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RESEARCH ARTICLE

Simulated climate warming and mitochondrial haplogroup modulate testicular small non-coding RNA expression in the neotropical pseudoscorpion, *Cordylochernes scorpioides*

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Abstract

Recent theory suggests that tropical terrestrial arthropods are at significant risk from climate warming. Metabolic rate in such ectothermic species increases exponentially with environmental temperature, and a small temperature increase in a hot environment can therefore have a greater physiological impact than a large temperature increase in a cool environment. In two recent studies of the neotropical pseudoscorpion, *Cordylochernes scorpioides*, simulated climate warming significantly decreased survival, body size and level of sexual dimorphism. However, these effects were minor compared with catastrophic consequences for male fertility and female fecundity, identifying reproduction as the life stage most vulnerable to climate warming. Here, we examine the effects of chronic high-temperature exposure on epigenetic regulation in *C. scorpioides* in the context of naturally occurring variation in mitochondrial DNA. Epigenetic mechanisms, including DNA methylation, histone modifications and small non-coding RNA (sncRNA) expression, are particularly sensitive to environmental factors such as temperature, which can induce changes in epigenetic states and phenotypes that may be heritable across generations. Our results indicate that exposure of male pseudoscorpions to elevated temperature significantly altered the expression of >60 sncRNAs in testicular tissue, specifically microRNAs and piwi-interacting RNAs. Mitochondrial haplogroup was also a significant factor influencing both sncRNAs and mitochondrial gene expression. These findings demonstrate that chronic heat stress causes changes in epigenetic profiles that may account for reproductive dysfunction in *C.*

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scorpioides males. Moreover, through its effects on epigenetic regulation, mitochondrial DNA polymorphism may provide the potential for an adaptive evolutionary response to climate warming.

Key words: climate warming; epigenetics; microRNAs; mitochondria; piwi-interacting RNAs; tropical ectotherm

Introduction

Adapted to already high and relatively constant temperatures, tropical terrestrial ectotherms are predicted to be at significant risk from climate warming [1–4]. In such ectothermic species, metabolic rate increases exponentially with ambient temperature [5–8], and a small temperature increase in a hot environment can therefore have a larger physiological impact than a large temperature increase in a cool environment [9]. Because tropical arthropods constitute the vast majority of animal species [10], the implications of the metabolic theory of climate warming for global biodiversity are profound [9, 11]. Unlike temperate zone species, tropical species often exhibit high levels of mitochondrial DNA sequence variation across small spatial scales even in the presence of gene flow [12–17]. Given the fundamental role of mitochondria in metabolism, sequence variation in the mitochondrial genome that influences OXPHOS activity, mitochondrial translational efficiency [18–20] and/or mitochondrial cellular concentration could be an important determinant of evolutionary responses to rising temperatures [21–24]. In addition, a growing body of theory and empirical evidence indicate that environmentally induced epigenetic modifications may be a critical factor in determining the fate of populations subjected to rapid environmental change [25–35].

Associated with the DNA scaffold is a system of somatically, intergenerationally and potentially transgenerationally heritable epigenetic marks [36, 37]. In conjunction with long [38] and short [39] non-coding RNAs (ncRNAs), DNA methylation [40, 41] and chemical modifications to core histone proteins affect how tightly DNA is packaged in chromatin [42, 43]. By providing differential access to underlying genetic information in a reversible, dynamic and inducible manner, epigenetic marks mediate the developmental pattern, tissue specificity and environmental context of gene expression [36, 37]. Among the various classes of ncRNAs, microRNAs (miRNAs) are conserved sequences that regulate gene expression at the post-transcriptional stage [44]. Because binding is generally restricted to a short 'seed' region at the 5′ end of the miRNAs [45], a single miRNA may bind to the mRNAs of numerous genes, and a single gene may be regulated by several miRNAs, resulting in complex regulatory networks that function in almost all developmental, physiological and disease-related processes [44, 45]. In contrast, piwi-interacting RNAs (piRNAs) are highly expressed in gonads where they function as a germline defence against transposable element (TE) activity [46–49]. In *D. melanogaster*, PIWI proteins are essential for male and female fertility [50], and, in the mouse, deficiency in PIWI proteins results in TE activation in testes and complete sterility [51]. There is also increasing evidence that piRNA functions extend beyond germline TE suppression to targeting genes involved in early development, *de novo* DNA methylation, and more generally as an epigenetic programming mechanism that guides other epigenetic factors to their targets [52].

Because of its unique reproductive biology and amenability to experimental manipulation (Fig. 1), the neotropical pseudoscorpion, *Cordylochernes scorpioides* (Linneaus 1758) (Pseudoscorpionida: Chernetidae), is a model tropical ectotherm for investigating mitochondrial and epigenetic responses to climate warming [53, 54]. In these arachnids, males transfer sperm to females in discrete packets via a stalked spermatophore deposited on the substrate. Matings can be interrupted following spermatophore deposition, and the sperm packet collected for assessment of sperm quantity and quality [53]. Females are viviparous, and nourish developing embryos in an external, transparent brood sac, overlying the genital aperture [53]. This 'external womb' form of viviparity facilitates embryonic epigenome editing using ncRNAs, and makes possible non-invasive monitoring of female reproductive status and embryological development. In addition, mitochondrial DNA sequence variation is extensive. Previous sequencing of the *C. scorpioides* mitochondrial *cox1* gene from populations in central and western Panamá identified three divergent lineages: one clade consisting predominantly of individuals from central Panamá (Clade A), and two sister clades (B1 and B2) which appeared to be restricted to western Panamá [55]. However, subsequent sequencing of the *ND2* gene from 66 *C. scorpioides* laboratory matrilines revealed that the B2 haplogroup co-occurs with the A haplogroup in central Panamá at estimated frequencies of 12% and 88%, respectively [53]. Next generation sequencing of the complete mitochondrial genomes of A- and B2-haplogroup individuals from our central Panamá laboratory population, as well as B2- and B1-haplogroup individuals collected from extreme western Panamá, revealed a mean divergence between the sympatric A and B2 haplogroups of 8.9% across the entire genome [56]. Further research has established that the two mitochondrial haplogroups differ markedly in the expression of nearly all the mitochondrial OXPHOS genes [Zeh et al., unpublished data]. Previous climate warming research on *C. scorpioides*, involving a single-step 3.5°C increase in temperature predicted for the end of the century by some climate-warming models, established reproduction as the Achilles' heel of this pseudoscorpion [53, 54]. Exposure to elevated temperature during development affected size, sexual dimorphism and survivorship only moderately but reduced sperm numbers by >50% and rendered males and females sterile [53].

