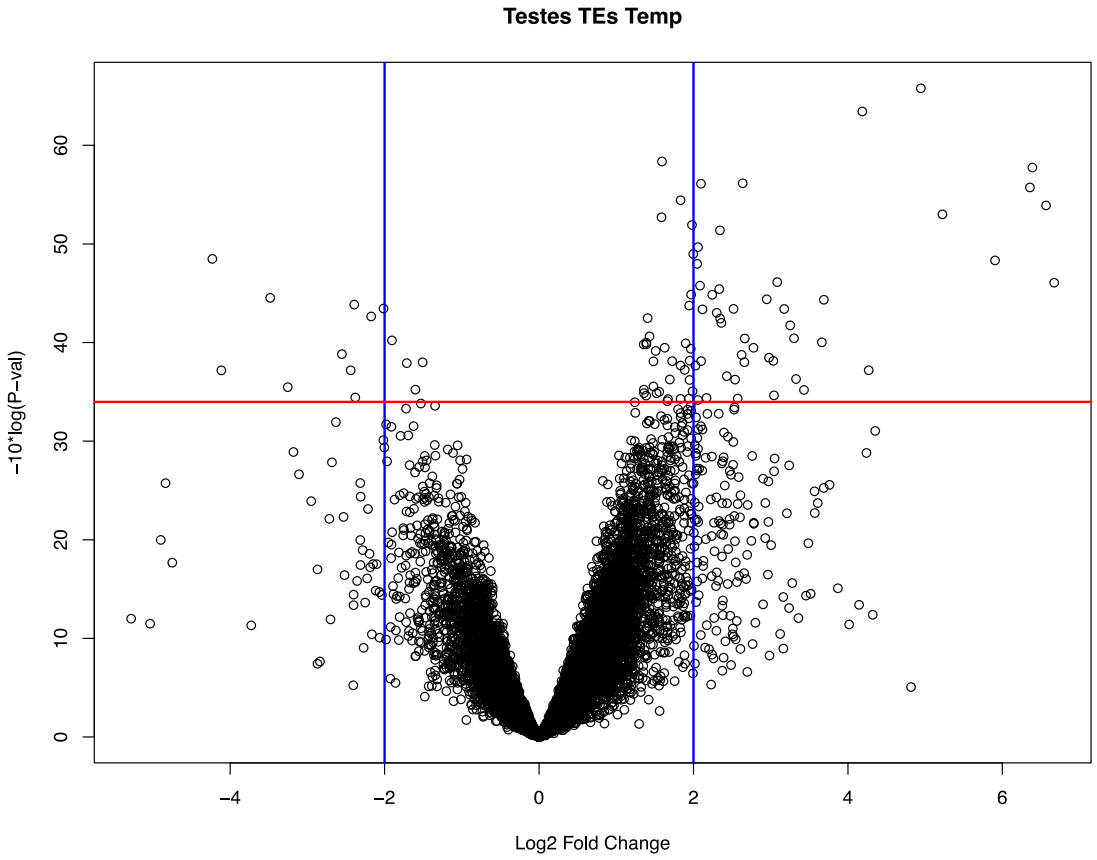

Figure 8. Volcano plot of differentially expressed transcripts in testicular transposon transcripts between control and high temperature males. Genes above the red line are significantly differentially expressed. Blue lines indicate genes with greater than two-fold differential expression.

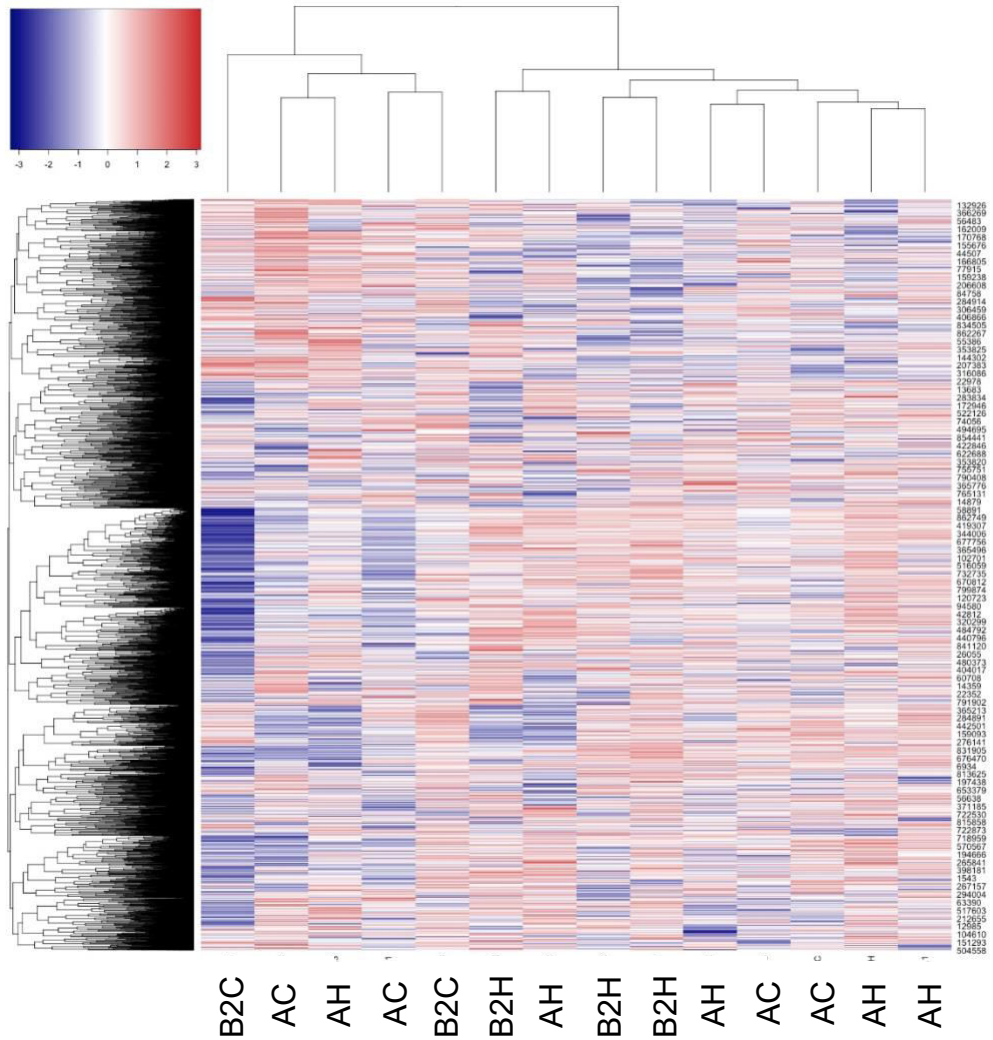

Figure 9. Heat map of 93 differentially expressed transposable elements. The right hand column consists of numbers representing the 93 differentially expressed genes. Replications consist of either “AC” or “B2C,” representing samples obtained from males treated with the control temperature regime from pooled A1/A2 haplogroups and the B2 haplogroup; or “AH” and “B2H”, representing samples obtained from males treated with the high temperature regime from pooled A1/A2 haplogroups and the B2 haplogroup. Similarities

among control and high temperature samples are not as consistent as with protein-coding expression.

According to heat map (Fig. 9), control and high transposon expression separation was not as consistent or apparent as that of protein-coding expression. Haplogroup analysis revealed a total of 31 significantly differentially expressed genes, with 20 being downregulated and 11 being upregulated between A and B2 haplogroups (Fig. 10 and Fig. 11). The majority of significantly different transcripts exhibited greater than a five-fold difference. Downregulated transposons in the B2 haplogroup consisted primarily of unknown classes, but the majority of upregulated transposons were classified as A-rich.

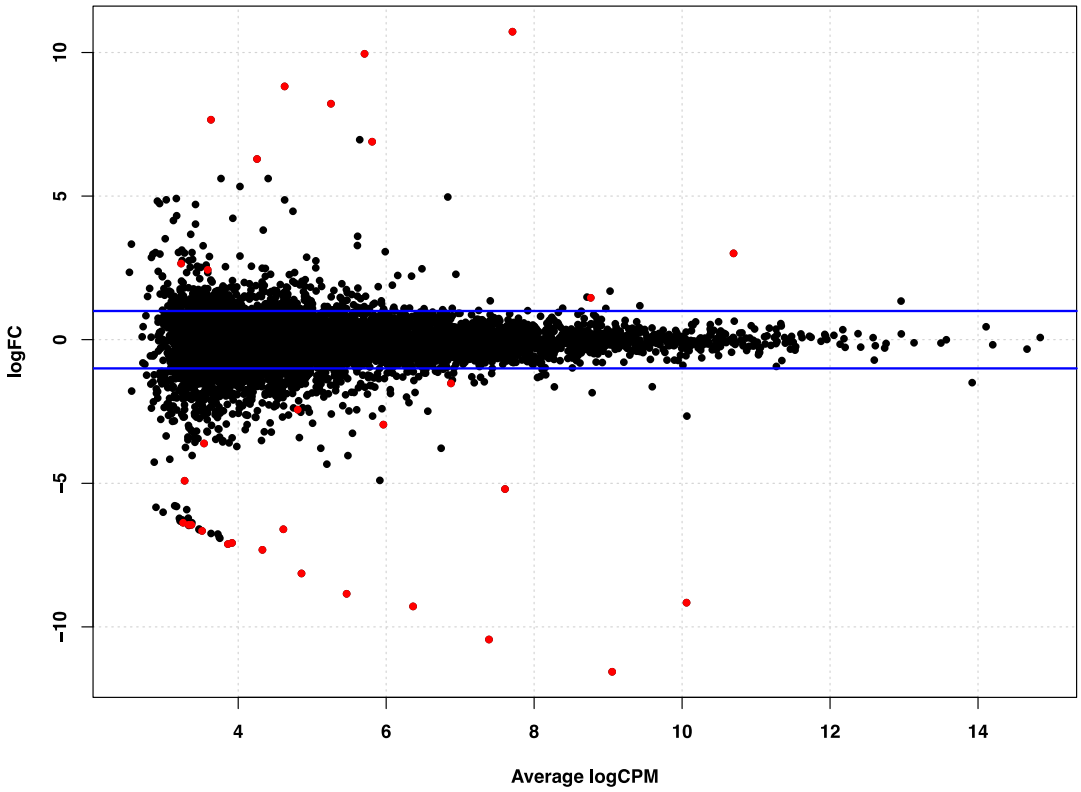

Figure 10. Smear plot of differentially expressed transcripts in testicular transposon transcripts between A1/A2 and B2 haplogroup males. Red genes are significantly differentially expressed. Blue lines indicate genes with greater than one-fold differential expression.

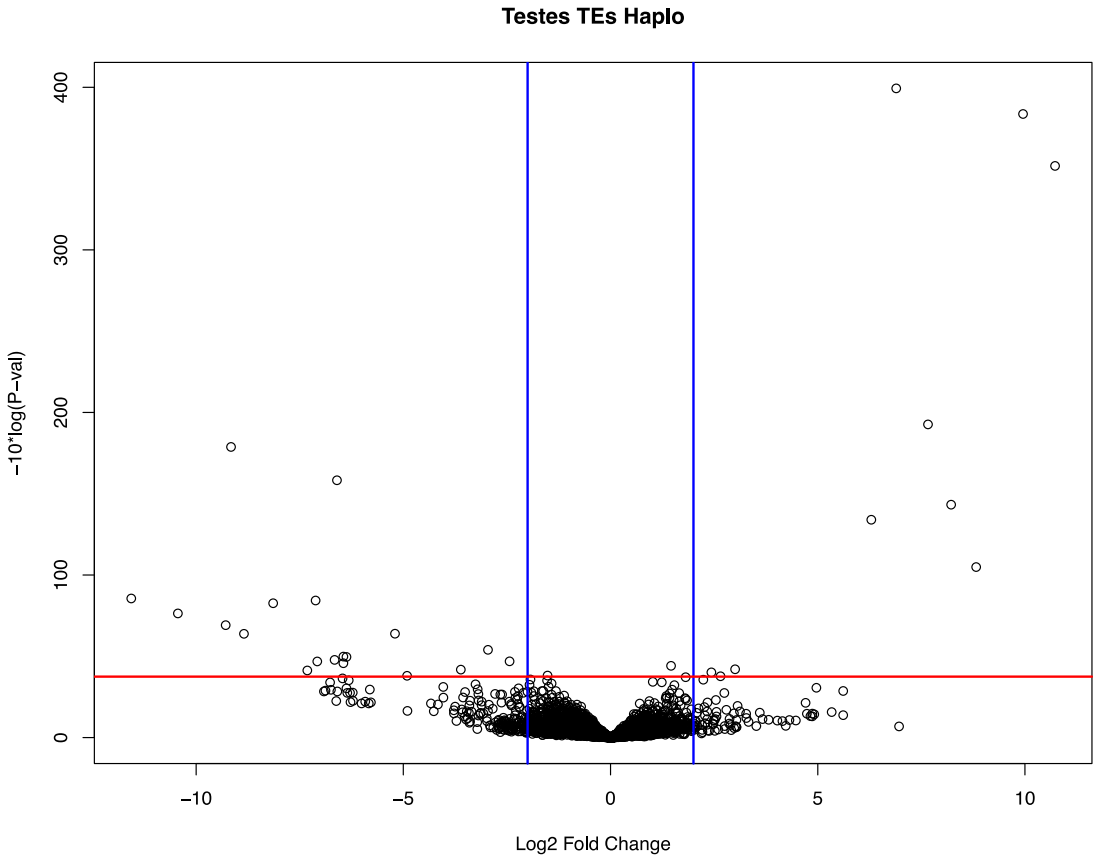

Figure 11. Volcano plot of differentially expressed transcripts in testicular transposon transcripts between A1/A2 and B2 haplogroup males. Genes above the red line are significantly differentially expressed. Blue lines indicate genes with greater than two-fold differential expression.

