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RESEARCH ARTICLE

Disproportionate role of nuclear-encoded proteins in organismal and mitochondrial thermal performance in a copepod

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ABSTRACT

Determining the mechanisms by which organisms evolve thermal tolerance is crucial to predicting how populations may respond to changes in local temperature regimes. Although evidence of relationships between mitochondrial background and thermal adaptation have been found, the presence of both nuclear-encoded and mitochondrial DNA (mtDNA)-encoded proteins warrants experiments aimed at parsing out the relative role of each genome in thermal adaptation. We investigated the relative role of mtDNA-encoded products in thermal tolerance between two divergent populations of *Tigriopus californicus* using first-generation (F_1) hybrids that vary in maternally inherited mtDNA but are heterozygous for population-specific alleles across nuclear loci. We tested two measures of thermal tolerance, (1) survivorship to acute thermal stress and (2) thermal stability of mitochondrial performance in Complex I-fueled ATP synthesis, both across a range of increasing temperatures. We found that the southern population (San Diego, CA, USA) outperformed the northern population (Strawberry Hill, OR, USA) in survivorship, and that both reciprocal F_1 hybrid crosses had intermediate survival. Mitochondria from the San Diego population displayed greater stability in ATP synthesis with increasing temperatures compared with those from Strawberry Hill. Interestingly, hybrids from both cross directions had synthesis profiles that were very similar to that of Strawberry Hill. Taken together, these results suggest that the relative role of the mtDNA in these phenotypes is negligible compared with that of elements encoded by nuclear DNA in this system.

KEY WORDS: Mitochondria, Hybridization, Adaptation, Thermal tolerance

INTRODUCTION

Heterogeneity in environmental temperature across a species' range creates the potential for distinctly different local environments. Allopatric populations, which are isolated from one another by geographical constraints and exhibit little gene flow, may experience selection at different intensities and on different sets of traits within these local temperature environments. This localized selection can lead to the rise of locally adapted alleles within allopatric populations that result in divergent thermal tolerance phenotypes (Hoekstra et al., 2006; Nosil et al., 2009; Sanford and Kelly, 2011), and plays a major role in determining the limits of a species' range (Deutsch et al., 2015; Pörtner, 2010; Sunday et al., 2011, 2012). Rising temperatures associated with global climate change can increase pressure on marine organisms to adapt or migrate.

Although thermal physiology and adaptation are commonly studied in many taxa, the role of mitochondrial genome evolution in organismal thermal tolerance has received less attention. Historically, the mitochondrial genome has been used as a neutral marker when investigating population genetics and phylogeography (Moritz et al., 1987). However, its involvement in important physiological functions and often increased rate of evolution suggest that mitochondrial genomes have the potential to respond to natural selection due to environmental pressures (Ballard and Kreitman, 1995; Ballard and Rand, 2005; Dowling et al., 2008; Rand, 2001). Indeed, evidence has recently accumulated that geographic variation in temperature regimes can sort mitochondrial genomes into distinct haplotypes among natural populations. Studies in multiple animal taxa, including mammals (Fontanillas et al., 2005), birds (Cheviron and Brumfield, 2009), gastropods (Quintela et al., 2014) and fish (Consuegra et al., 2015), have documented clear changes in mtDNA haplotype frequencies along temperature clines in nature. A few have also reported adaptive evolution at the amino acid level in mtDNA-encoded genes when sampled across populations varying in thermal environments (Foote et al., 2011; Ma et al., 2015; Morales et al., 2015). These patterns have similarly been documented in human populations (Balloux et al., 2009; Mishmar et al., 2003).

These results, however, are largely based on correlations between temperature gradients and mtDNA variation, which can be strongly influenced by non-selective processes. More importantly, such population genetic studies do not account for the parallel variation in nuclear-encoded mitochondrial proteins and their relative contributions to thermal tolerance phenotypes. Although mitochondrial DNA (mtDNA) encodes ∼13 protein-coding genes and ∼20–23 RNAs, the organelle houses an additional ∼500–1000 different nuclear-encoded gene products, which include ∼60–75 proteins in the oxidative phosphorylation (OXPHOS) pathway (reviewed in Burton and Barreto, 2012) and three heat shock protein chaperones (mtHSPs; Bender et al., 2011). Hence, the thermal sensitivity of mitochondrial function and its impact on organismal survival rely on the contributions of both the nuclear- and mtDNA-encoded proteins within the organelle. Adaptation in this context can occur on nuclear protein functions directly or on mitonuclear interactions. We argue that much of the geographic variation observed in organellar function may be due to the nuclear-encoded components that participate in thermal response, and that the relative contribution of each genome to local adaptation in mitochondrial function during thermal stress is still unresolved.

In a few natural systems, the presence of hybrids or admixed individuals have permitted comparisons of thermal tolerance between individuals carrying divergent mitochondrial genomes.

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Coyle et al. (2019) examined a sharp cline in mitochondrial *COI* sequences along Eastern North American populations of European green crabs (*Carcinus maenas*) and used diagnostic nuclear markers to identify individuals that vary in mtDNA but have nuclear genomes admixed between northern and southern populations. Laboratory assays then revealed that the presence of northern *COI* haplotypes in male (but not female) hybrids increased their ability to right themselves in cold water. Variation in the few nuclear loci examined was not associated with this cold-tolerance phenotype. In contrast, Healy et al. (2018) found no association between mitochondrial haplotypes and high thermal tolerance in Atlantic killifish (*Fundulus heteroclitus*) admixed along a sharp mtDNA latitudinal cline. A possible limitation of such studies is the technical inability to track nuclear-encoded mitochondrial genes in these admixed individuals. This leaves open the possibility that co-segregation of adapted nuclear loci contributes to some of the observed patterns, perhaps as a result of selection for mitonuclear coadaptation that can occur within natural hybrid zones (Nikelski et al., 2023; Wang et al., 2021).

Experimental work that can control for both nuclear and mtDNA backgrounds provide additional power for examining this issue. Experiments using natural genetic variation in Australian *Drosophila melanogaster*, for example, provide some of the strongest evidence that spatial distribution of mtDNA alleles themselves can result from natural selection by climactic heterogeneity. After documenting a latitudinal cline in mtDNA of Australian *D. melanogaster*, Camus et al. (2017) generated strains of flies that differed in mtDNA haplotypes (from 'warm' or 'cool' populations) but that shared an isogenic nuclear background from a distant subpopulation. Their experimental assays revealed that fly strains harboring haplotypes from warm populations had higher heat tolerance and lower cold tolerance than strains with cool-origin haplotypes. A laboratory evolution experiment by Lajbner et al. (2018) using admixed flies carrying the above regional mtDNA haplotypes further showed that long-term elevated temperatures caused a shift in frequency favoring the warm-adapted haplogroups.