In the study reported here, the impacts of elevated temperature and natural mitochondrial variation on sncRNA expression in *C. scorpioides* testicular tissue were investigated using a split-brood experimental design in which offspring from five A and five B2 mitochondrial haplogroup females were randomly assigned to either a diurnally fluctuating control (C) or a diurnally-fluctuating high temperature (H) regime for rearing from birth to the adult stage (for details, see [53]). Climate warming effects were simulated in the high temperature treatment by elevating the average temperature 3.5°C above the control temperature, which was estimated from long-term, daily high and low temperature records from *C. scorpioides*' native habitat in central Panamá [53]. edgeR [57] analyses demonstrated that 70 miRNAs and 14 piRNAs were significantly differentially expressed (DE) in response to either temperature or mitochondrial haplogroup. More generally, these results suggest that disrupted epigenetic profiles may account for climate-warming induced reproductive dysfunction in male *C. scorpioides*, and that mitochondrial DNA variation, through its

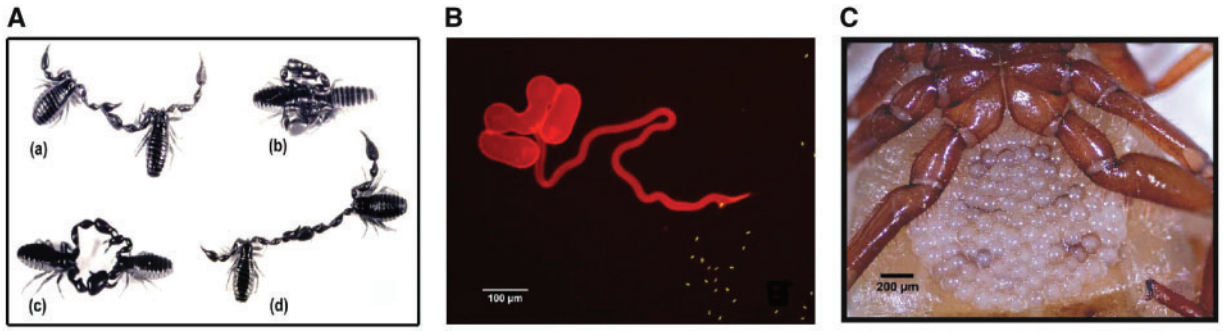

Figure 1: reproductive biology of *Cordylochernes scorpioides*. (A) Mating sequence in which male grasps female (a), produces and deposits spermatophore (b); reverses, pulling female over spermatophore (c) and maintains contact during sperm uptake phase (d). (B) Sperm packet (stained red) with the everted tube and evacuated sperm (stained green). (C) Ventral view of gravid female carrying a brood sac containing ~100 early-stage embryos. The images are reproduced from [56].

effects on the expression of sncRNAs, may provide the potential for an adaptive evolutionary response to climate warming.

Results

Cordylochernes scorpioides Males Abundantly Express a Diverse Set of miRNAs and piRNAs in Testicular Tissue

Interrogation with Sequery annotation software [58] of the 20 small RNA libraries from *C. scorpioides* testicular tissue detected 585 distinct piRNAs and 517 distinct miRNAs that met the threshold abundance of three or more normalized transcripts averaged across the 20 samples. Overall, miRNAs were more abundantly expressed than piRNAs, accounting for 7.53% of the total small RNA transcriptome compared with 0.53% for piRNAs. Total sncRNA transcripts were nearly six times more abundant than transcript fragments from the 13 OXPHOS genes of the mitochondrial genome (8.06% versus 1.43% of the total small RNA transcriptome). Plots of cumulative transcript abundance against abundance rank revealed that piRNAs were more evenly expressed (Supplementary Fig. S1) than miRNAs (Supplementary Fig. S2). The 20 most abundantly expressed miRNAs accounted for 68.5% of overall miRNA transcript abundance compared with a value of 24.7% for piRNAs.

Elevated Temperature Significantly Alters Expression of sncRNAs in *C. scorpioides* Testicular Tissue

Temperature exerted significant but opposite effects on overall miRNA and piRNA expression. In the case of miRNAs, average overall expression increased 37% at high temperature (Fig. 2; $P < 0.0001$). In contrast, the high temperature treatment decreased average overall piRNA expression by 32% (Fig. 3; $P < 0.0001$). Sixty-four of the 517 miRNAs (12.4%) were significantly DE by temperature. Despite increased overall miRNA expression, the vast majority ($N = 57$) of DE miRNAs was significantly downregulated at high temperature, with $\log_2$-fold changes ranging from –2.75 to 0.92 (Fig. 4a; Table 1). Most noteworthy were the miRNAs, mdo-miR-1547-5p, bmo-let-7-3p, hsa-miR-3116, mdv2-miR-M14-5p and bmo-miR-6496-3p, which were abundantly expressed at the control temperature but strongly downregulated at high temperature (Table 1).

A similar pattern of high temperature-induced downregulation was evident for piRNAs. Of the six significantly DE piRNAs, five were significantly downregulated at high temperature, with $\log_2$-fold changes ranging from –2.05 to 1.24 (Fig. 4b; Table 2). piRNAs that were abundantly expressed at the control temperature but strongly downregulated at high temperature included MIWI2-278992 and MIWI2-169803.

Natural Variation in Mitochondrial DNA Results in Differential Expression of sncRNA

Investigation of the effects of mitochondrial haplogroups on sncRNA expression revealed significant differential expression of a set of miRNAs and piRNAs distinct from those exhibiting temperature-dependent effects (Fig. 5a; Table 1). Six miRNAs were significantly DE between mitochondrial haplogroups. Four of these six miRNAs (dme-miR-315-3p, zma-miR395d-5p, cel-miR-253-5p and cbr-miR-253) exhibited significant and markedly increased levels of expression in the B2 haplogroup relative to the A haplogroup, with $\log_2$-fold changes ranging from 2.83 to 8.70. Interestingly, all eight significantly DE piRNAs exhibited markedly decreased B2 expression, with $\log_2$-fold changes ranging from –6.97 to –3.92 (Fig. 5b; Table 2). There were no significant interactions between mitochondrial haplogroup and temperature treatments, suggesting that mitochondrial haplogroup influences sncRNA expression, irrespective of temperature. A heatmap of significantly DE miRNAs and piRNAs separates the mitochondrial haplogroups into distinct clades independent of temperature effects (Fig. 6).

Discussion

Sequencing of replicate *C. scorpioides* small RNA libraries from A and B2 mitochondrial haplogroups at control and high temperatures revealed that testes sncRNA expression, particularly miRNA expression, is strongly influenced by simulated climate warming and natural mitochondrial variation in this neotropical ectotherm. Overall, temperature exerted significant effects on the expression of 64 miRNAs and six piRNAs. The majority of significantly DE miRNAs decreased in expression at high temperature, in some instances by more than two log folds. The down regulated aga-miR-13b and bma-miR-2a are members of the mir-2 miRNA family that is widespread in invertebrates and regulates cell survival by translational repression of pro-apoptotic factors [59]. Consequently, reduction in expression of these miRNAs may be causally linked to *C. scorpioides*' spermatogenic disruption at high temperature. Overall, piRNA expression at elevated temperature declined by 32%, and all DE *C. scorpioides* piRNAs mapped to piRNAs associated with MIWI2, a Piwi family member in mice. MIWI2, MIWI2-associated piRNAs and other protein complexes are essential components for spermatogenesis that repress TE activity in distinct ways [60–62].