Testes piwi-interacting RNA Results

In testes, a total of 122 piRNA loci were expressed in one or more experimental replications. Of these, only 2 piRNAs were significantly differentially expressed,

with both exhibiting greater than one-fold downregulation in high temperature *C. scorpioides* (Fig. 12 and Fig. 13). Between haplogroups, none of the identified piRNAs were significantly differentially expressed (Fig. 14).

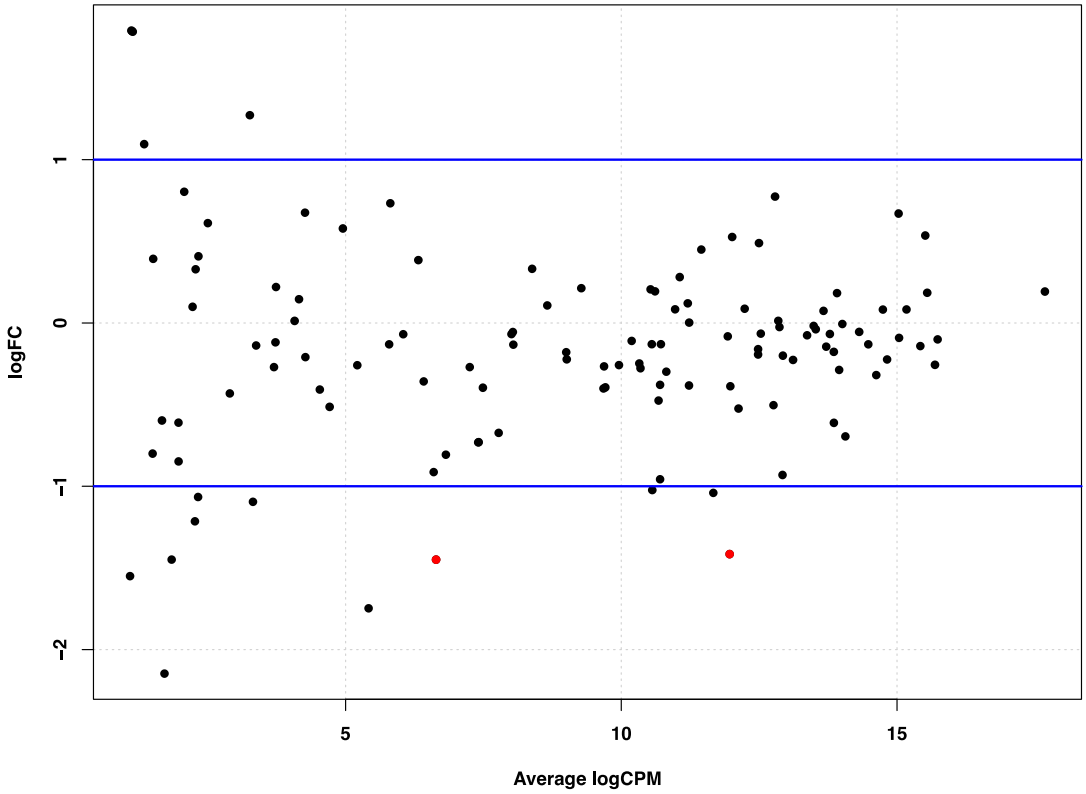

Figure 12. Smear plot of differentially expressed transcripts in testicular piRNA transcripts between control and high temperature males. Genes in red are significantly differently expressed transcripts. Blue lines indicate genes with greater than one-fold differential expression.

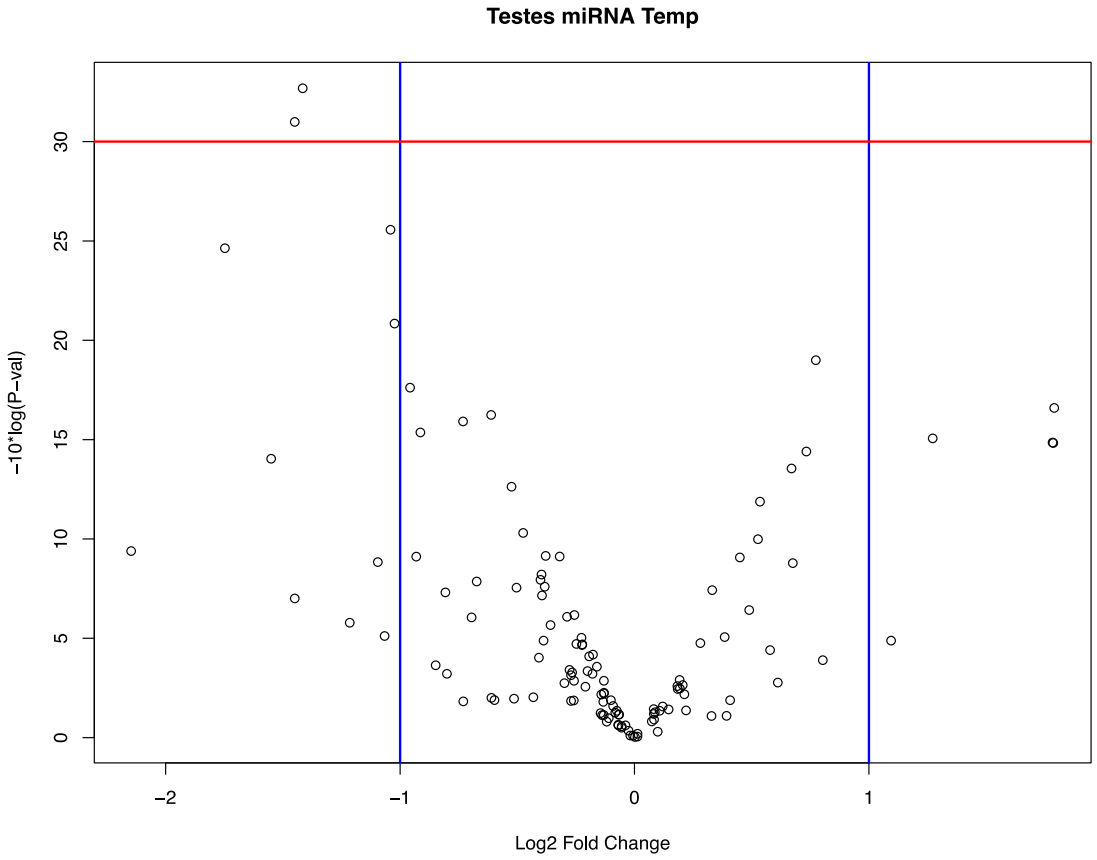

Figure 13. Volcano plot of differentially expressed transcripts in testicular piRNA transcripts between control and high temperature males. Genes above the red line are significantly differentially expressed. Blue lines indicate genes with greater than one-fold differential expression.

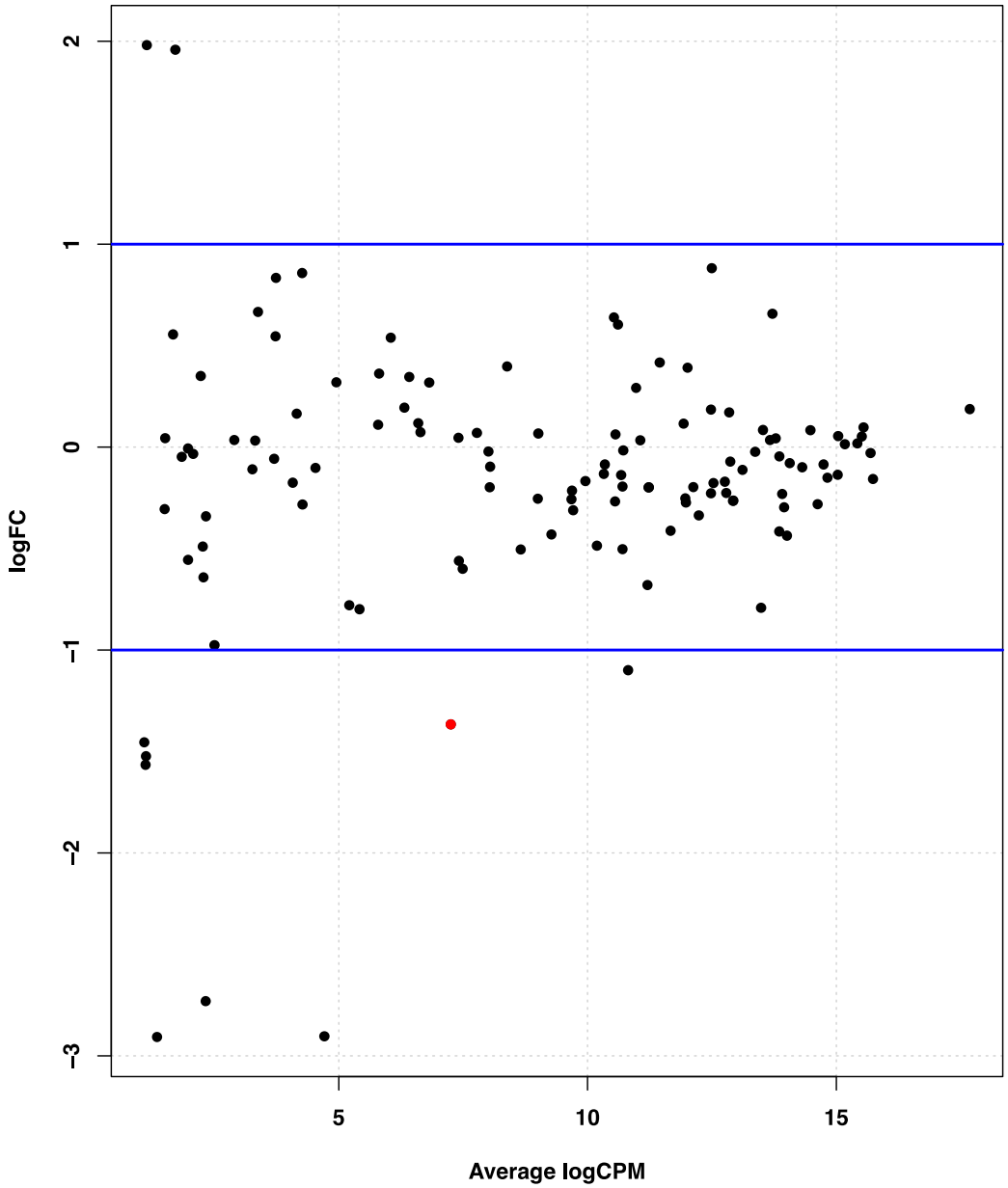

Figure 14. Smear plot of differentially expressed transcripts in testicular piRNA transcripts between A1/A2 and B2 haplogroup males. Gene in red was the

transcript with the lowest FDR value (FDR = 0.1). Blue lines indicate genes with greater than one-fold differential expression.

Sperm piwi-interacting RNA Results

Of the 102 piRNAs expressed in sperm samples between control and high temperature C. scorpioides, 17 were found to be differentially expressed, with 15 downregulated and 2 upregulated (Fig. 15). Haplogroup analysis revealed 6 differentially expressed piRNAs between the A and B2 haplogroups, with all 6 being significantly downregulated (Fig. 16).

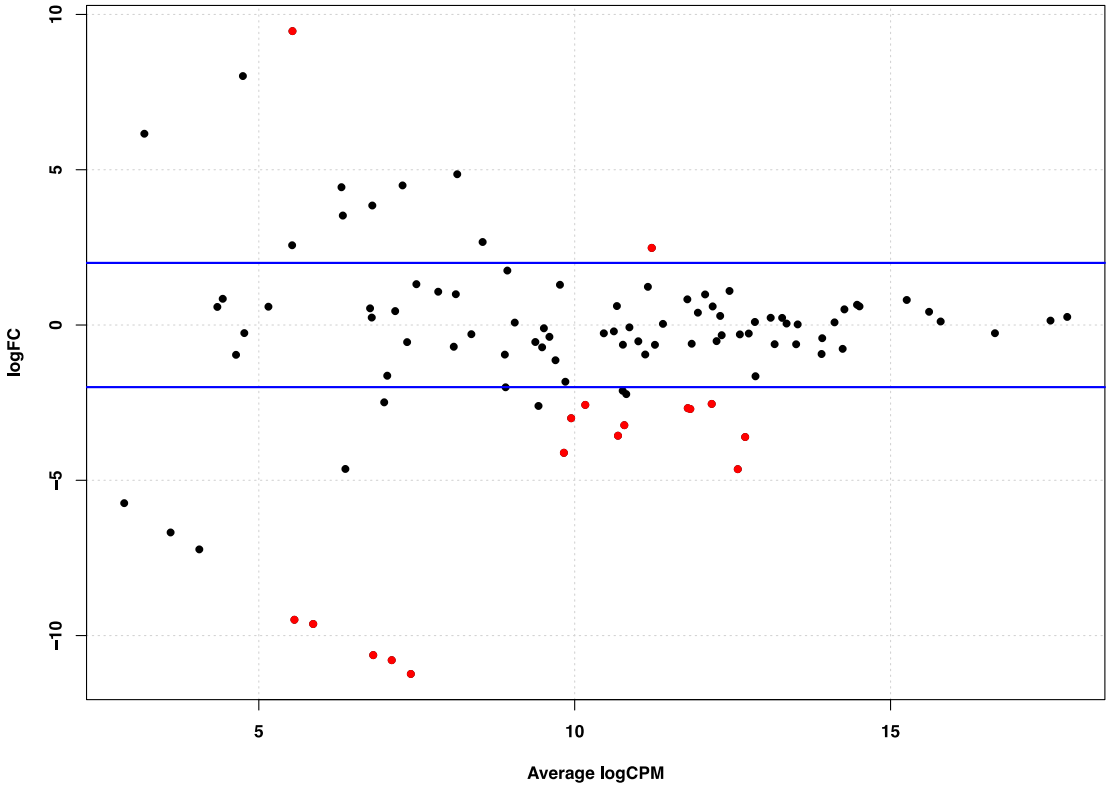

Figure 15. Smear plot of differentially expressed transcripts in spermatic piRNA transcripts between control and high temperature males. Genes colored red are significantly differentially expressed. Blue lines indicate genes with greater than one-fold differential expression.