These recent results thus suggest that evolution at the mtDNA level can indeed result from selection for thermal performance, but more work is needed to understand general processes of mitochondrial evolution and its contribution to variation in thermal performance. In the present study, we tested the relative role of mtDNA background in the local adaptation of thermal performance using the intertidal copepod *Tigriopus californicus*. *Tigriopus californicus* is a harpacticoid copepod that inhabits splash pools in the high intertidal and supralittoral zones along the western coast of North America, from southern Alaska, USA, to central Baja, Mexico (Burton, 1997). Because of its benthic life cycle and restriction to high rocky pools, there is very low connectivity between populations (Burton, 1997), and extreme levels of mtDNA divergence can occur (>20% divergence in some population comparisons; Barreto et al., 2018; Burton et al., 2007; Han and Barreto, 2021; Willett and Ladner, 2009). Additionally, local adaptation to high temperatures has been observed across populations of *T. californicus*, with upper thermal limits increasing broadly with decreasing latitude, but also with some mosaic patterns at finer scales (Kelly et al., 2012; Leong et al., 2018; Pereira et al., 2017; Willett, 2010; Willett and Son, 2018). At the level of the mitochondria, Harada et al. (2019) identified local adaptation in mitochondrial stability and ATP synthesis rates under thermal stress in this species, and Healy and Burton (2023) correlated loss of mitochondrial performance at high temperatures with organism upper thermal tolerance limits between populations. Together, these results suggest that local adaptation in mitochondrial function may play a role in the survival of the organism during thermal stress. Nevertheless, these studies cannot tell us the relative impact of nuclear- or mtDNA-encoded proteins in shaping variation in organellar performance at increasing temperatures.

Here, we performed a test of the role of mtDNA background in thermal tolerance by comparing organismal and organellar phenotypes among two divergent populations and their reciprocal first-generation (F_1) hybrid populations. Because reciprocal F_1 hybrids will have equivalent nuclear backgrounds, we can examine the impact of maternally inherited mtDNA by comparing the two hybrid crosses and how they differ from the parental-level phenotypes. Previous studies in F_1 hybrids of *T. californicus* have focused on comparisons with parental populations for survivorship phenotypes. Overall, they suggest that levels of F_1 survival following heat stress may depend on which populations are crossed; crosses between populations with greater differences in tolerance tend to produce F_1 hybrids with intermediate upper thermal limits (Griffiths et al., 2020; Pereira et al., 2014; Willett, 2012), whereas crosses between equally tolerant parents can produce F_1 hybrids with higher tolerance (Pereira et al., 2014; Willett, 2012). The present study characterized survival and mitochondrial function (change in ATP synthesis capacity) of two genetically and ecologically divergent populations of *T. californicus* and explored how their nuclear and mitochondrial genomes contribute to thermal performance using F_1 hybrids. We hypothesized that although F_1 hybrids may show intermediate phenotypes relative to their parental stocks, when compared with each other, hybrids with mtDNA from the more tolerant parent population will outperform those with the reciprocal organellar genome during thermal stress.

MATERIALS AND METHODS

Copepod collection and culture

Tigriopus californicus (Baker 1912) were collected from high rocky tidepools in Strawberry Hill Wayside, OR, USA (SH, 44.25°, −124.11°), and San Diego, CA, USA (SD, 32.45°, −117.25°). These populations exhibit high mitochondrial genetic divergence (11 to 35% across the encoded elements; Han and Barreto, 2021), and differ in thermal tolerance at the survivorship level (Kelly et al., 2012; Leong et al., 2018). Population stocks were reared in multiple 400-ml beakers containing 35 ppt salinity artificial seawater (ASW; Instant Ocean, Blacksburg, VA, USA) and fed a combination of live *Isochrysis* and *Nannochloropsis*, and ground dried *Spirulina* wafers (Hikari Sales USA, Hayward, CA, USA). Stocks and experimental plates were maintained at common garden conditions in incubators at 20°C, and with a 12 h:12 h light:dark cycle (Thermo Fisher Scientific, Marietta, OH, USA). Prior to use in any thermal exposure assays or in the creation of hybrid lines, population stocks were maintained in the laboratory for at least two generations.

We generated experimental populations of four genetic backgrounds: SH, SD and their reciprocal F_1 hybrid crosses (SH♀×SD♂ and SD♀×SH♂). To create non-hybrid populations from parental stocks, 100 mated females were collected from each stock population and mature egg clutches from those females were collected to found each experimental population. To create each hybrid population, unmated females were mated with males of the reciprocal population. Because females of *T. californicus* mate only once in their lifetime, we obtained 100 unmated females of each stock population by removing them from clasped pairs (formed between a mature male and a juvenile female) using a fine needle. Separated, unmated females of SH and SD were then mated with males from the reciprocal population. Egg clutches from these

mated females were collected in order to create the SH♀×SD♂ and SD♀×SH♂ populations. F_1 hybrid offspring from SH♀×SD♂ and SD♀×SH♂, and offspring from non-hybrid SH and SD experimental populations were reared in Petri dishes and then individuals were isolated into single wells of 24-well plates prior to reaching sexual maturity to prevent mating. Petri dishes and 24-well plates were dated to track age cohorts of offspring from picked egg clutches for each of the four populations.

Survivorship following acute thermal stress

Tolerance to a 1 h thermal stress at different temperatures was conducted using methods similar to those previously described for *T. californicus* (Pereira et al., 2014; Willett, 2010). Groups of 10 adult copepods of the same age cohort and sex for each population were transferred from plates to 15-ml tubes containing 5 ml of ASW. Tubes were acclimated in incubators (20°C) for 30 min before being submerged in a preheated water bath (VWR, Radnor, PA, USA) at one of four temperatures: 35, 35.5, 36 or 36.5°C. After 1 h at the stress temperature, tubes were returned to incubators and submerged in a 20°C water bath for at least 30 min for recovery. Copepods from each tube were then transferred to a well of a 6-well plate and kept under standard culturing conditions at 20°C. After 3 days, survivorship in each replicate was recorded as the proportion of surviving individuals in each group of 10. The full experiment encompassed each combination of the following factors: population (SH, SD, SH♀×SD♂ or SD♀×SH♂), sex (male or female), age cohort [20–25, 26–30, 31–35 or 36–40 days post-hatching (dph)] and stress temperature (35, 35.5, 36 or 36.5°C), with n=4 replicates (with 10 copepods in each) per combination of factors (for a total of 512 replicates).