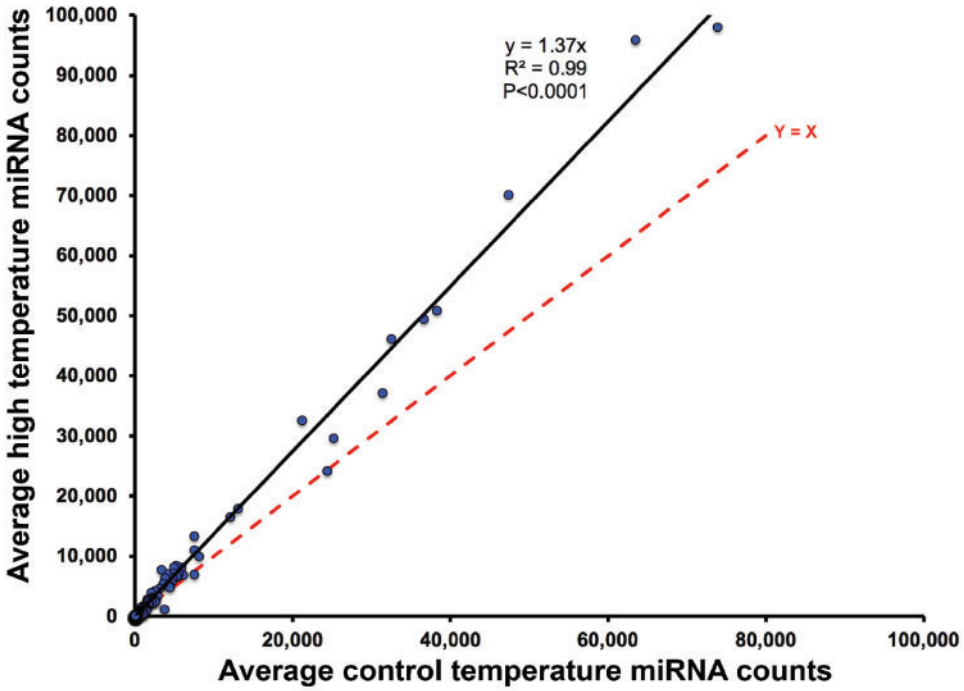

Figure 2: bivariate plot of average testes miRNA expression at high temperature versus control temperature. Overall miRNA expression increased 37% at high temperature. Red dashed line indicates equal miRNA expression at high and low temperature

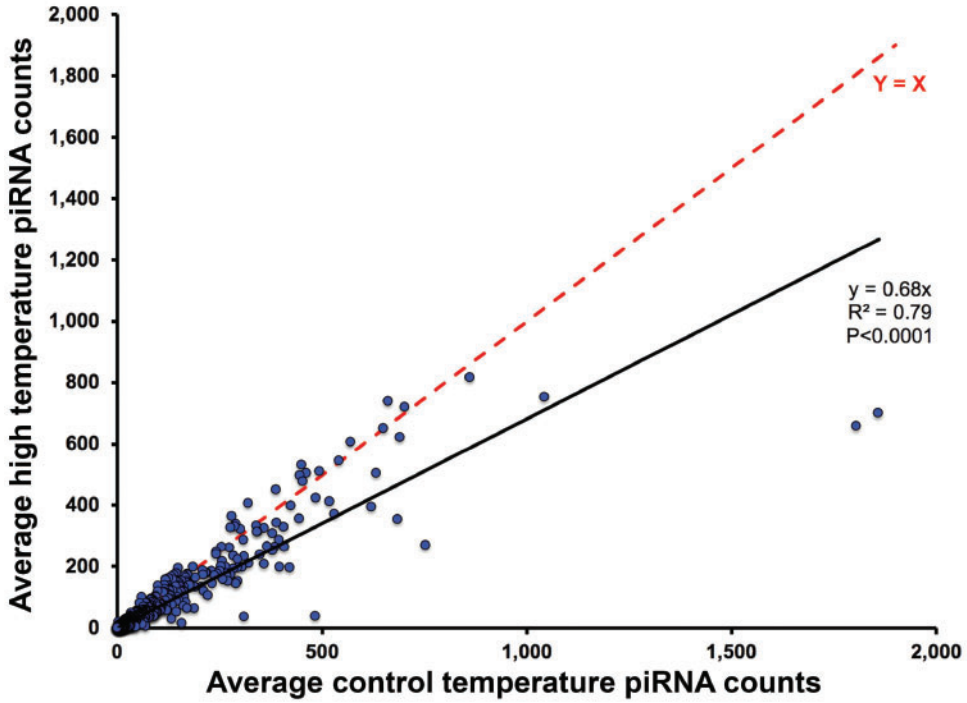

Figure 3: bivariate plot of average testes piRNA expression at high temperature versus control temperature. Overall piRNA expression decreased 32% at high temperature. Red dashed line indicates equal piRNA expression at high and low temperature

First, MIWI2 piRNA complexes function to repress TE activity by directing DNA methylation machinery to TE sites [60–62]. Second, MIWI, a small RNA-guided RNase, possesses catalytic activity which functions in genome defence by directly cleaving transposon messenger RNAs and prevents TE re-insertions that result in mutations and transposon-induced recombination [63]. Thus, the significant decrease in the expression of MIWI2-like piRNAs in *C. scorpioides* males subjected to high temperature may suggest that environmentally induced breakdown in the epigenetic regulation of TE activity contributed to the marked reduction in sperm produced by *C. scorpioides* males subjected to simulated climate warming [53, 54].

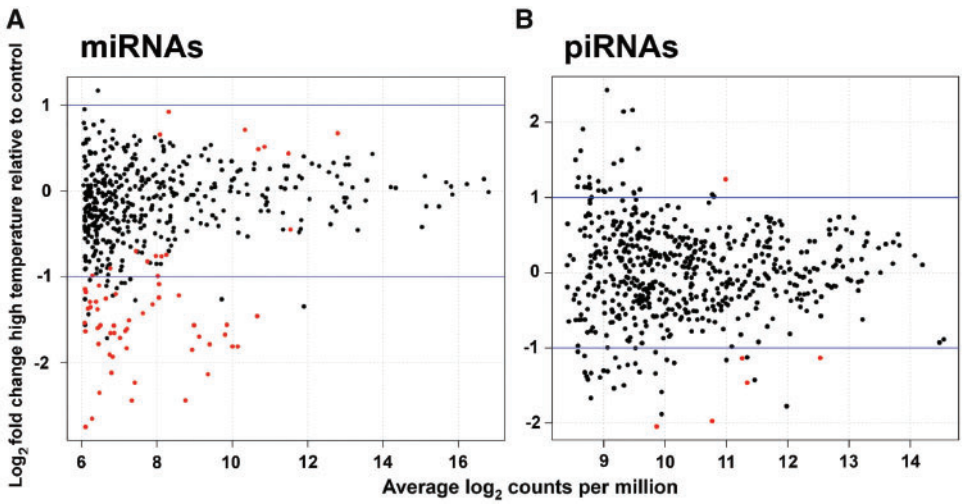

Figure 4: smear plots of (A) miRNAs and (B) piRNAs showing $\log_2$-fold changes in expression level at high temperature relative to control as a function of expression level. Significantly differentially expressed sncRNAs are indicated in red. Blue lines indicate a one log fold change for either increased and decreased expression at high temperature