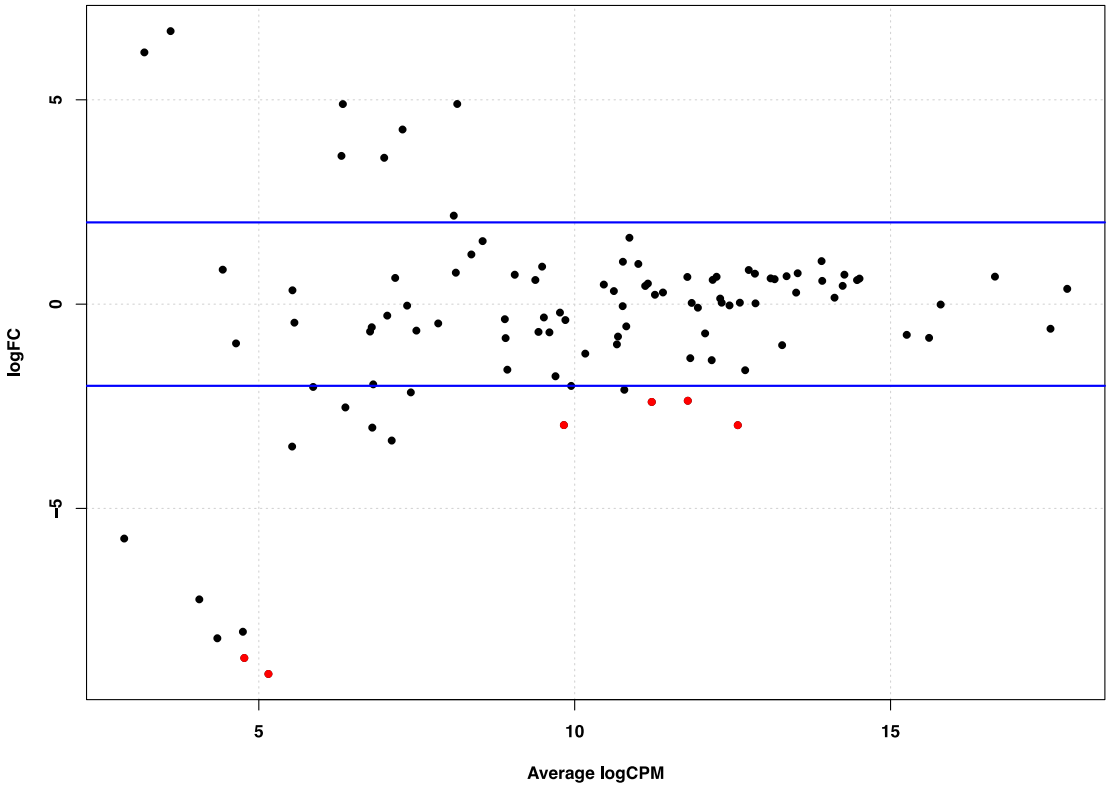

Figure 16. Smear plot of differentially expressed transcripts in spermatic piRNA transcripts between A1/A2 and B2 haplogroup males. Genes colored red are significantly differentially expressed. Blue lines indicate genes with greater than one-fold differential expression.

4 | DISCUSSION

Protein-coding genes in the testes were highly expressed in sperm, with 92.1% of all annotated genes expressed. While some upregulated proteins have been identified as resulting from heat shock, nearly 10% of upregulated genes in high

temperature-exposed males include genes related to transposon activity. Proteins derived from integrated transposons and proteins involved in the repression of active transposons, as well as proteins involved in epigenetic regulation were all upregulated. Proteins directly implicated in transposable element activity were detected in testicular tissue, with several significantly upregulated classes of transposable element transcripts identified as belonging to DNA transposons, LTR retrotransposons, and SINEs. Interestingly, temperature effects on piRNA expression in the testes was not pervasive, with only two transcripts significantly differentially expressed. However, high temperature treatment in sperm did have a major impact on piRNA expression, with downregulation of piRNA found in high temperature males. Though haplogroup analyses of testes protein-coding genes did yield significant upregulation of a protein phosphatase, potentially implicating increased histone modifications as a result of high temperature exposure, haplogroup effects were primarily undetected in protein-coding genes and piRNA expression, but the B2 haplogroup did display significant differential expression between the A1/A2 and B2 haplogroups in transposable element transcripts, with most transcripts being downregulated. Evidence suggests that the rare B2 haplotype originated at higher geographic elevations in Western Panamá, where it was subjected to cooler temperatures, thus it is likely that significant downregulation of piRNA from A1/A2 can be attributed to its evolution in cooler temperatures (Zeh, et al., 2003). The impact of this mitochondrial differential expression on transposon activity

remains to be determined, given the lack of data on the function of these transcripts.

These findings support the hypothesis that altered snRNA profiles in high temperature *C. scorpioides* have an impact on intragenerational altered epigenetic states that can be transmitted through sperm to affect the next generation. In vertebrate tissue, transposons expression and regulation mechanisms are remarkably varied throughout body tissues, except for the testes, where they exhibit consistently high expression, both from domesticated and active transposable elements (Pasquesi, et al., 2020). Heat stress has been shown to increase transposable element activity in the testes (Cappucci, et al., 2019), and has been linked to differential expression of protein-coding genes (Li, et al., 2009; Rockett, et al., 2001; Paul, et al., 2009; Wang, et al., 2015). The role piRNAs play in defending the genome against transposable elements has been well known for at least a decade (Aravin, et al., 2007; O'Donnell & Boeke, 2007; Siomi, et al., 2011). piRNAs silence transcription products by forming of an RNA-induced silencing complex (RISC) with PIWI proteins and serving as guides for the PIWI proteins to target TE mRNA, leading to cleavage of TE transcripts (Toth, et al., 2016). The PIWI/piRNA RISC plays essential roles in reproduction, with dysfunction of the piRNA/PIWI RISC, and deficiency of PIWI proteins (Dai, et al., 2019; Gou, et al., 2017; Hasuwa, et al., 2017) and piRNAs (Hong, et al., 2021) in the germline implicated in male infertility. Yet, the precise relationship between increased transposon expression and piRNA response requires further

study. Vandewege, et al. (2016), compared the evolutionary relationship between normal TE expression and associated piRNA expression in bats, horses, and dogs, and found that highly transcribed TE families in each species resulted in higher rates of piRNA activity. Current evidence on piRNA expression in response to heat stress supports the view that induction of stress-induced transposable element activity implicates loss of epigenetic control via piRNAs. In *C. elegans*, heat stress was found to induce transposable element activity in spermatocytes, resulting in infertility (Kurhanewicz, et al., 2020), with stressful environmental conditions modulating piRNA production, as exposure to heat shock resulted in reduced fitness of progeny and a downregulation of motif-dependent piRNA biogenesis. This resulted in reduced affinity of piRNA to TE transcripts and loss of piRNA-mediated silencing (Belicard, et al., 2018). Additionally, dysregulated piRNA production can lead to the mis-targeting of sequences marked for destruction, potentially causing erroneous destruction of essential transcripts (Frolows & Ashe, 2021). Here, upregulation of integrated and active transposable elements was associated with an increase of genes encoding epigenetic markers, indicating that transposon activity was increased in males exposed to heat stress, and a downregulation of piRNAs in sperm of high temperature males, indicating that inability of piRNA to regulate transposon suppression occurring in the spermatic tissue may contribute to negative intergenerational consequences of high temperature exposure experienced by the F_0 generation.

In previous work, significant temperature effects on directly treated *C. scorpioides* occurred in morphological, developmental, and reproductive traits (Bonham, 2021). Adults of both sexes with nymphal exposure to the high temperature regime developed faster and had reduced adult body size compared to adults exposed to the control temperature regime during nymphal development. High temperature-exposed females were less also likely to carry a brood to term and produced significantly less protonymphs than control females, and high temperature males had significantly reduced fertility (Bonham, 2021). The inheritance of these phenotypes varied in the F_1 generation, depending on whether the father or mother was exposed to high temperature. The strongest intergenerational effect by far in offspring with high temperature parents was the transmittance of decreased sperm count during mating (Chapter 2). Directly treated high temperature males produced a significant decrease in sperm count, with high males producing 59% less sperm. This significant decrease in sperm production was extended to the F_1 generation, as males with high temperature mothers produced 76.4% less sperm than males with control temperature mothers, and males with high temperature fathers produced 20.5% less sperm than did males with control temperature fathers. It is highly likely that transposon activation contributes to this loss of fertility function in directly treated males, resulting in inherited reduced sperm count in indirectly treated males.

One interesting pattern of intergenerational inheritance discovered was the transmission of reduced adult body size in solely male offspring through the

maternal line (Chapter 2). In the directly treated generation, high temperature exposure resulted in decreased adult body size, likely correlated with increased development time. This coupling of high-temperature-induced size dependence and increased developmental time is well established in the literature (Angilletta, et al., 2004; Gardner, et al., 2011; Sheridan & Bickford, 2011). However, in the F_1 generation, this relationship was severed. The sons, but not the daughters, of high temperature-exposed mothers were significantly smaller than their control counterparts, but did not significantly differ in developmental rate; and, the daughters, but not the sons, of high temperature-exposed fathers developed significantly faster than those of control fathers, but did not significantly differ in size. The most likely explanation for this phenomenon is due to the XX/XO system of sex determination in *C. scorpioides* (Šťáhlavský, et al., 2009). Since males have only a single X chromosome, they are more vulnerable than females to the negative consequences of disruption of X-linked gene expression, analogous to many human X-linked diseases, such as hemophilia A (Graw, et al., 2005), red-green color blindness (Neitz & Neitz, 2000) and Duchenne muscular dystrophy (Duan, et al., 2021). These diseases result from mutations in the DNA sequence of genes on the X chromosome, and, while females are capable of exhibiting effects of these mutations, disease phenotypes are largely restricted to males. However, unlike human counterparts, the cause of reduced size in the sons of high females observed in the intergenerational study was likely due to X-linked mutations in EMM, rather than traditional genetic mutations. Induced

changes in epigenetic regulation of X-linked gene expression that influence growth in the somatic and germline cells of exposed individuals occurred in the sons of dams exposed to high temperature during development, whereas daughters of high temperature dams exhibited no reduction in size due to the rescuing effect of the X chromosome they inherited from their control father. This interpretation is supported by preliminary RNA sequencing studies that have revealed significant differential X-linked gene expression of 115 genes in embryos with high temperature mothers, with 36.5% of genes related to transposon activity and the majority of those undergoing upregulation.

Despite the overwhelmingly negative effects in the directly treated generation, especially on reproduction (Bonham, 2021), several significant intergenerational effects were positive, including reduced female developmental time, faster mating initiation by males, and, most significantly, increased female reproductive success (Chapter 2). F_1 females sired by high temperature males gave birth to 22% more protonymphs than females sired by control males. Perhaps the most likely hypothesis for this fitness enhancement is selection, in which only the most genetically- or epigenetically-fit F_0 high temperature males, capable of increasing piRNA transcription and piRNA/PIWI RISCs to combat transposon activity, were able to successfully sire offspring. This suggests that, while climate change has an overall detrimental impact on *C. scorpioides* reproduction, some males may possess the epigenetic prowess to successfully adapt to new conditions and transmit those benefits to offspring.

Species in highly heterogeneous environments, such as arthropods, benefit greatly from intragenerational epigenetic mechanisms that allow them to rapidly change phenotype in response to environmental cues (Burggren, 2016), such as in the well-documented case of polyphenism of eusocial insects. In honeybees, queen and worker bees share genetic sequences, though they differ substantially in phenotype. Queen bees are larger than worker bees, and capable of reproduction, whereas worker bees have superior eyesight and can secrete royal jelly. Differentiation between worker castes and queen bee castes has been attributed to differential gene expression during the larval stage, with nutrition/diet (worker jelly versus royal jelly) causing differential methylation of the genome (Patalano, et al., 2012; Wang, et al., 2020). Whether transgenerational effects from epigenetic mutations in arthropods can be adaptive in the face of climate change is yet to be determined, as there's a discernable lack of studies involving the capacity for adaptive hereditary epigenetic effects in response to high temperature-induced stress (Hu & Barrett, 2017). Overall, a significant number of epigenetic factors have been found to cause transgenerational inheritance of diseased phenotypes. These include toxins, such as plastics and pesticides, nutrition, and environmental factors such as drought and temperature (Skinner, 2014). Theoretical evidence for the potential of adaptive transgenerational inheritance suggests that non-genetic inheritance can be adaptive under environmental conditions that change from generation to generation but maintain stability within generations (Yin, et al., 2019); and that parental effects produce

the strongest adaptive impact when offspring environment is auto-correlated with parent environment (Ezard, et al., 2014; Herman, et al., 2014). As anthropogenic change increases stochasticity in seasonal temperatures and precipitation, tropical regions face increasingly unpredictable futures, though males leave a legacy of their environment through germline epigenetic modifications. The adaptive potential of transgenerational inheritance and correlated epigenetic mechanisms influencing offspring fitness and associated transposon expression may be reduced to disastrous effects (Burgess & Marshall, 2014; Colicchio & Herman, 2020; Donelan, et al., 2020; McGuigan, et al., 2021). Nevertheless, plastic epigenetic changes are much more sensitive to environmental perturbations than is the genome itself (Furrow, et al., 2011). The frequency of epigenetic mutations is five orders of magnitude higher than that of genetic mutations (Skinner & Nilsson, 2021), therefore EMM provide a more direct way of inducing rapid phenotypic change in response to rapid environmental change (Bonduriansky & Day, 2009; Colicchio & Herman, 2020; Harrisson, et al., 2014). Empirical evidence is mixed, however, with adaptive responses being context-dependent. A meta-analysis by Yin et. al. (2019), found that transgenerational effects generally enhanced offspring performance in response to both favorable and stressful environments, and persisted over three successive generations.