ATP synthesis rate assays

The assay protocol used to measure thermal stability of ATP synthesis was similar to those from recent work in *T. californicus* (Harada et al., 2019; Healy et al., 2019). For each combination of population and sex, mitochondria were isolated from pools of copepods and assayed *in vitro* for their rate of ATP synthesis at five temperatures. Only copepods from the 26–30 dph age cohort were selected to avoid any age-related thermal tolerance effects identified during survivorship assays. For each assay replicate, mitochondria were isolated from groups of 24 copepods, and n=6–8 replicates were performed per combination of population and sex depending on the number of available individuals in each experimental population. The groups of 24 copepods were transferred to 1.5 ml microcentrifuge tubes and rinsed with 200 µl ice-cold homogenization buffer (400 mmol l^{-1} sucrose, 100 mmol l^{-1} KCl, 6 mmol l^{-1} EGTA, 3 mmol l^{-1} EDTA, 70 mmol l^{-1} HEPES, 1% w/v BSA, pH 7.6; Moyes et al., 1985) before being homogenized in 800 µl cold homogenization buffer with a plastic homogenizer. The homogenate was then centrifuged at 1000 ***g*** for 5 min at 4°C. The supernatant was transferred to new microcentrifuge tubes and then centrifuged at 11,000 ***g*** for 10 min at 4°C. The supernatant resulting from this second centrifugation was then discarded and the pellet was resuspended in 155 µl assay buffer (560 mmol l^{-1} sucrose, 100 mmol l^{-1} KCl, 10 mmol l^{-1} KH_2PO_4, 70 mmol l^{-1} HEPES, pH 7.6; modified from Moyes et al., 1985) and kept on ice until the assay was performed immediately following these steps.

ATP synthesis assays were conducted by incubating aliquots of mitochondrial isolates of each replicate simultaneously at five different temperatures (20, 30, 33, 36 and 39°C). For each replicate, 25-µl aliquots of mitochondrial isolate were dispensed into five wells across one row of a 96-well PCR plate, spaced so that each well could be exposed to a different temperature in a gradient-enabled thermocycler. Assays were performed simultaneously by adding 5 µl of a substrate cocktail to each well containing mitochondrial isolate, then incubating the entire plate in a thermocycler (Applied Biosystems, Waltham, MA, USA) for 15 min with each column incubated at a different assay temperature. The substrate cocktail used in the assay measured the ATP synthesis rate as a result of electron donation to OXPHOS Complex I (ADP, malate, pyruvate and glutamate) following Harada et al. (2019). Immediately following incubation at the target temperature, 25 µl of each assay mixture was transferred to a half-area plate well containing an equal volume of Celltiter-Glo (Promega, Madison, WI, USA), which halts ATP synthesis and allows for quantification of ATP concentration through luminescence. One additional 25-µl aliquot of mitochondrial isolate from each replicate was used to quantify 'initial' ATP concentrations in each assay prior to any incubation by combining the isolate with substrate cocktail and immediately transferring the mixture to a well of the half-area plate containing Celltiter-Glo. After all samples were added to the Celltiter-Glo, the half-area plate was incubated at room temperature for 10 min before being mixed by pipette and read for luminescence on a Tecan Spark plate reader (Tecan Genomics, Redwood City, CA, USA) alongside ATP standards ranging from 3.2 nmol l^{-1} to 10 µmol l^{-1}. Finally, ATP synthesis rates were calculated by subtracting the luminescence values of the 'initial' aliquots from those of the incubated aliquots. Rates were then normalized with protein content in the respective mitochondrial isolate, with protein concentrations quantified with NanoOrange Protein Quantitation Kit assays (Thermo Fisher Scientific, Waltham, MA, USA) using the remaining 5 µl of each mitochondrial isolate.

Statistical analysis

All statistical analyses were conducted in R (v3.6.1; https://www.r-project.org/) using the tidyverse package (v1.3.0; Wickham et al., 2019). All figures were generated using ggplot2 (v3.3.6; Wickham, 2016). We used generalized linear models (GLMs) with binomial distribution, ANOVA and Cohen's d effect size to identify and quantify statistically significant differences in survival after 1-h thermal stress using population, sex, age cohort and stress temperature as main factors. Effect size was calculated using the effectsize package (v0.6.0.7; Ben-Shachar et al., 2020). We used Tukey *post hoc* tests to make pairwise comparisons between levels in each variable. Survivorship data were also used to quantify median lethal temperature (LD_{50}) using the dose function in the MASS package (v7.3-51.4; Venables and Ripley, 2002).

To quantify differences in mitochondrial thermal stability, changes in ATP synthesis rate within each population were tested one at a time with temperature as a main factor in an ANOVA. *Post hoc* analysis was conducted using Tukey tests. Welch's two-sided t-tests and Cohen's d effect size tests were performed to compare the size of differences in mean ATP synthesis rates between adjacent temperature treatments within each population. All statistical tests were conducted with a threshold for statistical significance of α=0.05.

RESULTS

Survivorship following acute thermal stress

GLM and ANOVA detected a significant effect of increasing temperature on survival proportion across all populations, both sexes and all age cohorts (Table 1). Analysis of survivorship showed distinct differences among populations with regards to thermal tolerance. GLMs estimated that the median lethal temperature (LD_{50}), when examined across all sex and age categories, was

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Table 1. ANOVA for main effects of temperature, genetic background (population), sex and age on survivorship after heat stress in *Tigriopus californicus* copepods

Factor	d.f.	SS	MS	*F*	*P*
Temperature	3	59.83	19.944	457.785	$<2\times10^{-16}$
Population	3	12.41	4.137	94.964	$<2\times10^{-16}$
Sex	1	0.34	0.338	7.762	0.00554
Age	3	1.12	0.374	8.577	1.46×10^{-5}
Residuals	501	21.83	0.044		

highest in SD (36.35°C, s.e.=0.025), lowest in SH (35.41°C, s.e.=0.020) and intermediate in the hybrid crosses (SH♀×SD♂: 35.95°C, s.e.=0.016; SD♀×SH♂: 35.82°C, s.e.=0.018). Congruently, the ANOVA also detected population to be a significant main factor in survival (Table 1). *Post hoc* analyses suggested that SD had a significantly higher survival proportion than each of the other populations, whereas SH had significantly lower survival than the other populations, which is reflected in the high Cohen's *d* statistics of these comparisons (Table 2). The two hybrid populations were statistically intermediate to both parents and did not differ significantly from one another (P=0.12, d=0.13), suggesting that mtDNA background did not affect survival in the hybrids. Fig. 1 shows survival proportion in each population by exposure temperature, and reflects the intermediate survival seen in both hybrid populations compared with SH and SD parental populations. At 35°C and 35.5°C, both hybrids mirrored the survival of the more tolerant SD, but above these temperatures their survival dropped sharply and matched that of SH at the highest temperature (Fig. 1).