It is important to acknowledge two limitations of the study. First, reliance on publicly-available sncRNA databases due to the lack of a *C. scorpioides* reference genome is likely to have constrained our ability to detect significant temperature and haplogroup effects, particularly in the case of piRNAs. Across animal species, miRNAs are better characterized and more highly conserved than piRNAs [49], and in our study, miRNAs were more abundant, less variable and more likely to be DE. Absence of pseudoscorpion-specific piRNAs in the databases used to characterize piRNA expression could explain the relatively low abundance and reduced sensitivity of piRNAs to temperature. Unfortunately, *C. scorpioides* appears to possess a large (~2.5 Gb) and complex genome, and generating a reference genome will require substantial sequencing, assembly and annotation efforts.

Second, our experimental design did not permit determination of the precise mechanism of temperature-dependent differential expression in testes. Testes are composed of numerous germ and somatic cells types, and temperature induced differential expression could result either from changes in sncRNA expression at the cellular level, or from alterations in the cellular composition of the testes. In the absence of cell-specific expression data, these alternative mechanisms can be indirectly assessed based on comparison of overall mitochondrial gene expression at control versus high temperatures, as well as on published results on the effect of temperature on the number of mature sperm in spermatophores [53]. Recent single-cell RNA-Seq studies of mice demonstrate that mitochondrial gene expression varies extensively between testicular cell types and stage of spermatogenesis. Compared with other testicular cells, the relative abundance of mtDNA transcripts is greatly reduced in differentiating sperm cells, including meiotic spermatocytes, post-meiotic haploid round spermatids and elongating spermatids. This pattern stems from the major reduction in mitochondrial copy number as sperm mature [64], a relationship likely to hold across animal species. In our study, high temperature increased overall representation of mitochondrial transcript fragments in our small RNA libraries by 42% ($P = 0.0357$), suggesting a reduction in the proportion of differentiating sperm cells in the testes of high temperature males. This interpretation is consistent with significantly reduced sperm counts in high temperature males, who produce only 43% as much ejaculated sperm as control males [53]. It therefore seems likely that differential expression of sncRNAs at the level of testes stems at least partially from temperature-mediated depletion of differentiated sperm cells.

While the role of mitochondrial variation in influencing epigenetic regulation in nature remains poorly understood, mitochondria are known to be intimately involved in the establishment of epigenetic states through the conversion of calories to ATP, acetyl-CoA, NAD+ and SAM, the high-energy substrates essential for phosphorylation, acetylation, deacetylation and methylation [18], and through the synthesis of co-factors associated with active de/methylation and de/acetylation [19]. Experimental removal of functional mitochondria from cells alters patterns of DNA methylation that partially revert to their original state when mitochondria are re-introduced into cells [65]. Similarly, inactivation of mitochondria has been shown to lead to a 5-fold reduction in miRNA silencing efficiency [66, 67]. Recent findings also point to a key role for mitochondria in piRNA biogenesis. Evidence suggests that piRNA-cluster transcripts are exported from the nucleus to non-membranous cellular bodies, known as Yb-bodies in ovarian somatic cells and nuage in germline cells, that are closely associated with mitochondria and enriched with piRNA biogenesis factors [68].

In *C. scorpioides*, six miRNAs and eight piRNAs were significantly DE by the A and B2 mitochondrial haplogroups. Although natural mitochondrial variation accounted for far fewer DE sncRNAs than did high temperature, haplogroup-based differential expression was more extreme in magnitude, with $\log_2$-fold changes ranging from 6.97 to 8.7. Importantly, sncRNA differential expression by the two mitochondrial haplogroups cannot be attributed to differences in nuclear genetic background. The *C. scorpioides* individuals used in this study were mated randomly in the laboratory with respect to mitochondrial haplogroup for a minimum of 16 generations. Consequently, the two haplogroups became effectively homogeneous with respect to nuclear genetic background, greatly increasing the likelihood that any systematic differences between haplogroups in sncRNA expression are causally linked to differences in the mitochondrial genome. The coexistence of the highly divergent

Table 1: differentially expressed (DE) miRNAs in response to temperature treatment and mitochondrial haplogroup