Epigenetics retains the potential to not only cause intergenerational and transgenerational phenotypic change, but as a precursor for genetic evolution. Short-term plastic adaptations may result in selection of novel phenotypic traits,

leading to genetic mutations, a theory termed “genes as followers” (Nilsson, et al., 2018). Research has found that genetic mutations often require an “epigenetic precursor,” with EMM shown to increase the frequency of point and copy number mutations in the gene code (Skinner, 2015). Adaptive radiations also provide an example for EMM-mediated evolutionary change, as discovered, for example, in the adaptive radiation of Darwin’s finches in the Galapagos islands, where epigenetic variation, but not genetic variation, observed between species correlated with phylogenic relatedness (Skinner, et al., 2014). In addition, despite their negative effects in the individual, transposable elements can be beneficial on a population level as powerful facilitators of evolution. Loss of EMM in the control of transposable elements is capable of inducing wide-scale genomic re-structuring (Oliver & Greene, 2009; Wu & Lu, 2019), and massive duplication or recombination events initiated by transposon activity in germline cells are also known drivers of evolution (Cordaux & Batzer, 2009; Oliver & Greene, 2009; Viviani, et al., 2021).

Transposable elements can bring about a variety of substantial genomic changes through chromosomal rearrangements, gene duplication events, or rapid genome-wide dissemination of gene regulatory elements. Mutated domesticated transposons can contribute to the repertoire of functional protein-coding genes over evolutionary time. These large scale genetic events are subject to selection and can lead to major evolutionary change, facilitating speciation and taxon radiation as well as adaptation to survival in new habitats

(Lanciano & Mirouze, 2018). Consequently, while advantageous transgenerational epigenetic change can occur to potentially increase offspring fitness, disadvantageous epigenetic change may pave the way for transposon re-structuring events in the germline that allow species to reach new fitness peaks. Perhaps, therefore, hope exists for the future of this pseudoscorpion after all.

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Mitochondrial haplogroup mediates temperature effects on the gut microbiome of a model tropical arthropod

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Abstract

Host-microbe interactions are essential to many aspects of host health, with imbalances in the microbiome (dysbiosis) linked to multiple diseases. Gut microbial composition is highly variable across taxa and shaped by host and environmental factors. Host factors include not only nuclear genes, but also variation in mitochondrial DNA sequence, as reactive oxygen species (ROS) production during ATP synthesis correlates with microbial diversity. Short-chain fatty acids are produced by bacteria in the intestinal lumen and mediate host-microbiota interactions by modifying epigenetic states and influencing host gene expression. Environmental factors that influence the gut microbiome include diet and temperature. By midcentury, global temperatures are predicted to increase by 2ºC, threatening the stability of host/microbial symbioses. Terrestrial arthropods comprise the majority of species, but data are lacking on tropical arthropods likely to be disproportionately affected by climate change. Predicting the future of tropical ectotherms requires better understanding of the impact of elevated temperature on their microbiomes. Here, I report the effects of a 2.5ºC increase in ambient temperature on the gut microbial community of the neotropical pseudoscorpion, *Cordylochernes scorpioides*, a viviparous, predatory ectotherm exhibiting high mitochondrial variation, with three haplogroups (A1, A2, and B2) co-occurring in central Panamá. A factorial design with two temperature treatments and three haplogroups was used to investigate temperature,

haplogroup, and interaction effects on the diversity and community composition of the *C. scorpioides* microbiome. Elevated temperature was associated with increased microbial diversity but mitochondrial haplogroup accounted for the most pronounced effects, with higher diversity in A1/A2 haplotypes over B2 haplotypes. Twenty bacterial species differed significantly in abundance due to haplogroup by temperature interactions. Lack of appreciable temperature effects on the *C. scorpioides* microbiome coupled with significant haplogroup effects suggest that microbial composition changes are not responsible for detrimental phenotypic effects of a 2.5ºC temperature increase. Previous data suggest that high temperature-induced phenotypic disruption in this pseudoscorpion is largely due to epigenetic dysregulation. However, this does not exclude the possibility of gut microbial importance, since even brief exposure to some microbial species can induce long-lasting epigenetic changes.

1 | INTRODUCTION

Whereas historic perceptions of bacteria were largely negative with a focus on microbes as the primary cause for disease, attitudes have shifted in the past several decades as research into beneficial host-microbe interactions has gained traction. Indeed, host-microbe interactions are essential to many aspects of host health and, ironically, the prevention of disease (Dethlefsen et al., 2007). Microbial taxa that colonize animal species are collectively described as the microbiome, and include not only bacteria but also viruses and archaea, as well as eukaryotes, such as protozoan and fungal microbes (Colston & Jackson, 2016; McFall-Ngai et al., 2013). Humans, like all animal species, carry communities of microbes both on and within their bodies, particularly in the gastrointestinal tract and, in humans, gut microbiome diversity and composition have implications for a range of conditions. On the one hand, diverse and resilient “healthy” microbiomes play a vital role in host metabolism, physiology, nutrition, immune function and defense against pathogens (O’Hara & Shanahan, 2006; Sekirov et al., 2010). On the other hand, dysbiosis, that is, imbalance in the gut microbiome, has been linked to a variety of diseases, including metabolic disorders (Clemente, et al., 2012; Tremaroli & Backhed, 2012; Turnbaugh, et al., 2007), immune disorders (Levy, et al., 2017; Round & Mazmanian, 2009; Schippa & Conte, 2015), neurodegenerative disease (Cryan & Dinan, 2012;

Fung, Olson, & Hsiao, 2017; Sampson, et al., 2016), and psychological disorders (Leclercq et al., 2016; Luna & Foster, 2015).

Growing evidence suggests that crosstalk between the gut microbiome and the host is mediated by microbial-derived molecules acting as signals that induce changes in the complex system of epigenetic mechanisms (DNA methylation, histone modifications and non-coding RNAs) that regulates host gene expression (Louwies et al., 2020; Woo & Alenghat, 2017; Ye et al., 2017). For example, Kumar et al. (2014) found a strong correlation in pregnant women between the composition of their gut microbiomes and the DNA methylation profiles in their blood, while Heinonen et al. (2016) demonstrated that short-chain fatty acids generated by human gut microbiota inhibit histone deacetylase activity. Short-chain fatty acids, such as propionate, acetate and butyrate, are produced by bacteria in the intestinal lumen, and are increasingly recognized as major players in mediating host-microbiota interactions through their influence on the epigenome (Woo & Alenghat, 2017).

The composition of gut microbial communities is shaped by both host and environmental factors (Qin et al., 2022) and is highly variable both across animal taxa and between individuals within host species (Rudolph, et al., 2022; Van Rossum, et al., 2020; Youngblut, et al., 2019). Host factors associated with microbiome change include not only nuclear genes (Blekhman et al., 2015; Goodrich et al., 2017; Kreznar et al., 2017) and gender (Valeri & Endres, 2021) but also variation in mitochondrial DNA sequence (Aguilar-López et al., 2020).

Recent studies investigating the relationship between mitochondrial haplotype and the microbiome have found that reactive oxygen species (ROS) production, a byproduct of the oxidative phosphorylation process to generate ATP (Ballard & Towarnicki, 2020), correlates with microbial diversity (Yardeni, et al., 2019) and gut microbiota regulate key transcriptional co-activators involved in mitochondrial biogenesis through modulation of intestinal barrier function and mucosal immune responses (Clark & Mach, 2017). Other host factors that influence the microbiota are age and reproductive status. In elderly people over the age of 65 years, the core gut microbiota and level of diversity differ significantly from those of younger individuals (Biagi et al., 2010; Claesson et al., 2011). Similarly, pregnancy is associated with changes in gut microbiota composition and reduced microbial diversity in humans (Koren et al., 2012), laboratory mice (Elderman et al., 2018) and lizards (Trevelline et al., 2019). Among the environmental factors that shape gut microbiota, diet is considered to be one of the most important, with dietary effects on the microbiome documented in a broad range of hosts, such as ants (Sanders et al., 2017), spiders (Kennedy et al., 2020), fish (Ringø et al., 2016), voles (Li et al., 2021), mice (reviewed in Beam, Clinger & Hao, 2021) and humans (Latorre-Pérez et al., 2021).

Ambient temperature is another important environmental factor known to have an impact on gut microbiome composition and diversity (reviewed in Sepulveda & Moeller, 2020), and increased temperature is likely to pose an increasingly serious threat to the stability of host/microbial symbiont relationships

in the coming decades. The most recent report of the International Panel on Climate Change (IPCC 2021) predicts that temperatures will undergo an increase of 2ºC by midcentury, if zero efforts are taken towards mitigation of greenhouse gas emissions, and could potentially increase by 5.7ºC as a worst case scenario, if emission rates substantially increase globally. Terrestrial arthropods comprise the vast majority of species on Earth (Erwin, 1982), and are likely to be disproportionately affected by climate change. In ectotherms, temperature influences the rate of basic metabolic functions, and a single unit increase in temperature results in an exponential increase in metabolism (Dillon, et al., 2010; Gillooly, 2001; Klok & Harrison, 2013). This exponential relationship has major implications for tropical ectotherms, since they experience low seasonal variability in temperature and already live close to the upper limit of their thermal tolerances (Addo-Bediako, et al., 2000; Deutsch, 2008). Tropical arthropods have thus far responded to hotter conditions through behavioral adaptations such as phenology shifts (Forrest, 2016) and elevational range redistributions (Chen, et al., 2011), but a substantial increase in mean temperature will put tropical arthropods at risk of extinction. Species engaged in obligate mutualisms with their microbial symbionts are particularly vulnerable (Corbin et al., 2017; Kikuchi et al., 2016; Moran, 2016; Wernegreen, 2012; Wernegreen, 2017). However, there is currently a critical lack of data for arthropods inhabiting tropical regions (Renoz, et al., 2019). For some arthropod species, exposure to increased temperatures disrupts microbial activity causing dysbiosis. For example, the

bacterial species *Buchnera aphidicola* is an obligatory symbiont of aphids that is passed vertically and is responsible for providing nutrients to its host. Heat exposure causes the loss of *Buchnera*, resulting in decreased fecundity of the aphid host (Montllor, et al., 2002; Ohtaka & Ishikawa, 1991). Prado et. al. (2009) exposed the pentatomid bug *Nezara viridula* to higher temperatures and found substantial loss of its vertically transmitted gut symbiotic community, with this loss correlated with reduced development time and reduced fertility, but increased life expectancy. Consequently, predicting future responses of tropical ectotherms to climate warming requires more data and a better understanding of the effects of increased temperature on their gut microbiota.

Here, I report the results of a study that investigated the impact of a 2.5ºC increase in ambient temperature on the gut microbial community of the neotropical pseudoscorpion, *Cordylochernes scorpioides*, a viviparous, predatory ectotherm distributed throughout the rainforests of Central and South America (Zeh et al., (2003). *Cordylochernes scorpioides* directly exposed to an increase of 2.5ºC from ambient temperature in their native habitat during early stages of development experienced significant negative phenotypic effects, including reduced rate of survival, reduced adult body size, and severe handicaps in reproductive abilities. More specifically, males exposed to high temperatures experienced a 45% reduction in number of sperm produced and were overall 32% less successful at siring offspring compared to control temperature males, and females that developed at the higher temperature experienced a 12.5%

reduction in fecundity compared to control females and were 30% less likely to produce offspring. This arthropod species also exhibits substantial mitochondrial diversity, with differences between mitochondrial haplogroups having significant impacts on male reproductive function. While these detrimental phenotypes due to high temperature can be induced through other plastic means, such as epigenetic modifications, reduced microbial diversity in *C. scorpioides* exposed to high temperatures may have the potential to affect maladaptive phenotypic change.

2 | METHODS

2.1 | The Study Organism

Cordylochernes scorpioides is a small, flightless arthropod that inhabits decaying trees in the families, Moraceae and Apocynaceae, and uses the giant harlequin beetle, *Acrocinus longimanus*, as its dispersal agent (Zeh & Zeh, 1992). This pseudoscorpion is highly amenable to rearing in the laboratory, produces large numbers of offspring and has a unique reproductive biology that makes it a model organism for non-invasive study of the effects of climate warming on a wide range of developmental, morphological and reproductive traits. Males of this species transfer sperm to the female by means of a discrete stalked spermatophore, placed directly on the substrate to be retrieved by the female.

Females display an “external womb” form of viviparity, where developing embryos are nourished by the mother in a transparent brood sac overlying her genital aperture. This species also exhibits high levels of mitochondrial variation, with three mitochondrial haplogroups, A1, A2, and B2 co-occurring in populations in central Panamá. Identified through next-generation sequencing, the A1 and A2 haplogroup sequences diverge by 1.9% from each other across the mitochondrial genome, and by 8% from the B2 haplogroup (Padua, et al., 2014; Zeh et. al., unpublished).

Pseudoscorpions for this study were descended from 297 individuals collected from seven decaying *Ficus* trees in Central Panamá in July 2017 (for locations, see Chapter 1). A laboratory population was perpetuated by outcrossing isofemale lines randomly with respect to mitochondrial haplogroup for multiple generations, thereby establishing a relatively uniform nuclear genome across haplogroups. Individuals for this experiment were generated as described elsewhere (see Chapter 2), as part of a larger study aimed at investigating the effects of a 2.5ºC increase on developmental, morphological and reproductive traits in *C. scorpioides*. Briefly, virgin females from the laboratory population were randomly mated to an unrelated male to generate A1, A2 and B2 haplogroup families. A split-brood rearing design was then used, in which protonymphs (first-stage nymphs) from each family were randomly assigned at birth, 20 to a control (C) incubator and 20 to a high (H) incubator. The control temperature diurnally-oscillating regime was based on temperature data collected by iButton loggers

attached to the seven decaying trees from which the founding members of the *C. scorpioides* laboratory population were collected (see Chapter 1). The high temperature regime involved an increase of 2.5ºC above the control regime. Pseudoscorpions go through three nymphal (protonymph, deutonymph and tritonymph) molts prior to the adult stage, at which point the pedipalps and cephalothorax become fixed in size and gender is morphologically visible (Weygoldt, 1969). Nymphs were reared in individual vials and fed a weekly diet of *Drosophila melanogaster* larvae during the first two nymphal stages, and then switched to *Tribolium confusum* larvae for the tritonymph and adult stages, as described elsewhere (Zeh et. al., 2005). Development time to adulthood at the high (+2.5ºC) temperature typically takes a minimum of 31 days, so daily visual inspection of vials for adult emergence commenced 28 days after birth. After developing to the adult stage, individuals were sexed and one high-temperature and one control female from each of three A1, three A2 and three B2 families (N = 18 females) were selected and stored at -80 ºC for subsequent DNA extraction.