When sex and age were included as main factors, an ANOVA detected that they also showed significant variation in survival (Table 1), but this was only after accounting for other factors in the model. In single factor models, neither sex (P=0.18) nor age (P=0.11) were significant contributors to survival. With regards to sex, *post hoc* analysis of the full ANOVA model revealed that females had a slightly higher survival compared with males, across populations and ages combined (Table 2, Fig. S1). Variation across age groups occurred at only one cut-off: the youngest age group (20–25 dph) had a higher survival when compared with all other age groups, whereas the other three age groups were not found to be significantly different from one another (Table 2, Fig. S2).

Table 2. *Post hoc* pairwise comparisons of survivorship in *T. californicus* copepods

Comparison	Difference	Adjusted *P*-value	*d*
Genetic background			
SH – SH♀×SD♂	−0.264	1.39×10^{-10}	0.63
SH – SD♀×SH♂	−0.206	1.39×10^{-10}	0.49
SH – SD	−0.436	1.39×10^{-10}	1.19
SD – SH♀×SD♂	0.172	8.54×10^{-10}	0.45
SD – SD♀×SH♂	0.229	1.39×10^{-10}	0.59
SH♀×SD♂ – SD♀×SH♂	0.058	0.120	0.13
Sex			
Males – females	−0.051	0.0055	0.12
Age cohort (dph)			
[20–25] – [26–30]	0.101	7.11×10^{-4}	0.24
[20–25] – [31–35]	0.101	6.79×10^{-4}	0.24
[20–25] – [36–40]	0.118	4.28×10^{-5}	0.28
[26–30] – [31–35]	0.000	0.999	0.00
[26–30] – [36–40]	0.017	0.910	0.04
[31–35] – [36–40]	0.017	0.915	0.04

Shown are *P*-values from Tukey multiple comparisons tests (α=0.05) and Cohen's *d* effect size test statistics for each comparison. SD, San Diego; SH, Strawberry Hill; dph, days post-hatching.

Thermal stability in ATP synthesis rates

Within each population, we compared changes in *in vitro* Complex I-driven ATP synthesis rates across a gradient of temperatures to determine whether mitochondrial physiological function at stressful temperatures was affected by mtDNA background in hybrid populations. ANOVA tests within each population indicated that temperature had a significant effect on ATP synthesis rate (SH: $P=1.84\times10^{-4}$, SD: P=0.0554, SH♀×SD♂: $P=9.24\times10^{-5}$, SD♀×SH♂: $P=3.54\times10^{-5}$). Results of the Welch's two-sided *t*-tests and Cohen's *d* effect size tests comparing mean ATP synthesis rates at adjacent temperatures within each population are summarized in Table 3. Fig. 2 shows mean ATP synthesis rates for each population at each assay temperature and reflects the differences in rates across assay temperatures. Between 20°C and 30°C, ATP synthesis rates increased in all four populations (Table 3); however, SD showed the lowest effect size for this change and Welch's *t*-test did not suggest the increase was statistically significant in this population (P>0.05). Between 30°C and 33°C, ATP synthesis rates did not change meaningfully in any population, with the largest effect size in SD♀×SH♂ (d=0.33). Between 33°C and 36°C, ATP synthesis rates dropped for SH (d=0.47), SH♀×SD♂ (d=0.41) and SD♀×SH♂ (d=0.43), but not for SD (d=0.04). Although none of these changes were statistically significant within each population (P>0.2; Table 3), these effect size estimates suggest that SD was least affected by the increase to 36°C compared with the other experimental populations. Between 36°C and 39°C, ATP synthesis rates in all four populations showed decreases (Table 3). Notably, however, the decrease in SD had a lower effect size than other populations and Welch's *t*-test suggested that this decrease was not statistically significant (P>0.05).

The two hybrid populations also displayed higher ATP synthesis rates compared with both parental populations at a benign (20°C) temperature, suggesting that hybrids have higher baseline Complex I-driven ATP production than parentals (Fig. 2). When compared at 20°C using Cohen's *d* effect size test and Welch's two-sided *t*-test, SH♀×SD♂ had higher ATP synthesis rates than SH (d=0.97, P=0.018) and SD (d=1.56, $P=4.34\times10^{-4}$). SD♀×SH♂ also had higher ATP synthesis rates at 20°C compared with SH (d=1.17, $P=4.00\times10^{-3}$) and SD (d=1.61, $P=2.14\times10^{-4}$).

DISCUSSION

The role of mtDNA variation in the evolution of thermal tolerance phenotypes has only recently started to be examined. Quantifying the fitness impact stemming from genetic variation in mtDNA is made difficult by the presence and importance of nuclear-encoded proteins within the organelle and its function (Wolff et al., 2014). Studies investigating this complex question vary among three general approaches: (1) capturing the covariation between mtDNA or its encoded protein sequences and geographic clines in temperature, (2) measuring the impact of mitochondrial haplotypes in individuals admixed in hybrid zones between natural cline edges, and (3) experimentally controlling nuclear and mtDNA backgrounds in hybrid strains to isolate effects from variation in each genome on thermal tolerance. Our experiment with *T. californicus* is one of few studies using the latter approach, and it provides a set of contrasting results that will be useful in further generalizing such evolutionary processes.