miRNA Identity	Haplogroup FDR	Temperature FDR	Log fold change	Average HA counts	Average CA counts	Average HB2 counts	Average CB2 counts	Known function
aae-miR-13-3p	–	4.38E–02	–1.59	3.49	17.54	28.64	40.13	–
aae-miR-14	–	3.08E–02	–1.56	128.40	513.86	220.80	462.52	–
aae-miR-275-3p	–	8.05E–03	0.71	530.76	195.21	828.72	417.93	–
aga-miR-13b	–	5.68E–03	–1.71	12.13	44.60	28.05	66.23	Regulates cell survival by translational repression of proapoptotic factors
aga-miR-14	–	3.04E–02	–1.24	46.49	120.41	63.53	118.93	–
aly-miR4233	–	3.76E–02	–0.99	11.19	19.31	18.12	26.81	–
ame-miR-317	–	1.00E–02	–0.45	1038.84	969.57	1118.94	1253.75	–
ame-miR-3751	–	4.50E–03	–1.64	4.93	16.38	12.01	27.89	–
api-miR-275	–	2.46E–02	0.52	845.67	345.67	982.88	671.13	–
bbe-miR-10a-5p	–	2.14E–03	–0.99	62.79	95.23	71.25	111.71	–
bdi-miR7725a-3p	–	3.42E–02	–0.90	23.47	35.75	25.61	34.29	–
bfl-miR-9-5p	–	3.04E–02	0.49	770.39	376.33	860.98	516.28	Suggested to regulate neuronal differentiation
bma-miR-2a	–	8.00E–03	0.44	1231.73	652.19	1603.27	895.87	Regulates cell survival by translational repression of proapoptotic factors
bmo-let-7-3p	–	4.85E–05	–2.14	21.62	151.97	340.25	361.82	–
bmo-miR-2762	–	5.50E–03	–1.64	14.70	38.40	22.43	89.05	–
bmo-miR-279a	–	8.00E–03	0.67	3420.90	1627.81	4274.12	1798.56	–
bmo-miR-2808a-3p	–	1.43E–02	–1.66	4.75	33.85	47.06	45.85	–
bmo-miR-3392	–	6.84E–04	–1.84	8.40	43.30	39.37	80.00	–
bmo-miR-6495-3p	–	4.11E–03	–1.61	19.95	48.86	34.82	70.39	–
bmo-miR-6495-5p	–	1.25E–02	–1.32	25.81	69.25	122.85	85.16	–
bmo-miR-6496-3p	–	3.08E–02	–1.79	42.23	145.98	73.20	577.86	–
bmo-miR-6497-5p	–	8.00E–03	–1.57	5.16	21.64	25.97	40.20	Transposable element-associated small RNAs
cbr-miR-253	1.63E–17	–	8.70	0.89	0.79	492.03	659.48	–
cbr-miR-74b-5p	–	2.89E–02	–1.15	11.90	18.90	10.61	19.21	–
cca-miR6117	–	8.41E–04	–1.46	148.21	421.67	731.36	948.41	–
cel-miR-253-5p	1.06E–16	–	6.83	0.70	0.24	111.82	125.99	–
cgr-miR-1973	–	3.04E–02	–1.20	19.88	48.85	28.20	36.44	–
cla-miR-1994	–	9.87E–03	–1.68	42.91	228.95	735.07	262.13	–
dme-miR-279-5p	–	1.50E–02	–0.76	59.59	79.69	84.86	106.26	–
dme-miR-315-3p	1.11E–05	–	2.83	2.76	3.85	30.18	26.14	–
gga-miR-1753	–	1.46E–02	–1.43	12.48	40.93	110.28	83.01	–
gga-miR-6577-3p	–	1.19E–02	–1.29	5.02	15.62	17.64	31.40	–
ggo-miR-198	–	3.78E–03	–1.37	7.75	19.88	12.19	25.99	–
ggo-miR-4520b	–	2.24E–02	–2.24	16.15	76.92	24.22	83.04	–
gma-miR395h	–	3.08E–02	–1.18	7.65	15.28	13.44	26.07	–
gma-miR6300	–	2.40E–02	0.92	87.33	41.82	279.51	59.01	–
hsa-miR-1973	–	4.57E–03	–1.09	57.95	101.58	72.42	107.18	–
hsa-miR-3116	–	2.46E–07	–1.82	183.19	542.86	208.73	556.81	–
hsa-miR-3662	–	1.12E–02	–1.36	6.40	14.44	15.71	34.62	–
hsa-miR-660-3p	–	2.78E–03	–1.85	20.19	107.52	367.56	168.96	–
hsa-miR-6891-3p	–	2.89E–02	–1.57	28.10	120.50	384.50	161.85	–
hvu-miR6203	–	5.06E–06	–2.35	9.59	29.96	6.75	43.92	–
mdo-miR-1547-5p	–	4.61E–07	–2.44	20.18	126.94	130.67	304.67	–
mdo-miR-7286-3p	–	2.28E–05	–2.12	7.04	44.76	16.58	54.72	–
mdo-miR-7360-5p	1.28E–04	–	–2.49	29.04	23.62	7.49	1.69	–
mdv2-miR-M14-5p	–	3.62E–05	–1.81	99.47	485.26	237.90	531.00	–
mml-miR-607	–	1.16E–02	–1.60	3.93	29.92	20.47	31.73	–
mmu-miR-468-5p	–	8.05E–03	–1.29	8.26	19.86	19.95	35.76	–
mmu-miR-6240	–	7.98E–04	–1.22	96.91	151.37	80.90	181.26	–
mmu-miR-7078-3p	3.22E–06	–	–2.82	37.66	21.51	2.12	5.53	–
mse-miR-2a	–	4.47E–02	–0.70	45.31	53.50	50.30	70.35	–
mse-miR-750	–	3.68E–02	–0.76	54.83	96.39	110.63	103.77	–
oan-miR-1341	–	4.57E–03	–2.45	4.71	46.62	26.39	122.53	–
oan-miR-1344	–	1.04E–02	–1.91	6.31	21.08	15.89	71.74	–
osa-miR1425-3p	–	4.61E–07	–2.66	5.60	21.47	5.08	43.00	–

continued

Table 1:. (continued)

miRNA Identity	Haplogroup FDR	Temperature FDR	Log fold change	Average HA counts	Average CA counts	Average HB2 counts	Average CB2 counts	Known function
pma-miR-4626	–	8.36E–05	–1.70	53.64	168.18	221.63	313.69	–
rlcv-miR-rL1-5-5p	–	1.58E–03	–1.79	5.32	24.58	18.01	40.34	–
sme-let-7c-5p	–	3.08E–02	–0.82	48.82	66.37	64.92	92.93	–
sme-miR-133b-3p	–	3.08E–02	–0.74	96.99	111.34	77.53	115.92	–
sme-miR-87a-3p	–	3.97E–02	0.66	121.83	44.08	133.30	74.32	–
tca-miR-12-5p	–	2.84E–06	–2.75	1.02	19.73	7.11	33.94	–
tca-miR-279c-3p	–	4.63E–03	–1.51	13.38	46.88	43.64	80.54	–
tca-miR-279e-3p	–	1.02E–02	–1.26	11.55	30.85	25.05	39.95	–
tca-miR-2944a-3p	–	1.17E–02	–1.38	7.03	24.70	18.17	37.52	–
tca-miR-31-5p	–	4.57E–03	–1.54	5.40	19.70	11.27	24.93	–
tca-miR-3828-3p	–	3.08E–02	–1.57	6.85	29.45	50.84	43.43	–
tur-miR-12a-5p	–	1.81E–04	–1.93	7.37	35.53	21.43	63.20	–
tur-miR-34-5p	–	1.79E–02	–1.10	15.28	24.88	17.69	33.55	–
tur-miR-5738-5p	–	7.98E–04	–1.66	10.52	28.14	19.59	60.43	–
zma-miR395d-5p	8.78E–14	–	5.11	1.94	0.21	66.67	50.32	Thought to target mRNAs coding for ATP sulphurylases, upregulated in *Arabidopsis* during sulphate-limited conditions

A total of 64 DE miRNAs were detected between temperature treatments and six DE miRNAs between mitochondrial haplogroup. FDRs represent the level of significance determined by *edgeR*. Positive and negative log fold changes indicate increased and decreased expression, respectively. For miRNAs with temperature FDRs, log fold change represents high temperature relative to control temperature. For miRNAs with haplogroup FDRs, log fold change represents B2 haplogroup relative to A haplogroup. Average number of counts are noted for: high temperature A mitochondrial haplogroup (HA); control temperature A mitochondrial haplogroup (CA); high temperature B2 mitochondrial haplogroup (HB2) and control temperature B2 mitochondrial haplogroup (CB2). Although most miRNAs in miRBASE do not have a known function, functions that have been suggested are included in the table.