2.2 | Sequencing and Diversity Analysis

A 2x3 factorial design with two temperature treatments (C and H) and three mitochondrial haplogroups (A1, A2 and B2) was used to investigate temperature, haplogroup and temperature by haplogroup effects on the diversity and community composition of the *C. scorpioides* microbiome. There were three

replicates per treatment for a total of 18 samples, with each individual per treatment randomly selected for microbial analysis. Genomic DNA was extracted from each sample using the Invitrogen Chargeswitch gDNA Micro Tissue Kit (ThermoFisher Scientific, MA, USA) and NanoDrop 2000/2000c spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA) was used to quantify the A260 ratio of genomic DNA present for each sample. Genomic samples were stored in 98 ul volume of 10mM Tris-HCl buffer at -20ºC and mailed to RTL Genomics (Lubbock, TX, USA) for PCR amplification and sequencing. Assays chosen for amplification correspond to the V1-V3 region of the bacterial 16S rRNA gene and the internal transcribed spacer (ITS) region of the nuclear genome for fungi, using, respectively, 28F primers (28F - GAGTTTGATCNTGGCTCAG forward and 519S – GTNTTACNGCGGCKGCTG reverse) and ITS1 primers (ITS1F - CTTGGTCATTTAGAGGAAGTAA forward and ITS2aR GCTGCGTTCTTCATCGATGC reverse). Library preparation and next-generation RNA sequencing were carried out by RTL Genomics. Sequencing was performed for each sample on an Illumina Miseq using 10k paired-end reads, and results were processed in-house. Raw sequencing reads were merged, quality trimmed using RTL Genomics in-house algorithm, then sent through Robert Edgar's USEARCH (Search and clustering orders of magnitude faster than BLAST, 2010) to cluster unique sequences. Large numbers of clusters were classified into OTUs using UPARSE OTU selection algorithm (Edgar, 2013) by first removing chimeric sequences using UCHIME (Edgar, et

al., 2011). Sequences were aligned with consensus sequences to correct base-pair call errors and controlled based on primer length and expected alignment and condensed into a single FASTA file with quality scores for microbial diversity analysis. Taxonomic identification was performed through a combination of USEARCH global search algorithm on an internally maintained NCBI database of both bacteria and fungi and an internally developed Python program to determine taxonomic assignment, as described by Nicholas Bokulich (Bokulich, et al., 2015). A table was developed in which each cluster was matched with taxonomic identification to create an OTU table, resulting in a trimmed and full file with taxonomic information for each cluster, containing the number of sequences for each match, the percentage for each taxonomic match per sample, and the corresponding trimmed versions.

2.3 | Alpha Diversity and Differential Abundance Analyses

Various diversity and differential abundance analyses were performed in R v4.03 (R Core Team, 2020) by the Nevada Bioinformatics Center. Rarefaction curves were obtained to determine whether bacterial samples from each pseudoscorpion were adequately sampled. Measures of alpha diversity, including Chao, Shannon, and Simpson diversity indices, were calculated and plotted; most abundant phyla for temperature and haplogroups were plotted; and

significantly different genera between H and C and A1, A2 and B2 haplogroups were calculated and plotted according to relative aggregated abundance.

Principal component coordinates were obtained and visualized for both bacterial and fungal datasets to determine the extent to which haplogroup or temperature treatment samples clustered. Next, top genera were calculated from both bacterial and fungal data based on aggregated relative abundance. Significantly different genera were determined by performing a Kruskal-Wallis test of significance to determine whether aggregated relative abundance could be explained by differences in haplogroup or temperature. A pairwise Wilcox test was then performed on the list of genera to determine which treatments differed from each other. Resulting p-values for the different genera were adjusted using the Benjamini-Hochberg method for multiple comparisons. The top 10 most abundant genera for both temperature and haplogroup and the top 10 relative aggregated abundances for temperature and haplogroup were calculated and plotted and heatmaps of differences between haplogroups and temperature were generated using log-transformed values of aggregated relative abundance.

DESeq2 (Love, Huber, & Anders, 2014) is primarily used for determining significantly different levels of gene expression using read counts, but here was adapted for differential bacterial sequence counts using a Likelihood Ratio test, as described in Love et al. (2014). The Adonis test in the R vegan package (Oksanen, et al., 2020) was used to perform further differential count analysis with a Permutational Multivariate Analysis of Variance, which uses distance

matrices. Analysis of fungal diversity was also calculated by determining aggregated relative abundance. The top 8 most abundant fungal genera for both haplogroup and temperature treatment were plotted and relative aggregated abundances of fungal genera were plotted by temperature and haplogroup.

3 | RESULTS

A total of 403,400 sequences were obtained and used for taxonomic analysis. Of these sequences, 373,233 were classified into bacterial OTUs, for 115 distinct taxa between the haplogroup and temperature treatment replications. Unknown or unclassified bacterial sequences represented 30,167 of the total sequences generated. One sample was removed from further analysis due to suspected contamination, as visualization revealed that it was the only sample to contain a large bloom of the genus *Kocuria* (2.01% of reads). Rarefaction curves plotted for each *C. scorpioides* sample demonstrated that the amount of reads taken adequately captured bacterial diversity.

The bacterial microbiome was dominated by the phylum *Proteobacteria,* with a median aggregated relative abundance of 73.1%, followed by *Actinobacteria* (3.53%) and *Bacteroidetes* (3.26%) (Fig.1). When classified by genus, the most abundant taxa discovered were Acetobacter (39.1%), *Pseudomonas* (16.5%), and unclassified reads (8.3%) (Fig. 2). The next ten taxa identified, *Arthrobacter, Luteimonas, Serratia, Lactobacillus, Acinetobacter, Sphingobacterium,*

Chitinophaga, Comamonas, Elizabethkingia, and *Mucilaginibacter*, were all present in relative abundances between 5% and 1% and the remaining 102 taxa identified were present in abundances below 1%.

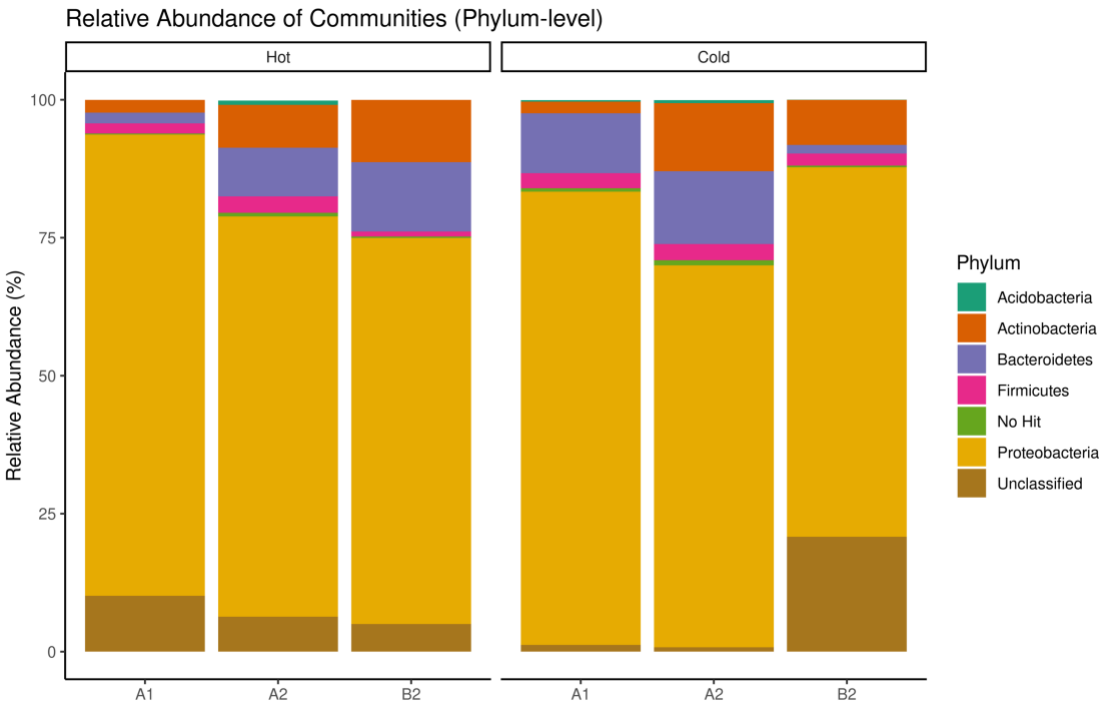

Figure 1. Relative bacterial abundance of each haplogroup by temperature for observed phyla.

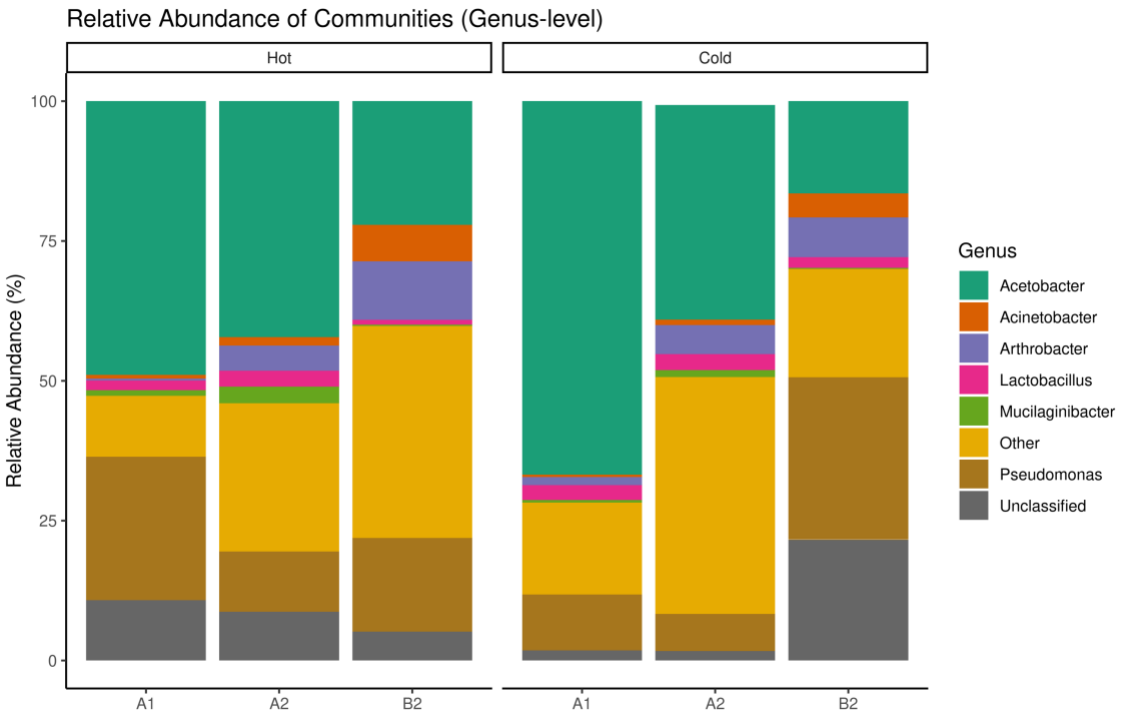

Figure 2. Relative bacterial abundance of each haplogroup by temperature for observed genera.

3.1 | Effects of Temperature on Community Composition of *C. scorpioides* Microbiota

There were six significantly different genera between temperature treatments according to the Kruskal-Wallis test of significance (Table 1), and all of these significantly different genera were relatively rare (< 5% relative abundance). None of the top 10 most abundant genera was significantly different between temperature groups (Fig. 3). DESeq2 analysis of differentially abundant bacteria

revealed 20 significantly differentially abundant taxa between haplogroup and temperature treatment interactions (Fig. 4). Of these genera, *Acetobacter, Pseudomonas, and Sphingobactium* were the most highly over expressed between interaction groups, while most species were significantly under expressed. A multivariate PERMANOVA analysis did not reveal any statistically significant relationships between temperature treatments, haplogroups, or temperature by haplogroup interactions, with temperature explaining 2% of total observed variance (P = 0.971), and the temperature by haplogroup interaction explaining 7% of variance (P = 0.893) (Fig. 5).