Our findings in F_1 hybrids suggest that the relative contribution of mtDNA-encoded elements in organismal thermal response physiology is weak or negligible in this system. Concordant with

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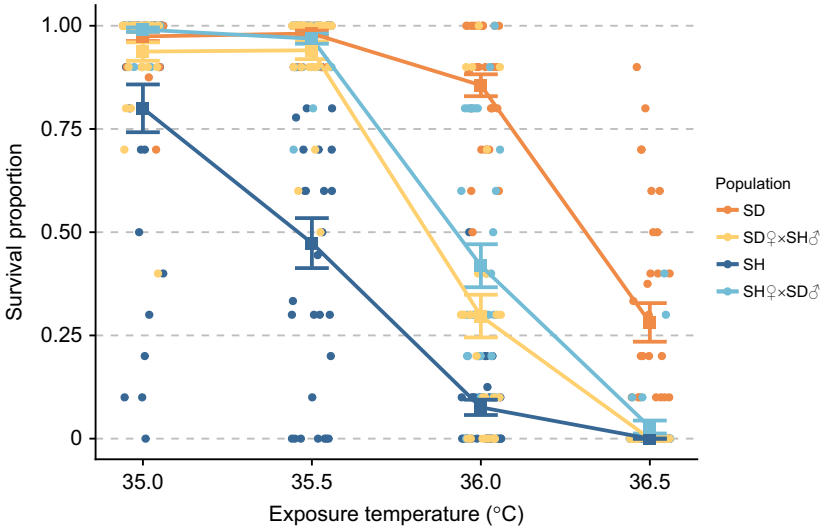

Fig. 1. Acute thermal tolerance of *Tigriopus californicus* copepods assessed by survivorship after 1 h heat stress. Colors represent four genetic backgrounds of parental populations from San Diego, CA, USA (SD), and Strawberry Hill, OR, USA (SH), and their reciprocal first-generation hybrids. Data are presented as individual point replicates with whisker plots indicating means±s.e.m. Each point represents the survival proportion of one replicate of 10 copepods subjected to a target stress temperature. Points were jittered along the *x*-axis to improve visualization. For each genetic group, n=32 replicates (16 of each sex) were tested at each stress temperature (n=512). Statistically significant differences among populations were detected by two-way ANOVA tests followed by Tukey *post hoc* tests (P<0.05) and Cohen's d effect size (see also Tables 1 and 2).

previous findings in this system, the more southern SD population showed elevated survival at higher temperatures compared with the northern SH (Kelly et al., 2012; Leong et al., 2018; Pereira et al., 2017; Willett, 2010; Willett and Son, 2018). We took advantage of this phenotypic divergence to test the relative effects of nuclear and mitochondrial DNA backgrounds on thermal tolerance. Based on our acute thermal stress tests, we found that the two hybrid lines both had intermediate survival relative to the parental populations. Further, the two hybrids did not differ from one another in survival, suggesting that they display no organismal level differences in acute thermal stress tolerance based on mtDNA background. If local adaptation in mitochondrial DNA has a direct impact on organismal survival, we predicted the SD♀×SH♂ population would display higher survival than the SH♀×SD♂ population, owing to the mtDNA background it inherited from SD. Because the reciprocal hybrids had nearly identical patterns of survivorship across temperatures, it is unlikely that temperature-driven local adaptation in the mitochondrial genome is contributing to the tolerance differences between populations of *T. californicus* at the organismal level. Nuclear-encoded genes hence appear to account for most or all of the survival variation under thermal stress between SH and SD.

Table 3. Comparison of ATP synthesis rates between adjacent assay temperatures in *T. californicus* copepods

Comparison	*P*	*d*
SH		
20°C vs 30°C	0.019	0.96
30°C vs 33°C	0.936	0.03
33°C vs 36°C	0.224	0.47
36°C vs 39°C	0.0072	1.16
SD		
20°C vs 30°C	0.059	0.75
30°C vs 33°C	0.721	0.14
33°C vs 36°C	0.919	0.04
36°C vs 39°C	0.070	0.72
SH♀×SD♂		
20°C vs 30°C	0.0156	1.06
30°C vs 33°C	0.830	0.09
33°C vs 36°C	0.310	0.41
36°C vs 39°C	0.0117	1.08
SD♀×SH♂		
20°C vs 30°C	0.0044	1.13
30°C vs 33°C	0.381	0.33
33°C vs 36°C	0.253	0.43
36°C vs 39°C	0.0186	0.92

Analyses were performed for each of the four genetic backgrounds (SD, San Diego; SH, Strawberry Hill). Shown are *P*-values obtained via Welch's two-sided *t*-tests and Cohen's *d* effect size.

Mitochondria isolated from the pure SH population displayed higher Complex I ATP synthesis rates than those from the pure SD population at temperatures below 36°C, including the benign 20°C temperature. These inherent differences in ATP synthesis rate alone should not be interpreted as differences in fitness, as these populations evolved baseline ATP synthesis rates independently under different environmental conditions. We make no effort to impose whether high ATP synthesis rates represent good or bad energetic performance. The diagnosis of mitochondrial performance in this manner would require greater physiological and cellular context than our methods provided (Brand and Nicholls, 2011). Because ATP synthesis rates vary intrinsically between populations (Harada et al., 2019; Healy and Burton, 2023), we argue that it is the change in ATP synthesis rate with increasing temperature that is an indication of thermal stability and may be adaptive, rather than the rates themselves. We found that mitochondria from pure SD individuals may be less sensitive to changes in temperature than those from pure SH individuals, as reflected in the comparatively flattened curve for SD (Fig. 2) and low effect sizes between adjacent temperatures (Table 3). We predicted that if the SD mtDNA background is contributing heavily to thermal stability of ATP synthesis rate, then the presence of SD mtDNA in SD♀×SH♂ hybrids would impart higher thermal stability relative to the reciprocal cross (SH♀×SD♂). Instead, thermal stability of isolated mitochondria from both hybrid crosses were similarly sensitive and showed marked drops at the high end of stress temperatures, a pattern more similar to that of the pure SH population. Overall, these patterns suggest that the difference in ATP synthesis stability between parental populations is more likely the result of adaptative divergence in nuclear-encoded gene products present within the organelle, such as Complex I components or mitochondrial heat shock proteins (mtHSPs).