Table 2: differentially expressed (DE) piRNAs in response to temperature treatment and mitochondrial haplogroup

piRNA Identity	Haplogroup FDR	Temperature FDR	Log fold change	Average HA counts	Average CA counts	Average HB2 counts	Average CB2 counts
MIWI2-169803	–	0.0011	–1.46	17.86	99.16	46.50	88.91
MIWI2-169803	–	–	–1.27	19.88	65.10	39.41	57.44
MIWI2-169819	0.0291	–	–5.45	25.82	54.16	0.00	0.00
MIWI2-189701	–	0.0134	–1.13	50.63	173.49	146.45	247.78
MIWI2-251905	–	0.0011	–2.05	6.75	69.09	0.00	0.00
MIWI2-265104	0.0010	–	–4.52	342.08	673.85	12.99	11.04
MIWI2-278992	–	0.0413	–1.97	13.96	153.56	0.91	3.85
MIWI2-278992	0.0047	–	–3.92	13.96	153.56	0.91	3.85
MIWI2-291701	0.0042	–	–4.86	37.22	307.10	1.11	3.41
MIWI2-370324	0.0291	–	–5.58	27.26	61.73	0.00	0.00
MIWI2-407429	–	0.0172	–1.14	18.10	77.70	44.53	92.16
MIWI2-521209	–	0.0011	1.24	58.97	30.72	41.25	29.30
MIWI2-521209	–	–	1.14	57.36	24.17	30.32	28.73
MIWI2-531856	0.0410	–	–4.53	20.55	22.42	0.00	0.23
MIWI2-614295	0.0123	–	–6.19	77.93	79.35	0.31	0.00
MIWI2-622507	0.0030	–	–6.97	39.37	482.45	0.79	0.00

A total of eight DE piRNAs were detected between haplogroups and six between temperature treatments. FDRs represent the level of significance determined by *edgeR*. Positive and negative log fold change indicate increased or decreased expression, respectively. For piRNAs with temperature FDRs, log fold change represents high temperature relative to control temperature. For piRNAs with haplogroup FDRs, log fold change represents B2 haplogroup relative to A haplogroup. Average number of counts are noted for: high temperature A mitochondrial haplogroup (HA); control temperature A mitochondrial haplogroup (CA); high temperature B2 mitochondrial haplogroup (HB2) and control temperature B2 mitochondrial haplogroup (CB2).

A and B2 haplogroups in central Panamá is particularly intriguing given the strong effect of haplogroup on sperm competitive ability. In two-male sperm competition experiments, DNA profiling has demonstrated that B2 males sire 264% more offspring than A males, and this B2 competitive advantage cannot be explained by female mitochondrial haplogroup or male nuclear genetic background [56]. RNA sequencing of large RNA libraries implicates differential expression of 11 mitochondrial oxidative phosphorylation genes in the B2 competitive advantage, including a >20-fold upregulation of *atp8* in B2 males [Zeh et al., unpublished data]. Whether differential expression of sncRNAs contributes to the B2 sperm competitive advantage remains to be investigated.

Recent studies suggest that the microbiome of a species can modify epigenetic regulation in host cells, including the expression of ncRNAs [69]. While it was confirmed that none of the males used in our sncRNA expression analyses was infected with the common bacterial endosymbiont, *Wolbachia*, it is

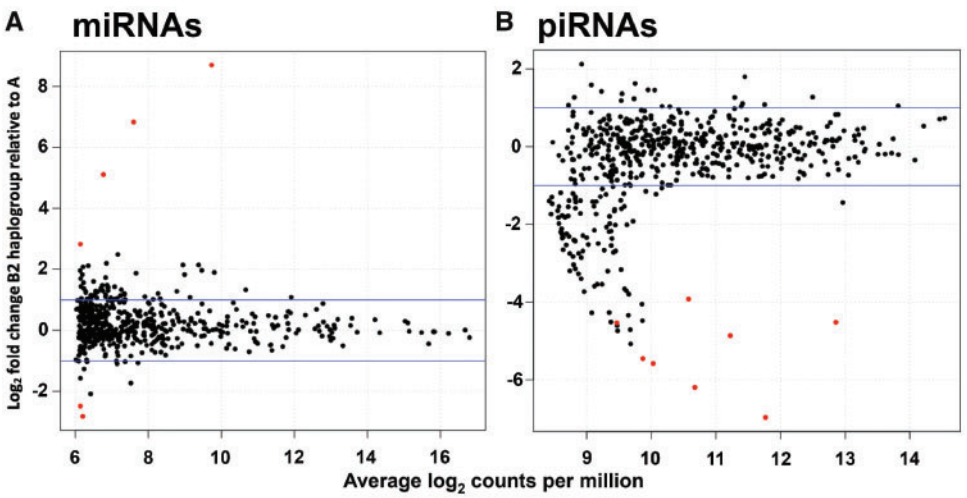

Figure 5: smear plots of (A) miRNAs and (B) piRNAs showing $\log_2$-fold changes in expression level for the B2 mitochondrial haplogroup relative to the A haplogroup as a function of expression level. Significantly differentially expressed sncRNAs are indicated in red. Blue lines indicate a one log fold change for either increased and decreased expression of B2 relative to A

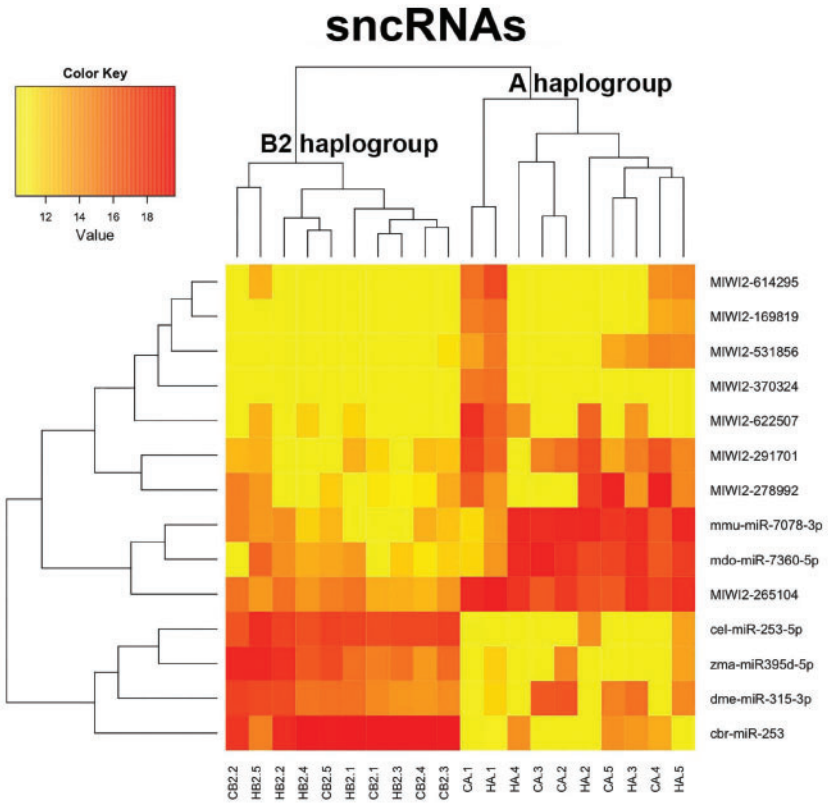

Figure 6: a heatmap showing expression levels of six significantly differentially expressed (DE) miRNAs and eight DE piRNAs in B2 and A haplogroups. For these 14 DE sncRNAs, the A and B2 haplogroup samples cluster into two distinct clades as indicated in the dendrogram

important to recognize that temperature-mediated changes in the species composition and abundance of the *C. scorpioides* microbiome could be a factor contributing to the differential expression of sncRNAs detected in this study.