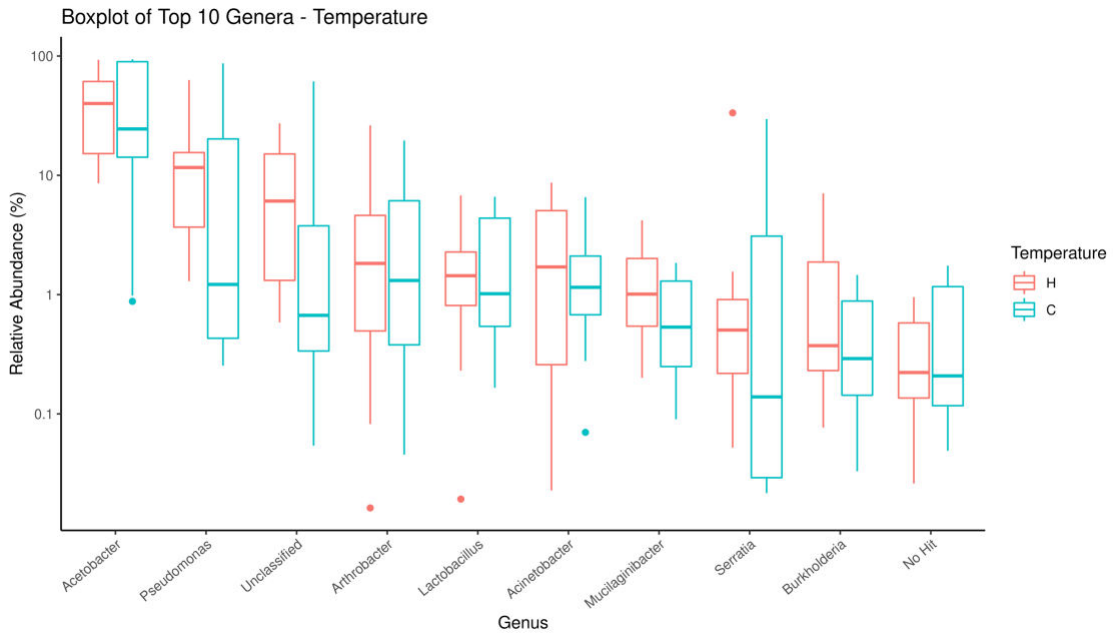

Figure 3. Box and whisker plot of top 10 bacterial genera by aggregated relative abundance for each temperature group.

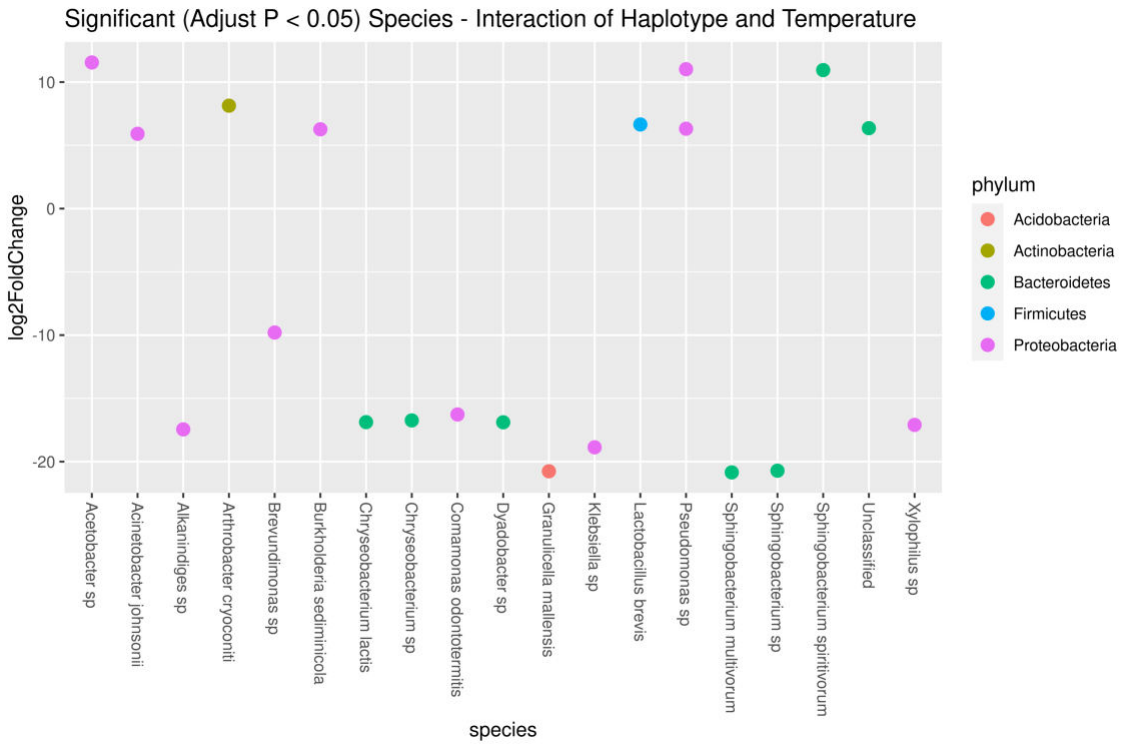

Figure 4. Figures lists log fold differences for the twenty significantly differentially abundant genera due to temperature and haplogroup interactions. Abundance values for genera was log transformed for comparisons.

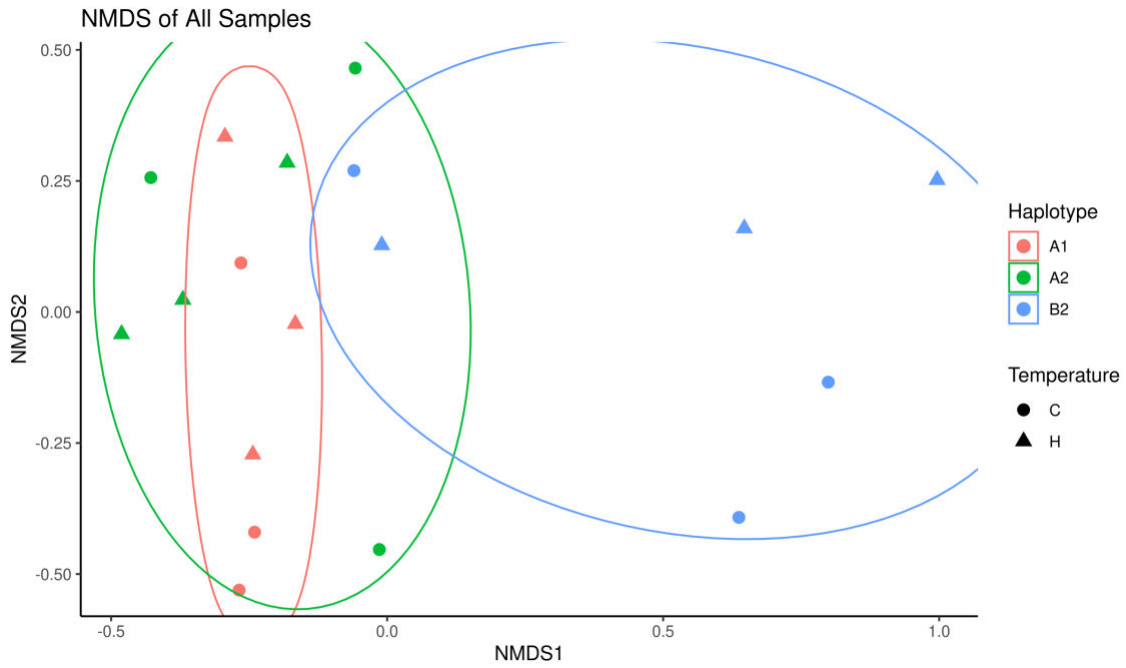

Figure 5. Multivariate PERMANOVA analysis results for haplogroup and temperature aggregations. Temperature treatments cluster randomly, but the B2 haplogroup forms a clear, distinct cluster from either the A1 and A2 haplogroups.

Table 1. Differently abundant genera between temperature comparisons according to Kruskal-Wallis.

Genus	Group 1	Group 2	Average Abundance	Adjusted P value
Achromobacter	H	C	0.458517262	0.042078
Elizabethkingia	H	C	1.135041229	0.015932
Janthinobacterium	H	C	0.054031934	0.035418
Legionella	H	C	0.017623296	0.045676

Variovorax	H	C	0.661534826	0.047849
Xylophilus	H	C	0.104807353	0.033861

Table 2. Multivariate PERMANOVA results between temperature treatments, haplogroups, and temperature and haplogroup interaction.

	Df	Sum of Squares	R2	P-value
Temperature	1	.0955	.01920	.9710
Haplogroup	2	.9059	.18204	.1415
Temperature x Haplogroup	2	.3394	.06819	.8936
Residuals	12	3.6357	.73057	
Total	17	4.9766	1.0000	

3.2 | Effects of Haplogroup on Community Composition of *C. scorpioides* Microbiota

There were 12 significantly different genera between haplogroups according to the Kruskal-Wallis test of significance (Table 3). *Acinetobacter, Chryseobacterium, Pedobacter, Pelomonas*, and *Salinibacterium* were all

significantly differentially abundant for more than one haplogroup comparison set. Of the top 10 most abundant genera identified, three genera, *Acinetobacter*, *Mucilaginibacter*, and *Serratia* were significantly differentially abundant between haplogroups (Fig. 6). None of the significantly different genera overlap between temperature and haplogroup comparisons.

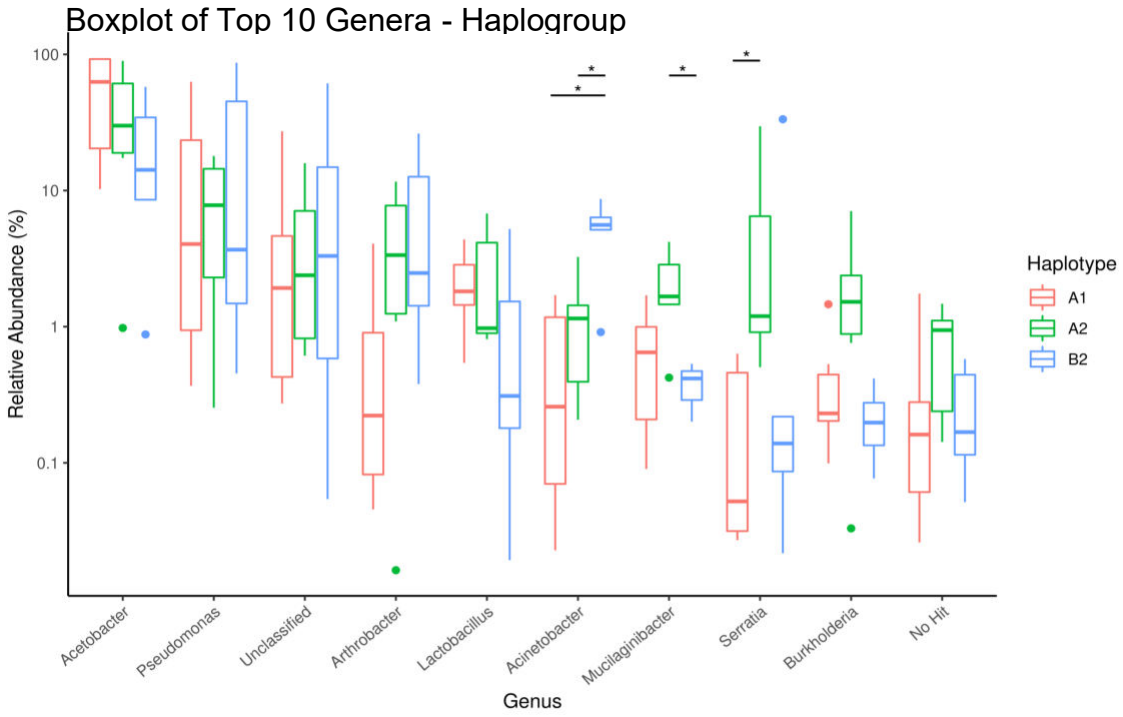

Figure 6. Box and whisker plot of top 10 bacterial genera by aggregated relative abundance for each haplogroup. Asterisks indicate significantly different abundance between haplogroups.

Table 3. Differently abundant genera between haplogroup comparisons according to Kruskal-Wallis.

Genus	Group 1	Group 2	Average Abundance	Adjusted P value
Acinetobacter	B2	A1	2.183374296	0.025974
Acinetobacter	B2	A2	2.183374296	0.038961
Chryseobacterium	A2	A1	0.700603453	0.038961
Chryseobacterium	B2	A2	0.700603453	0.013011
Mucilaginibacter	B2	A2	1.044086373	0.023387
Pedobacter	B2	A1	0.24070683	0.008335
Pedobacter	B2	A2	0.24070683	0.042661
Pelomonas	B2	A1	0.312152646	0.028865
Pelomonas	B2	A2	0.312152646	0.042661
Salinibacterium	B2	A1	0.097136182	0.028865
Salinibacterium	B2	A2	0.097136182	0.042661
Serratia	A2	A1	2.553944582	0.012987

Results from multivariate PERMANOVA analysis demonstrate that haplogroup had the greatest effect on microbial diversity, able to explain 18% of the observed variance (P = 0.142). However, NDMS visualization suggests that grouping each haplogroup independently did not provide the best explanation for differences between groups, thus further analysis was conducted by pooling A1 and A2 haplogroups. This step did not result in changes in abundance in the

most predominant species identified, but it did result in significant differences between A1/A2 and B2 haplogroups (P = 0.042), with haplogroup now explaining 13.5% of microbial composition variance, and haplogroup and temperature interaction explaining 4.4% of variance observed (Fig. 7). This also revealed several new significantly differentially abundant genera between haplogroup comparisons in the Kruskal-Wallis test (Table 4.) Significantly different species between A1/A2 and B2 haplogroups from revised DESeq2 analysis are shown between *C. scorpioides* treated with high temperature (Fig. 8) and control temperatures (Fig. 9).