Our findings in *T. californicus* are in stark contrast to similar experimental work in *D. melanogaster*, mentioned above (Camus et al., 2017; Lajbner et al., 2018), and *Saccharomyces* yeasts (Baker et al., 2019; Li et al., 2019). In both of these systems, hybrid strains that shared the same nuclear genome content showed a significant

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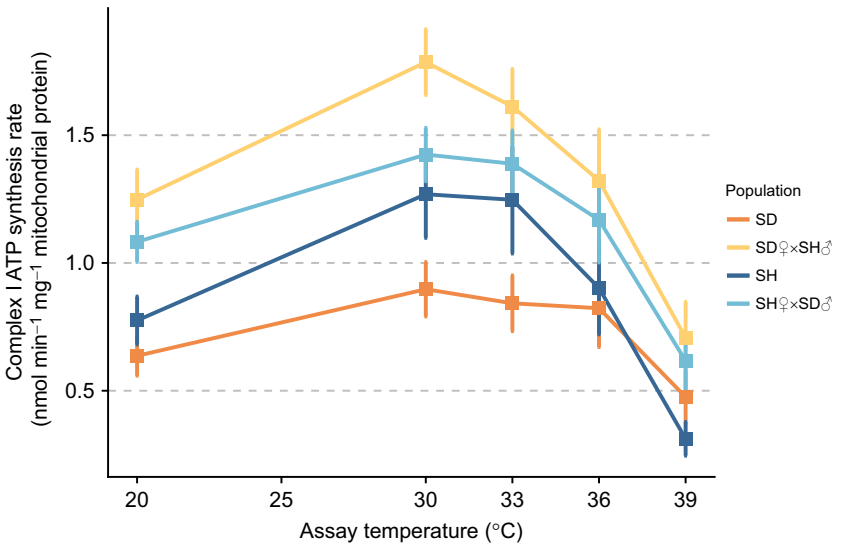

Fig. 2. Complex I ATP synthesis rates quantified *in vitro* from isolated mitochondria of *T. californicus* copepods. Colors represent four genetic backgrounds of parental populations from San Diego, CA, USA (SD), and Strawberry Hill, OR, USA (SH), and their reciprocal first-generation hybrids. Whisker plots represent means±s.e.m. Mitochondria were isolated from pools of 24 copepods and aliquoted evenly between stress temperatures, with n=14 replicates for SH, n=14 for SD, n=13 for SH♀×SD♂ and n=15 for SD♀×SH♂. Statistically significant changes in rate between adjacent stress temperatures were detected by Welch's two-sided t-tests (P<0.05) and by Cohen's d effect size tests for each population (see also Table 3).

effect of mtDNA haplotype on thermal tolerance. Li et al. (2019) provides perhaps the most direct experimental comparison to our study, as they tested cold and heat tolerance in F_1 diploid strains created reciprocally between wild-type forms of two species of yeast that naturally differed in their thermal adaptations. They found that hybrids carrying mtDNA from warm-adapted *S. cerevisiae* grew larger colonies at 37°C than the reciprocal carrying mtDNA from *S. uvarum*, a cold-adapted species; the opposite pattern was observed when tested at 4°C (Li et al., 2019). These recent studies even detected the influence of variation at finer genetic scales. For instance, haplotypes associated with thermal tolerance variation in hybrid flies differed by only 15 synonymous substitutions (Camus et al., 2017), whereas Li et al. (2019) traced a strong effect to species divergence at the mtDNA-encoded *COI* gene. By contrast, the *T. californicus* populations we hybridized have diverged by as much as 35% in some mtDNA-encoded genes (Han and Barreto, 2021).

Although reciprocal F_1 crosses have the same pattern of interspecific or interpopulation heterozygosity across all nuclear loci, results from these types of experiments may still be partly due to dominance patterns between nuclear alleles. In *T. californicus*, it is unclear how SD and SH alleles are expressed in hybrids during thermal stress. When examined across experimental temperature gradients, survivorship in F_1 hybrids may indicate complex patterns of genetic dominance. Both reciprocal crosses showed survivorship at the level of SD at the low end of the stress gradient, but their tolerance decreased sharply, nearing that of SH, as temperature was increased (Fig. 1). Willett (2012) reported a broadly similar pattern in hybrids of two different *T. californicus* crosses. For example, in a cross between the southern, more tolerant Abalone Cove (AB) population and the northern, more sensitive Santa Cruz (SCN) population, F_1 hybrids survived to the same extent as AB at 37°C, but decreased to SCN levels of survivorship at 38°C. We hypothesize that these phenotypic patterns of survival in F_1 hybrids may reflect changes in expression dominance across increasing temperatures, with SD alleles being expressed at the lower end of the temperature gradient, and more SH alleles at the higher ends. This complex pattern of expression may be driven by interactions between *cis*- and *trans*-regulatory elements in gene expression that have diverged between these allopatric populations. Alternatively, both SD and SH alleles may be expressed equally across temperatures, with warm-adapted SD alleles, even in the presence of SH proteins, being fully competent at the lower stress temperatures frequently encountered by SD. An increase to nearly lethal temperatures, when SD alleles are pushed to the limit, may then expose the lesser ability of SH alleles to contribute to the stress response. Li et al. (2019) proposed a similar mechanism of dominance to explain their observed effects of mitochondrial background, wherein mtDNA-*COI* from the sensitive *S. uvarum* can function with *S. cerevisiae* nuclear-encoded proteins under heat stress, but loses heat tolerance when interacting with *S. uvarum* proteins that are dominant to *S. cerevisiae* in hybrids.

Similarly, both SD×SH hybrid crosses in our study showed stability of Complex I ATP synthesis *in vitro* that was more similar to that of their less stable parent SH. These patterns suggest that having a full complement of warm-adapted (SD) alleles in the nucleus is essential for improved thermal stability of ATP synthesis in the mitochondria. SH nuclear alleles may have dominant expression (i.e. more highly expressed) to SD alleles, despite their potentially reduced capacity to mitigate high temperature stress. This study, however, lacks the power to examine the impact of expression dominance of nuclear alleles on the observed phenotypes. Quantification of allele-specific expression in F_1 hybrids (e.g. Tangwancharoen et al., 2020) across a temperature gradient would be a powerful way of testing these hypotheses.

F_1 hybrids in *T. californicus* are known to exhibit hybrid vigor in many life-history traits (Burton, 1990; Edmands, 1999; Edmands and Deimler, 2004; Ellison and Burton, 2008). We found no evidence for heterosis in survival during thermal stress in SD×SH hybrids. Our result contributes to the broader observation in this system that hybrid vigor in thermal tolerance appears to vary by cross, but that it is more likely to be manifested in crosses between southern populations. Both Willett (2012) and Pereira et al. (2014) observed thermal tolerance vigor in F_1 hybrids of at least some crosses between SD and other populations from southern California. In contrast, those studies, along with Griffiths et al. (2020) and the present study, never detected this phenotype when SD was crossed with populations from cooler latitudes. In the latter cases, F_1 hybrids were either intermediate or similar to one of the parents. In tests of mitochondrial thermal stability, mitochondria isolated from both hybrid crosses displayed much higher *in vitro* Complex I ATP synthesis rates than those from either the pure SD or pure SH populations across all stress temperatures. However, they did not show improved stability of rate compared with SH or SD. Combined

with their lack of heterosis in survival, these results further highlight that capacity for high rates of Complex I ATP synthesis are not predictive of overall fitness and should not alone be interpreted as a form of heterosis. As suggested by Chung and Schulte (2020), links between mitochondrial function and overall thermal tolerance fitness vary in the strength of interpretation based on the metric used to quantify them. Our results support their assertion that loss of ATP synthetic capacity (the result of thermal instability) seems a strong contender for a functional link between mitochondrial physiology and critical thermal maximum at the organism level, and suggest that the ability to maintain ATP synthesis with increasing temperature is more indicative of fitness than the amount of ATP produced prior to losing function.