In theory, *C. scorpioides* individuals could avoid temperature-induced epigenetic disruption by strategically exploiting relatively cool microhabitats, a topic briefly considered elsewhere [53]. Ongoing research, employing temperature loggers to record diurnal temperature fluctuations in the range of habitat types utilized by *C. scorpioides* in central Panamá, indicates that mean daily temperatures in all of these microhabitats exceed those in full shade by at least 1°C. However, diurnal fluctuations vary greatly with host tree type (standing versus fallen), microhabitat type (under bark on the top, side or underneath of branches, or in accumulated wood-boring beetle frass), and tree exposure to solar radiation (closed versus open canopy). Diurnal temperature fluctuations are maximal on the top surfaces of fallen trunks in open canopy habitats where daily maximum

temperatures can exceed 43°C and diurnal fluctuations can exceed 20°C. Diurnal temperature fluctuations are minimal in standing trees in closed canopy habitats and in the frass microhabitats of all trees. While tree and microhabitat effects on diurnal temperature regimes appear to have important implications for *C. scorpioides* abundance, it seems unlikely that microhabitat selection could mitigate the long-term effects of global warming on this tropical ectotherm.

In *C. scorpioides*, exposure to a large and abrupt increase in temperature severely compromised reproductive function [53, 54] and was associated with altered, and apparently disrupted, sncRNA expression. More generally, a fundamental question in evolutionary biology is whether altered epigenetic regulation augments or hinders adaptive evolution [26–30, 34, 70]. Epigenetic 'mutations', that is, changes in DNA methylation, chromatin states and the diversity and/or abundance of ncRNAs, typically exhibit mutation and reversion rates orders of magnitude greater than DNA sequence based (genetic) mutations [27, 71]. In a model in which epigenetic variation was assumed to be random with respect to fitness, epigenetic mutations exerted three qualitatively different effects on adaptation, depending on their stability and fitness effects relative to genetic mutations [70]. Large-effect epigenetic mutations tended to slow adaptation and result in lower equilibrium fitness, whereas small-effect epigenetic mutations either slowed early adaptation but allowed populations to attain higher final fitness or, alternatively, accelerated early adaptation at the cost of increased mutational load and lower final fitness [70]. Finally, in the model simulations, epigenetic changes were ultimately replaced by genetic mutations, suggesting that epigenetic mutations are most important in early responses to environmental change ([70]; see also [27]). Interestingly, recent empirical studies provide evidence of epigenetic mutations in the initial steps of divergence between populations of birds [72], fish [73] and mammals [74]. In addition, it has now been established that environmentally induced epigenetic mutations can precipitate genetic changes [75].

The random-walk model [70] discussed above represents only the tip of the iceberg with respect to the possible consequences of epigenetic inheritance for evolutionary dynamics. Other modelling frameworks assume that heritable epigenetic changes are environmentally induced, based on accumulating evidence that parents bestow an environmental legacy on offspring by transmitting acquired epigenetic changes through eggs and sperm [76–79]. Parental exposure to environmental stimuli that modify epigenetic states can directly affect both somatic and germline tissues, resulting in intergenerational epigenetic effects [80]. In gravid, viviparous pseudoscorpion females, direct environmental induction impacts three generations simultaneously: maternal somatic tissue in the F0 generation; foetal somatic tissue in the F1 generation, and foetal primordial germ cells that will contribute to the F2 generation, as it does in gestating female mammals [81]. In males, direct exposure is restricted to two generations, paternal somatic tissue and sperm. Models positing environmentally induced intergenerational and transgenerational epigenetic effects indicate that the adaptive value of epigenetic inheritance depends on the pace of environmental change, the level of environmental heterogeneity, life history characteristics, and the correlation between environmental conditions experienced by offspring and parents [26, 27, 29, 33, 34] These theoretical predictions are supported by empirical studies, demonstrating the adaptive value of transgenerational phenotypic plasticity as a buffer against environmental change [25, 30, 32]. For example, in *Arabidopsis*, exposure of the parental generation to high temperature resulted in a 5-fold increase in fitness in the F3 generation [25]. Similarly, in experiments carried out on *D. melanogaster*, offspring fitness increased linearly with the temperature experienced by their parents over a range of constant temperatures from 18°C to 29°C [82].

It is important to recognize, however, that not all intergenerational epigenetic effects are adaptive, as evidenced by numerous studies of humans and model organisms, demonstrating stress-induced transmission of altered epigenetic states that disrupt the phenotype, reduce fertility and increase disease susceptibility [76, 77, 83, 84]. Epigenetic mechanisms involve fundamental metabolic pathways that are fuelled by mitochondria and are energetically expensive to maintain [18, 19, 85]. Exposure to extreme environmental conditions can disrupt the intricate processes involved in the epigenetic regulation of gene expression and attenuate the suppression of TE sequences [86]. Future theoretical and empirical studies should seek to identify the conditions under which epigenetic modifications facilitate or impede adaptive responses to environmental challenges.

Conclusion

Our study demonstrates that chronic heat stress causes changes in testicular epigenetic profiles that may at least partially account for reduced reproductive function in *C. scorpioides* males exposed to increased temperature, and that mitochondrial DNA polymorphism, through its effects on epigenetic regulation, may provide the potential for an adaptive evolutionary response to climate warming. Since tropical terrestrial arthropods constitute the vast majority of animal species, the findings of this study have important implications for understanding the consequences of climate change for global biodiversity.

Materials and Methods

Study Organism and Experimental Design

Experimental pseudoscorpions were drawn from a large laboratory population established from 350 *C. scorpioides* adults and nymphs collected in 2006 and 2008 from six locations spanning a 60 km region in central Panamá [53]. In this laboratory population, pseudoscorpions were reared and maintained in individual vials to ensure virginity, and matings were staged to maintain a large number of field-collected matrilines. Each generation, no matings were carried out between full siblings, half siblings or first cousins in order to minimize inbreeding. Within these constraints, pairs for mating were chosen randomly without regard to mitochondrial haplogroup. Because random mating between haplogroups was performed for a minimum of 16 generations, individuals from the two haplogroups were effectively homogenized for nuclear genetic background. To avoid possible confounding effects of *Wolbachia* infection on sncRNA expression, *Wolbachia*-specific MLST PCR assays were used to confirm that all *C. scorpioides* matrilines used in the study were uninfected with *Wolbachia* (for methodology, see [87]).

To investigate the effects of climate warming and mitochondrial haplogroup on sncRNA expression, a split-brood experimental design was used in which 40 first-stage nymphs (protonymphs) from five A and five B2 mitochondrial haplogroup females were randomly assigned to either a diurnally fluctuating control (C) or a diurnally fluctuating high temperature (H) regime for rearing from birth to the adult stage (for details, see [53]). Climate warming effects were simulated in the

high temperature treatment by elevating the average temperature 3.5°C above the control temperature. The control temperature was estimated from long-term, daily high and low temperature records from *C. scorpioides*' native habitat in central Panamá [53].