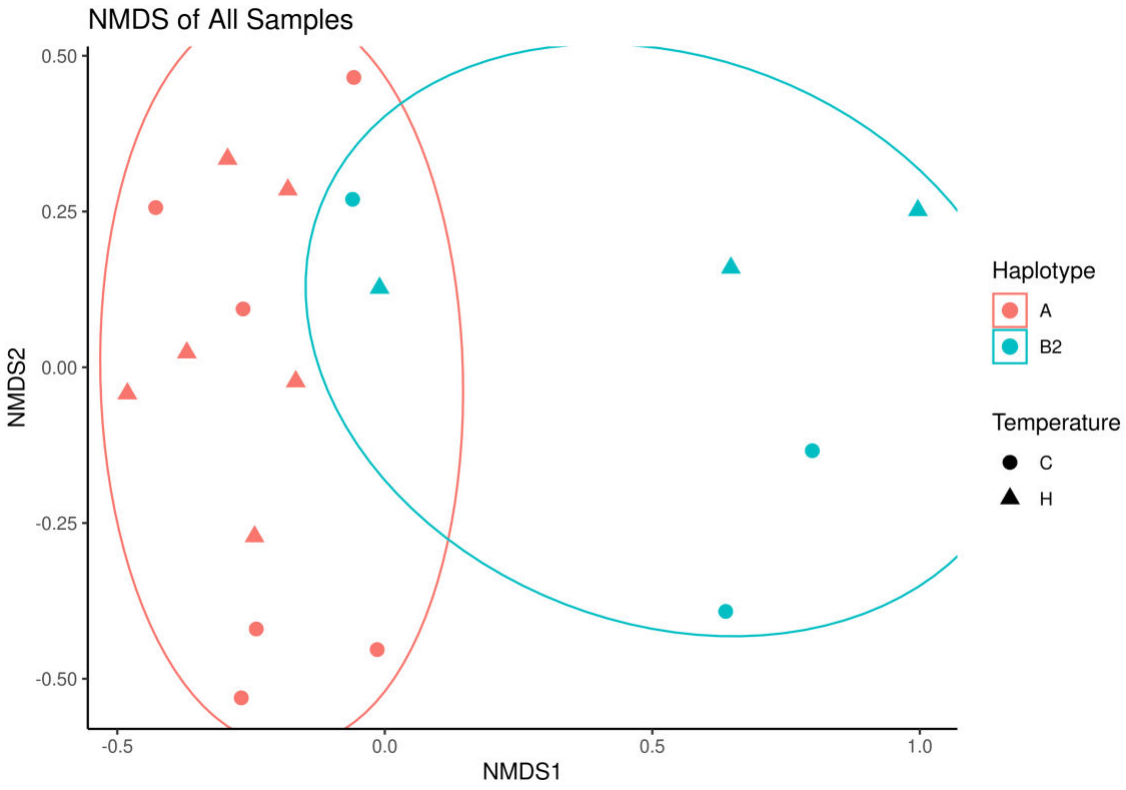

Figure 7. Multivariate PERMANOVA analysis results for haplogroup and temperature aggregations with A1 and A2 samples pooled. Pooling A1 and A2 haplogroup creates a clear cluster significantly different from B2 haplogroup.

Table 4. Differently abundant genera between pooled A1/A2 and B2 haplogroup comparisons according to Kruskal-Wallis.

Genus	Group 1	Group 2	Average Abundance	Adjusted P-value
Acinetobacter	B2	A	2.082796232	0.0032
Aeromicrobium	B2	A	0.272186415	0.0204
Alkanindiges	B2	A	0.034705007	0.0116
Brevundimonas	B2	A	0.675260288	0.0397
Burkholderia	B2	A	0.752354983	0.0276
Chryseobacterium	B2	A	0.590976698	0.0054
Dyadobacter	B2	A	0.039662866	0.0485
Marmoricola	B2	A	0.11502231	0.0402
Mucilaginibacter	B2	A	0.942984631	0.0074
Nevskia	B2	A	0.069410015	0.0402
Pedobacter	B2	A	0.162369856	0.0038
Pelomonas	B2	A	0.417451661	0.0080
Salinibacterium	B2	A	0.073128409	0.0080

Stenotrophomonas	B2	A	0.379771939	0.0402

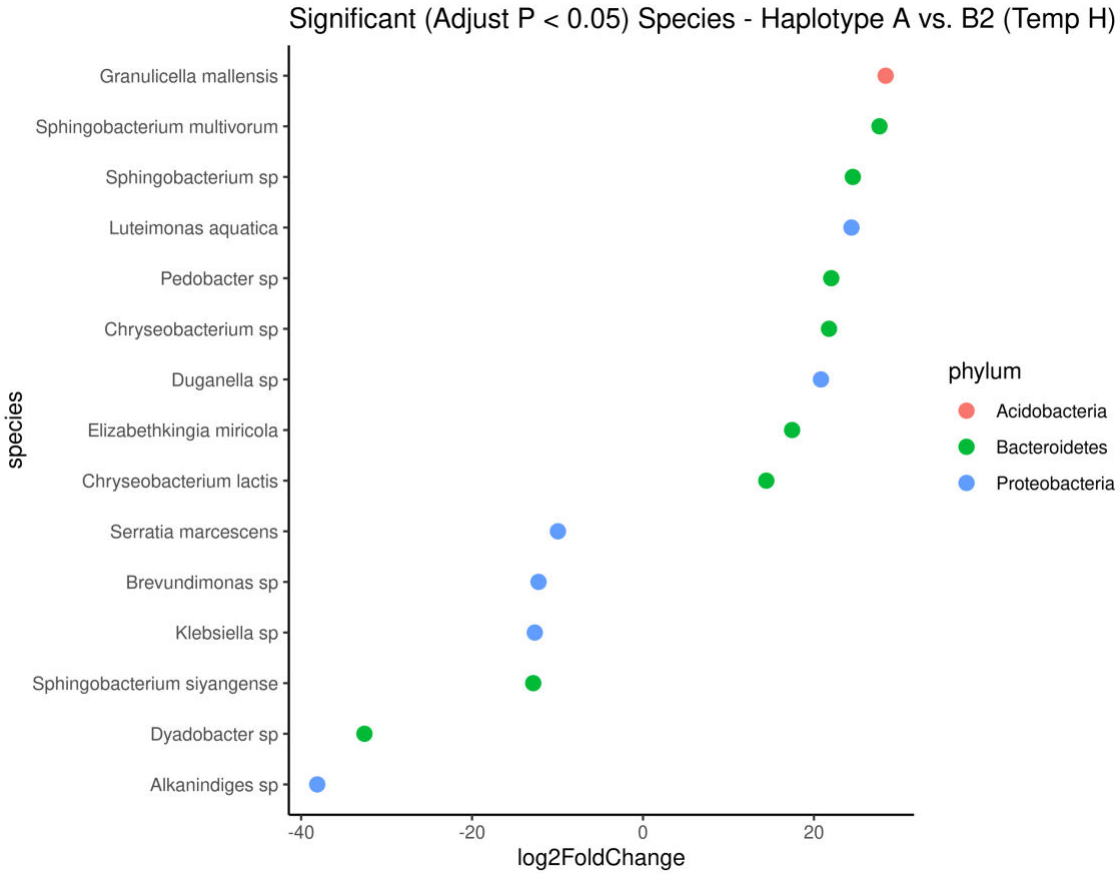

Figure 8. Significantly differentially abundant genera for between A1/A2 haplogroup and B2 haplogroup for all high temperature treated *C. scorpioides* samples. Abundance values for genera was log transformed for comparisons.

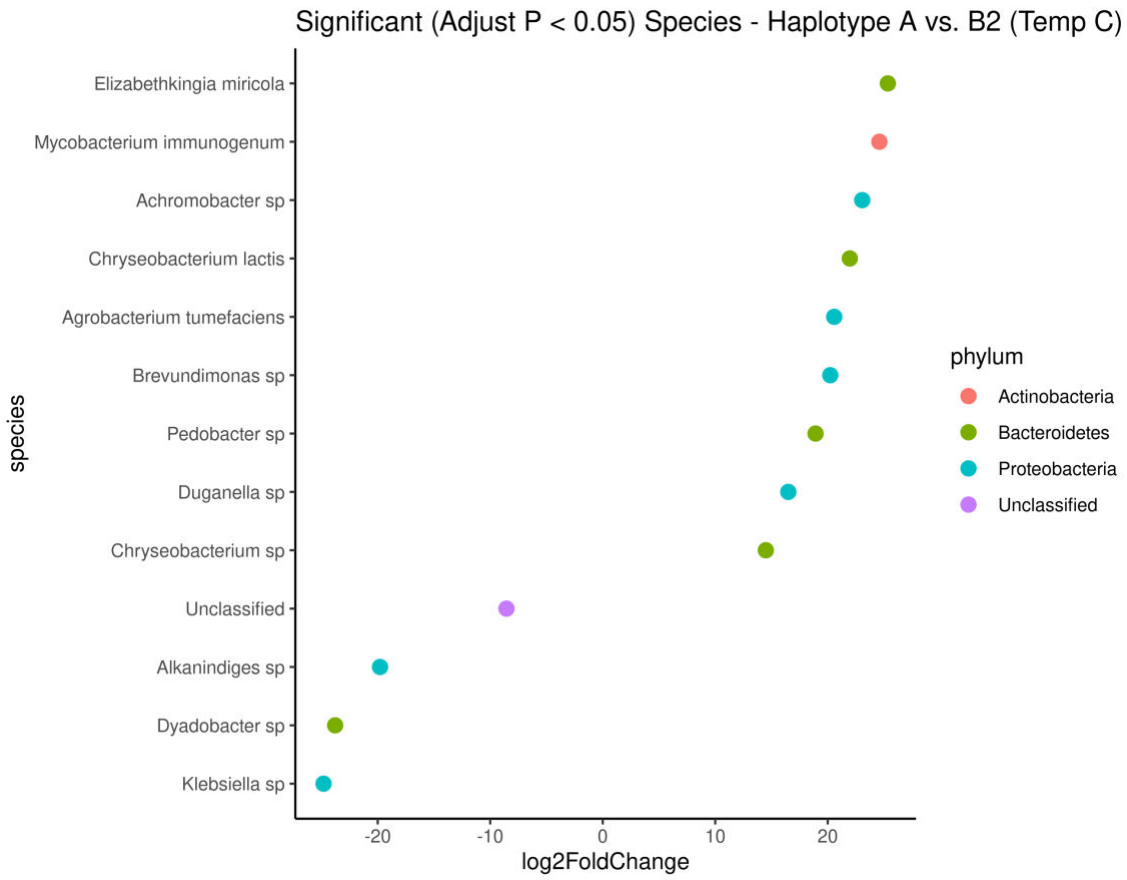

Figure 9. Significantly differentially abundant genera for between A1/A2 haplogroup and B2 haplogroup for all control temperature treated *C. scorpioides* samples. Abundance values for genera was log transformed for comparisons.

3.3 | Effect of Temperature and Haplogroup on *C. scorpioides* Microbial Diversity

Microbial diversity was greater for the high temperature treatment over the control temperature treatment for all measures of alpha diversity (Fig. 10). For the Chao, Shannon-Weaver, and Simpson indices, diversity among high

temperature treated pseudoscorpions averaged, relatively, $\bar{x}$ = 71.67, $\bar{x}$ = 1.95, and $\bar{x}$ = 0.64, whereas the respective averages for each index measured $\bar{x}$ = 66.1, $\bar{x}$ = 1.58, and $\bar{x}$ = 0.54 for control temperature treated pseudoscorpions. However, two tailed Student's T-test did not show significance for any of the indices between control and high temperature treatment indices.

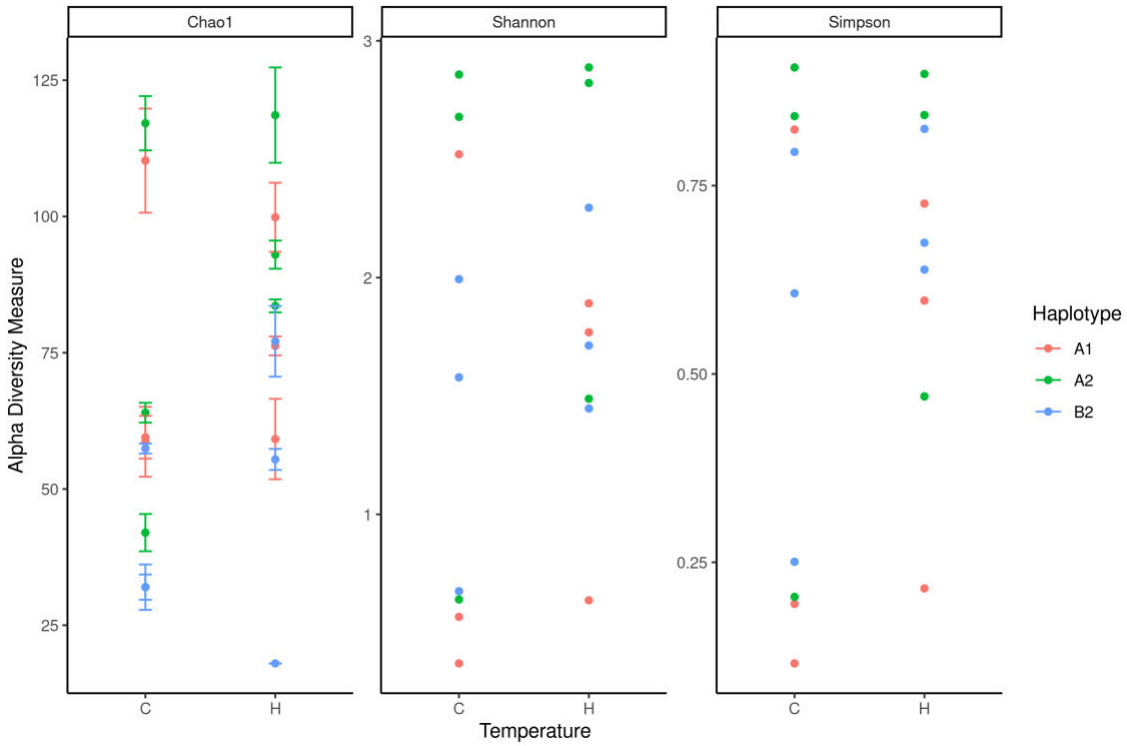

Figure 10. Bacterial alpha diversity metrics for Chao1, Shannon-Weaver, and Simpson indices between haplogroups by temperature.

For haplogroup effects, alpha diversity varied greatly depending on index used. According to the Chao, Shannon, and Simpson indices, A2 haplogroup individuals exhibited the highest average diversity ($\bar{x}$ = 86.4 for Chao; $\bar{x}$ = 2.2 for Shannon; and $\bar{x}$ = 0.7 for Simpson), but according to both the Shannon and Simpson indices, A1-haplogroup individuals possessed the lowest average diversity ($\bar{x}$ = 1.3 for Shannon and 0.4 for Simpson). However, the Chao index calculated the B2 haplogroup as having the lowest average diversity ($\bar{x}$ = 45.3). While the relationship between A1, A2, and B2 haplogroups was not significant for any of the three indices, when A1 and A2 haplogroups were pooled, it was revealed that B2 haplogroup *C. scorpioides* adults did have a significantly lower Chao index than the A1/A2 haplogroup, according to two-tailed Student's T-test (P = 0.009).