Hybridization between divergent taxa can result in the breakup of coadapted mitonuclear interactions, particularly when admixture continues beyond the F_1 generation. This form of hybrid breakdown is known to occur in *T. californicus* (Burton et al., 2006), among several other systems (reviewed in Rand et al., 2004; Wolff et al., 2014). How temperature regimes shape the process of mitonuclear coevolution is still poorly understood. Experiments in parasitoid wasps in the genus *Nasonia* show that elevated temperatures can exacerbate the already reduced fitness of F_2 hybrids known to be the result of mitonuclear incompatibilities (Koevoets et al., 2012). In contrast, Willett (2012) found that in *T. californicus*, differences in fitness between F_2 hybrids and their parental populations decreased under heat stress, consistent with an alleviation of fitness losses caused by mitonuclear incompatibilities. These studies support the potential for the coevolution between nuclear- and mtDNA-encoded elements to play a role in thermal tolerance phenotypes alongside evolution of thermal tolerance in each genome, independently. Mitonuclear coadaptation may play a role in our observed patterns of ATP synthesis in F_1 hybrids, if codominance of expression of nuclear SD and SH alleles generate chimeric protein complexes in the OXPHOS pathway and exposes incompatibilities during thermal stress.

Our study is one of few to explicitly test for the relative impacts of nuclear and mitochondrial genome backgrounds on thermal tolerance. Survival following acute thermal stress for the two parental populations investigated is consistent with previous studies (Kelly et al., 2012; Leong et al., 2018; Pereira et al., 2017; Willett, 2010; Willett and Son, 2018) and shows clear population level divergence and local adaptation. We argue, however, that previous evidence of local adaptation at the level of mitochondrial function in *T. californicus* (Harada et al., 2019; Healy and Burton, 2023) did not address the relative importance of evolution at nuclear and mitochondrial genomes. Our results suggest that mitochondria-encoded products, relative to nuclear-encoded ones, may play a limited role in thermal tolerance phenotypes in *T. californicus*. Experiments studying this question vary in which phenotypes are used to assess tolerance, and hence interpretations are still difficult between studies and across systems. Although our results suggest that local adaptation in mtDNA plays only a small role in thermal tolerance, determining variation in expression of mitochondria-associated nuclear gene products may be an important future step toward characterizing mitochondrial thermal stability and the role of mitonuclear coevolution in thermal adaptation. These results contribute to a relatively small number of studies that have attempted to isolate the role of the mitochondrial genome in thermal tolerance. In contrast to previous studies, we found no evidence of a link between mtDNA and thermal tolerance, suggesting that the role of mitochondria in thermal tolerance is yet to be resolved.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: K.K., F.S.B.; Methodology: K.K.; Validation: K.K.; Formal analysis: K.K.; Investigation: K.K.; Resources: F.S.B.; Data curation: K.K.; Writing - original draft: K.K.; Writing - review & editing: K.K., F.S.B.; Visualization: K.K.; Supervision: F.S.B.; Project administration: K.K.; Funding acquisition: K.K., F.S.B.

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Data availability
Data associated with copepod survivorship and ATP synthesis can be found in Datasets 1 and 2, respectively.

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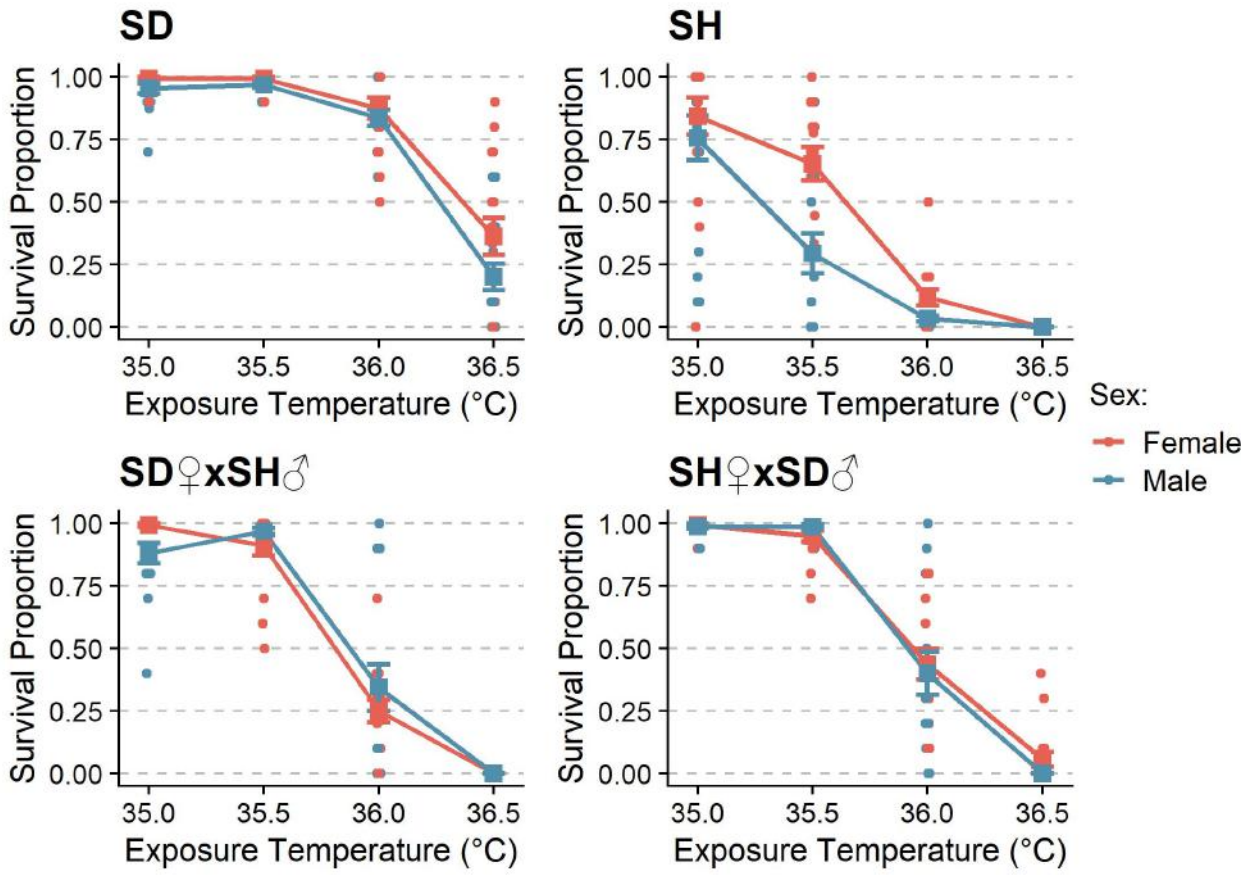