Testicular Tissue Dissection and RNA Extraction

Five full-sibling families for each of the two mitochondrial haplogroups were randomly selected for testicular tissue dissection and RNA extraction. To obtain sufficient RNA, dissected testes from four young adult males were pooled within control and high temperature treatments for each of the 10 full-sibling families. Males were frozen in liquid nitrogen, dissected under 20–40× magnification, and the testes surgically removed [88]. Total RNA was purified into small (<200 nucleotides) and large (>200 nucleotides) fractions using a PureLink® miRNA Isolation Kit in combination with a PureLink® RNA Mini Kit (ThermoFisher Scientific, Waltham, MA, USA). Purified RNA samples were stored at –80°C for further downstream processing prior to sequencing.

Small RNA Library Preparation and Next Generation Sequencing

The 20 small RNA fractions were first analysed using an Agilent Small RNA Analysis kit (Agilent Technologies, Inc., Santa Clara, CA, USA) to determine total concentration of small nucleic acid sequences (6–150 nt), and the percentage of miRNA within each sample. All sequenced samples exceeded the minimum requirement for small RNA library preparation of 1 ng of miRNAs in at most 1 μg of total RNA in a 3 μl volume. One control temperature and one high temperature small RNA testes sample from each of five A-haplogroup and five B2-haplogroup families were submitted for sequencing in two blocks of ten samples each. In total, there were five replicates of each temperature treatment and mitochondrial haplogroup combination: high temperature A-haplogroup (HA.1–HA.5), control temperature A-haplogroup (CA.1–CA.5), high temperature B2-haplogroup (HB2.1–HB2.5) and control temperature B2-haplogroup (CB2.1–CB2.5). Because of shallow sequencing depth, the second block of samples was re-submitted for sequencing. Small RNA library preparation and sequencing were carried out by the Nevada Genomics Center at the University of Nevada, Reno. Samples were first concentrated by speed vac prior to library preparation. Since the lowest amount of small RNA in all 20 samples was 7 ng, libraries were prepared, starting with 7 ng of small RNA, according to manufacturer's protocols for small RNA samples using the Life Technologies' Ion Total RNA-Seq Kit v2 Library Kit (ThermoFisher Scientific, Waltham, MA, USA). In order to distinguish sample identities, each sample was barcoded with a unique adaptor sequence using the Ion Express™ RNA-Seq Barcodes, following manufacturer's protocols. Library size verification and quantitation were performed using the Agilent High Sensitivity DNA Kit (Agilent Technologies, Inc., Santa Clara, CA, USA). Templated Ion Sphere Particles (ISPs) were prepared using Life Technology's Ion PI Template OT2 200™ Kit version 2. Sequencing was performed on a Life Technologies Ion Torrent Proton Sequencer using the Life Technology's Ion PI™ Sequencing 200 Kit™ version 2 and Life Technologies' Ion PI™ v2 Chip™. Across the 20 small RNA samples sequenced, the mean read length was 20 bp and the mean sequencing depth was 2.7 M reads, with a range of 0.5–4.7 M reads.

sncRNA Transcriptome Analysis

sncRNA annotation and expression analyses were adapted from the computer-assisted annotation pipeline and Sequery, an annotation software program [58]. Since the first sequencing run from the second block of small RNA samples resulted in shallow sequencing depth, the first sequencing run and the second sequencing run for the second block were combined. Sequencing files from the second block were trimmed to provide an average of 3.5 M reads, the same average from the first block of samples submitted for sequencing. Raw sequencing files were trimmed of their adapters and separated by barcode using the Torrent Suite 4.0.2 software. Sequences <15 nt were removed and single files were split into sub-files containing, at most, 500 000 sequences to accommodate limitations in Sequery. Since Ion Torrent sequencing quality scores are >Q20 until approximately the 75th base addition, we did not perform quality trimming, given the targeted sequence lengths of 24–32 nucleotides. The fastq files for the 20 small RNA sequencing libraries have been deposited in the NCBI Short Read Archive under accession numbers (SRR7971701–SRR7971720).

For sncRNA identification and expression, sequenced reads were compared with deduplicated reference files containing ~30 000 identified miRNA sequences from miRBase [89] and ~1 000 000 piRNA sequences from piRNA clusters [58] under a 1–2 nucleotide substitution allowance, non-exhaustive match parameter using Sequery [58]. Sequence comparison/annotation output files contained the abundance of each matched sequence, a sncRNA sequence identity, where the match occurred in the reference sequence, and the percentage difference between each match [58]. The miRNA and piRNA reference databases are available as Supplementary Files S01–S05.

Output files were converted to count tables containing only sequence identity and abundance of sequenced reads and sequence counts were normalized to counts per million (CPM) based on sequencing depth alone [90]. To eliminate pseudocounts or false positives, the total abundance for each sncRNA from all 20 sequenced samples was sorted from highest to lowest, summed from one identity to the next, and plotted against the cumulative number of sncRNAs (Supplementary Figs S1 and S2). Very low abundance sequences, likely to represent false positives, were eliminated by excluding sncRNA identities that did not contain at least ten sequenced counts in any of the 20 sequenced samples, and did not meet a threshold abundance of three or more normalized transcripts averaged across the 20 samples. These threshold abundance criteria eliminated sequences contributing negligibly to overall transcript abundance (Supplementary Figs S1 and S2), and resulted in the inclusion of 517 miRNAs and 585 piRNAs for differential expression analyses. A description of the complete Sequery analysis pipeline is provided as Supplementary Fig. S3. Python scripts for editing sequence files are available as Supplementary Files S06–S11.

Statistics and Bioinformatics

Separate files containing total miRNA and piRNA sequence counts were loaded into R statistical software and tested for significant differential expression using edgeR, a Bioconductor software package that allows for examination of differential expression of replicated count data [57]. This program uses an overdispersed Poisson model to account for biological and technical variability and empirical Bayes methods to moderate the degree of overdispersion across transcripts [57]. Analyses were performed using generalized linear models to examine the

effects of temperature increase, mitochondrial haplogroup and temperature × haplogroup interactions on sncRNA expression. Analyses that examined expression differences between temperature treatments and interactions with mitochondrial haplogroups included family identification as a blocking factor. Significant DE sncRNAs and genes were represented with a false discovery rate (FDR) equal to or >0.05, which controlled for the expected proportion of incorrectly rejected null hypotheses in multiple comparisons. Average abundances of miRNAs and piRNAs were tested for significance against temperature treatment, mitochondrial haplogroup and the interaction between temperature treatment and mitochondrial haplogroup. The edgeR commands for carrying out the above analyses are available as Supplementary Fig. S3.

Supplementary Data

Supplementary data are available at *EnvEpig* online.

Conflict of interest statement. None declared.

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