3.4 | Fungal Diversity

Fungal diversity was more difficult to quantify, as most fungal sequences resulted in "No Hit." The most abundant genera for both temperature and haplogroup were "No Hit," *Phoma*, and *Coniophora* (Fig. 11). Principal component analysis did not reveal any significant patterns between haplogroup or temperature treatment groups.

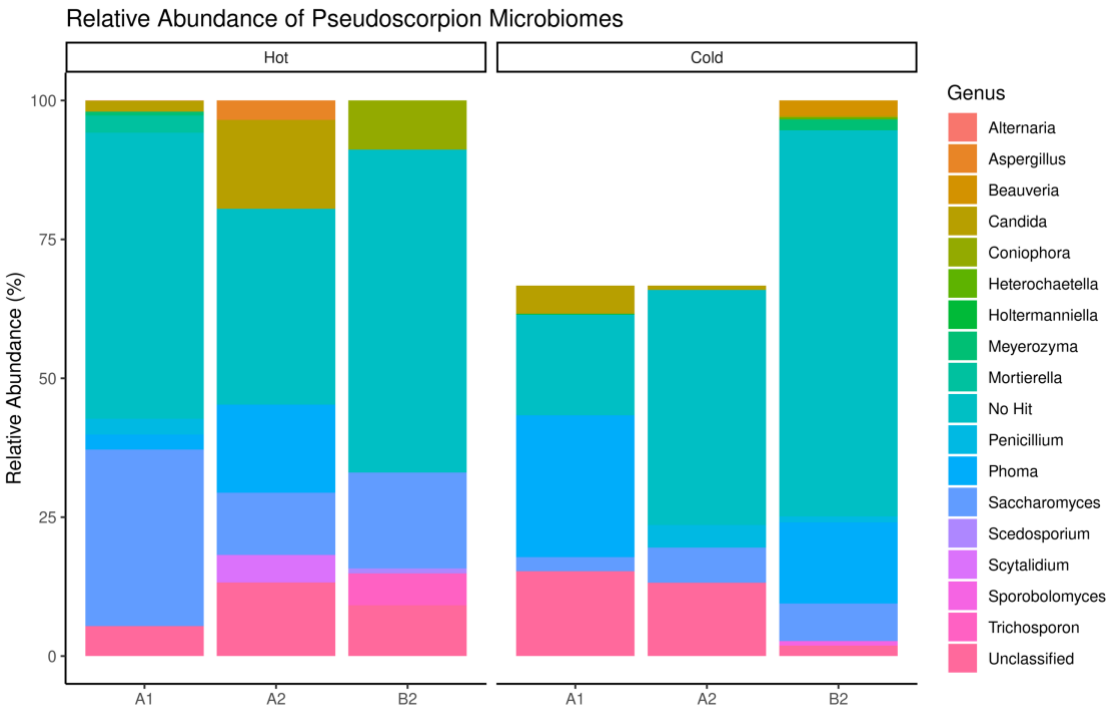

Figure 11. Relative abundance of each haplogroup by temperature for observed fungal phyla.

4 | DISCUSSION

Overall, mitochondrial haplogroup accounted for the most pronounced effects on microbial diversity between treatment groups, with A1/A2 haplotypes having overall higher measures of alpha diversity than B2 haplotypes. Analysis of bacterial sequence counts revealed statistically significant temperature by haplogroup interactions, with twenty bacterial species differing significantly in abundance between interaction groups. Statistically significant differences were observed in three genera, *Acinetobacter*, *Mucilaginibacter*, and *Serratia*, which were also within the top 10 most abundant genera found when analyzing haplogroups.

Not only have mitochondrial mutations been implicated as the source of a wide range of maladies, including cancer (Guerra, et al., 2017), infertility (Boguenet, et al., 2021) and degenerative diseases in tissues with high metabolic requirements, such as the brain (Fiocchetti, et al., 2021) the heart (Poznyak, et al., 2020), and as a cause of aging (Son & Lee, 2019), but both mitochondrial mutations and dysbiosis of the microbiome have been linked to the development of disease. Several studies have begun to characterize the relationship between mitochondria and the microbiome. Yardeni et al. (2019) discovered that, in mice, mitochondrial haplotype modulates ROS production, with increased ROS production directly correlated with decreased microbial diversity, and that mice with similar mitochondrial haplotypes developed similar

microbiome profiles shortly after birth, despite initially inheriting differing microbial communities from their mothers. In a study of *C. elegans*, Han et al. (2017) directly correlated gut *E. coli* with aging, with five bacterial mutants increasing longevity through production of polysaccharide colanic acid, a compound known to regulate mitochondrial function. Finally, Gniansky et al. (2021) demonstrated a link between mitochondrial function and microbial diversity in *D. melanogaster*, by showing that removal of gut bacteria reduces metabolites involved in the production of ATP and prevents flies from developing oocytes, thereby inhibiting reproductive capacity. Thus, the significant relationship presented in this study between mitochondrial haplogroup and microbial diversity is unsurprising.

However in this study, alpha diversity was unexpectedly found to be higher in *C. scorpioides* individuals exposed to higher temperatures than in those that developed under the control temperature regime, and temperature effects on microbial diversity were less pronounced than mitochondrial haplogroup effects. The aim of this study was to investigate whether changes in the gut microbiome can account for deleterious phenotypic effects observed in *C. scorpioides* exposed to high temperatures during early development (Bonham 2021), but increased microbial diversity, often associated with healthy gut microbiomes, in high temperature-exposed *C. scorpioides* runs counter to the strong negative effects of temperature on *C. scorpioides* life history traits observed in a previous simulated climate warming study of *C. scorpioides* (Bonham, 2021).

The absence of appreciable temperature effects on *C. scorpioides* microbiome composition and diversity coupled with significant mitochondrial haplogroup effects on bacterial relative abundance are not consistent with the hypothesis that changes in the gut microbiome underlie the previously documented detrimental phenotypic effects of a 2.5ºC temperature increase (Bonham 2021) in this pseudoscorpion. In this tropical ectotherm, response to fluctuating environmental conditions associated with increasing global temperature will depend on genetic variability for adaptation to novel environments and adaptive phenotypic plastic responses until genetic fixation occurs. Mitochondrial variation is a poorly understood aspect of such genetic variability, as most biomedical studies are focused on characterizing specific mitochondrial mutations responsible for human pathologies but rarely consider the evolutionary implications of mitochondrial adaptation (Ballard & Pichaud, 2013). In *C. scorpioides*, mitochondrial haplogroup proved to have a substantial impact on gut microbial community composition and diversity. Differential mitochondrial gene expression by divergent mitochondrial haplogroups in this pseudoscorpion has previously been shown to exert profound effects on male reproductive traits (Zeh, et al., 2019; Padua, et al., 2014). However, the potential for adaptive evolutionary response to this haplogroup-dependent fitness effect is limited by the maternal mode of mitochondrial inheritance (Leeflang, et al., 2021; Vaught & Dowling, 2018), which creates a sex-selective sieve that makes detrimental and/or beneficial mitochondrial mutations in males invisible to

selective pressures, a phenomenon dubbed the “mother’s curse,” or “sister’s curse” (Padua et al., 2014).

A host and its microbial community share an intricately linked relationship. Host species have not only domesticated bacteria as organelles such as mitochondria, but they have also incorporated microorganisms as endosymbionts. Consequently, understanding how species will respond to climate warming requires a more holistic view of organisms and microbes as “holobionts” that function as integrated units (Feldhaar, 2011; Groussin, et al., 2020). Selection for adaptive evolution of the holobiont depends on the host genome, microbial genomes, and host/microbial genome interactions (Perreau & Moran, 2022). In some instances, these interactions can be antagonistic, as occurs in host sex-ratio manipulation by the bacterial cellular endosymbiont, *Wolbachia* (reviewed in Werren, et al., 2008; Koop, et al., 2009). However, novel complex traits, such as metabolic improvements, can be acquired through mutualistic interactions, and these beneficial traits, spread through lateral transfer followed by vertical transmission, can reach fixation in a population faster than genetic mutations (Feldhaar, 2011; Moran, et al., 2008; Zilber-Rosenberg & Rosenberg, 2008). Changes in microbial composition may have complex effects on host fitness, influencing nutrient absorption (Ridley, et al., 2012), pathogen resistance, and life history traits (Bahrndorff, et al., 2016; Sepulveda & Moeller, 2020). Although microbial composition is an often-overlooked component of plasticity, there is growing evidence linking it to arthropod thermal tolerances (Arango, et al., 2021;

Jaramillo & Castaneda, 2021; Salsbery & DeLong, 2021; Zhu, et al., & Hong, 2021). Thermal tolerance and microbial diversity can be directly linked, as in the case of ice nucleating bacteria, whose presence is responsible for regulation of freezing in overwintering insects or to prevent freezing in cold temperatures (Andreadis & Athanassiou, 2016; Ferguson, et al., 2018; Lee, et al., 1991), or indirectly linked when presence of certain microbial lineages facilitates or prevents a physiological response involving tolerance to thermal stress (Moghadam, et al., 2018; Zhang, et al., 2018; Yang, et al., 2021). Such molecular interactions between hosts and symbionts are highly variable and species dependent (Corbin, et al., 2017). A notable example of microbial tolerance to heat stress occurs in obligatory symbiont *Rickettsia* bacteria in the sweet potato whitefly, *Bemisia tabaci* (Brumin, et al., 2011). The presence of *Rickettsia* bacteria in whiteflies confers resistance to mortality in flies exposed to temperatures above 32ºC. The *Rickettsia* produce stress-related proteins that indirectly contribute to thermotolerance when ambient temperature increases. On the other hand, some endosymbiotic bacteria exhibit a reduction in abundance at high temperatures, thus reducing tolerance to environmental stress and heat exposure (Wernegreen, 2012). A notable example of this also occurs in species infected by parasitic *Wolbachia,* as some *Wolbachia* strains modify host behavior to prefer cooler temperatures, inhibiting host thermal tolerance, which Hague et al. (2020) suggests is a survival strategy to increase *Wolbachia* titers and promote bacterial replication.

Survival of *C. scorpioides* in the face of increased global temperature will likely result from plastic epigenetic modifications. While both microbial lineages and epigenetic modifications are heritable and have the capacity to affect fitness, data on gene, transposable element and small noncoding RNA expression suggest that phenotypic disruption at high temperature in this pseudoscorpion is largely due to temperature-induced epigenetic dysregulation of transposable elements (see Chapters 2 and 3), Transposable elements are mobile genetic elements capable of copying and/or excising themselves and inserting into new locations in the genome, resulting in a range of structural and functional effects from genomic instability and gene disruption to enhanced gene expression and even the evolution of novel genes and genetic regulatory networks (Bourque, et al., 2018; Casacuberta & Gonzalez, 2013; Chuong, et al., 2017). Nearly 70% of the *C. scorpioides* genome consists of repetitive sequences/transposable elements, an unusually high percentage for an arthropod species, and heat stress may temporarily disable the system of epigenetic mechanisms that suppress transposable element activity, resulting in phenotypic evidence of epigenetic dysregulation (Werren et al., 2011; Yi & Goodisman, 2021; Zeh et al., 2009.). Indeed, previous RNA-seq analysis of *C. scorpioides* testicular tissue revealed that high temperatures caused upregulation of 11 differentially expressed genes derived from transposable elements (Bonham, 2021), suggesting breakdown in epigenetic control of transposable elements.

The discovery of significant differences in the abundance of 20 microbial genera due to haplogroup by temperature interactions combined with the differential abundance of several genera between high and control temperatures in this study suggests the potential for adaptive evolutionary response to heat stress in *C. scorpioides*. While the majority of the genera affected by temperature were low in relative abundance, the potential of these bacteria to confer physiological modifications in response to heat stress could be disproportionately large. Many studies investigating the role of microbial communities focus on characterizing highly abundant species, with species present in less than one percent abundance frequently filtered out, but “keystone” microorganisms may influence metabolic functions in the host, despite their low abundance (Alves de Cena, et al, 2021). Minor differences in bacterial abundance are known to affect epigenetic modifications, influencing gene expression due to production of short chain fatty acid bacterial metabolites capable of modulating epigenetic markers involved in immune system function and the integrity of the intestinal epithelial barrier (Woo & Alenghat, 2017). In addition, opportunistic pathogenic species present in low abundances may contribute to compositional or functional shifts towards dysbiosis. For example, in humans, the species *Bacteroides fragilis* has been found to be present in low abundances, but is capable of promoting oncogenic effects due to its ability to secrete compounds that alter colonic epithelial cells and mucosal immune system function (Alves de Cena, et al, 2021). While the primary aim of this experimental study was to investigate the

potential for temperature to influence the *C. scorpioides* microbiota, future research should therefore include more in-depth characterization of the gut microbial community, including characterization of the microbiome from individuals collected in the wild.

However, given its carnivorous diet, the *C. scorpioides* microbiome may not involve a stable set of resident microbes but instead entails temporarily borrowing microbes from its predatory diet, and is highly transient depending on prey consumed, as has been demonstrated in other predatory arachnids (Kennedy, et al., 2020). Though most species are assumed to have a necessary resident system of gut endosymbionts, this view is largely influenced by the discovery of the human gut microbiome, and may not be universally true for arthropod species (Esposti & Romero, 2017), though this does not exclude the possibility of gut microbial importance, since even brief exposure to some microbial species can induce long-lasting epigenetic changes (Louwies, et al., 2019). Though microbial diversity may have implications for survival in many species, epigenetics is likely the primary mechanism underlying adaptive or maladaptive phenotypic change in response to climate warming for this tropical ectotherm.

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