Fig. S1. Acute thermal tolerance of male and female *T. californicus* copepods assessed by survivorship after 1-hr heat stress. Panels are separated by genetic backgrounds of parental populations from San Diego, California (SD) and Strawberry Hill, Oregon (SH), and for their reciprocal first-generation hybrids. Data are presented as individual point replicates and with whisker plots indicating means ± s.e.m. Each point represents the survival proportion of one replicate of 10 copepods subjected to a target stress temperature. For each genetic group, n = 16 replicates were tested for each sex at each stress temperature (n = 512 total). Statistically significant differences between sexes were detected by two-way ANOVA tests followed by Tukey *post hoc* tests ($P < 0.05$) and Cohen's d effect size tests (Tables 1 and 2 in the main text).

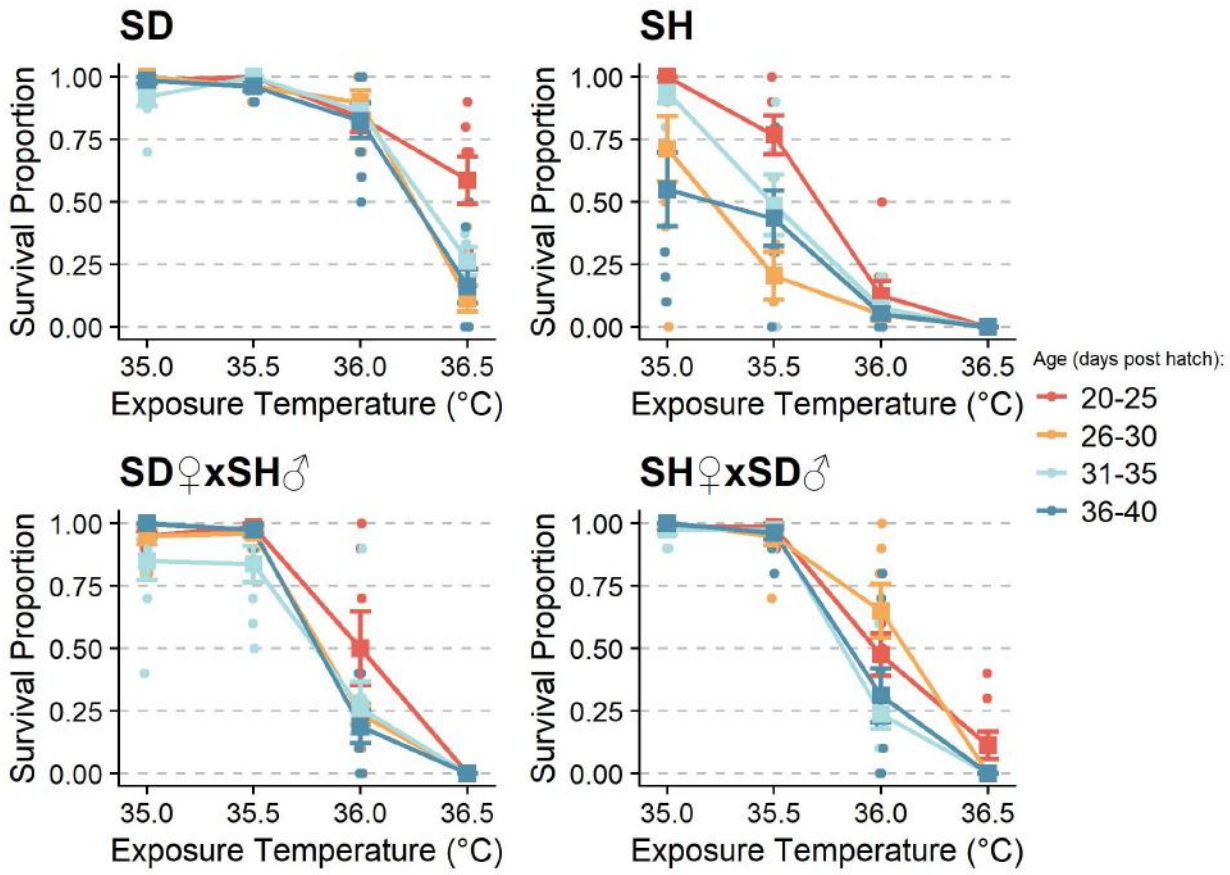

Fig. S2. Acute thermal tolerance of four age cohorts of *T. californicus* copepods assessed by survivorship after 1-hr heat stress. Panels are separated by genetic backgrounds of parental populations from San Diego, California (SD) and Strawberry Hill, Oregon (SH), and for their reciprocal first-generation hybrids. Data are presented as individual point replicates and with whisker plots indicating means ± s.e.m. Each point represents the survival proportion of one replicate of 10 copepods subjected to a target stress temperature. For each genetic group, n = 8 replicates were tested for each age group at each stress temperature (n = 512 total). Statistically significant differences among age groups were detected by two-way ANOVA tests followed by Tukey *post hoc* tests ($P < 0.05$) and Cohen's d effect size tests (Tables 1 and 2 in the main text).

Dataset 1. Survivorship following acute thermal stress in *T. californicus*. Replicates were exposed to stress temperature for 1-hr and given 3 days to recover. "SHSD" refers to SH♀xSD♂ (same for SDSH) and is abbreviated for ease of analysis.

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Dataset 2. *in vitro* Complex I ATP synthesis rates across temperature gradients in *T. californicus*. Mitochondria were isolated from pools of 24 individuals. Sample IDs describe population, replicate number within that population, and stress temperature. "SHSD" refers to SH♀xSD♂ (same for SDSH) and is abbreviated for ease of analysis.

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