UNIVERSITY OF CALIFORNIA SAN DIEGO

Variation and mechanisms of thermal tolerance among populations of *Tigriopus californicus*

A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy

in

Marine Biology

by

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2019

The Dissertation of Alice Elizabeth Harada is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California San Diego

2019

DEDICATION

To Mom and Dad,

thank you for your unconditional love, unwavering guidance, and constant support.

EPIGRAPH

"Nothing in Biology makes sense except in the light of evolution."

-Theodosius Dobzhansky

"It is our choices, Harry, that show what we truly are, far more than our abilities."

-J.K. Rowling, Harry Potter and the Chamber of Secrets

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Chapter 3, in full, is published at Physiological and Biochemical Zoology: Harada AE, Burton RS (2019). Ecologically relevant temperature ramping rates enhance the protective heat shock response in an intertidal ectotherm. The dissertation author was the primary investigator and author of this paper.

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Harada AE, Lindgren EA, Hermsmeier MC, Rogowski PA, Terrill E, Burton RS. (2015) Monitoring Spawning Activity in a Southern California Marine Protected Area Using Molecular Identification of Fish Eggs. *PLoS ONE* 10(8): e0134647.

Fields of Study

Evolutionary genetics
Molecular ecology
Evolutionary physiology
Marine biology

ABSTRACT OF THE DISSERTATION

Variation and mechanisms of thermal tolerance among populations of *Tigriopus californicus*

by

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Doctor of Philosophy in Marine Biology

University of California San Diego, 2019

Professor Ronald Burton, Chair

As the climate warms, the response of organisms to rising temperatures has become an area of increasing interest. Much research is focused on understanding the basis for thermal tolerance, which can help us predict the capacity for adaptation or shifting range limits in response to changes in climate. Of particular interest are intertidal ectotherms, which experience highly variable environmental conditions. One such organism is *Tigriopus californicus,* a copepod found in high rocky tidepools along the west coast of North America. Its relatively low gene flow contributes to genetic isolation of populations, allowing the study of local adaptation along a latitudinal gradient. Previous studies have shown that southern populations have higher

survivorship following heat stress than northern populations, which is correlated with higher upregulation of important heat shock protein (HSP) genes. However, the physiological mechanisms and gene regulation patterns underlying thermal tolerance are not fully understood. In order to address these questions, thermal performance under acute and chronic thermal stress conditions and at abrupt and gradual ramping rates was assessed in three populations of *T. californicus* distributed from south to north. Additionally, gene regulation during the heat shock response was examined using knockdown of the heat shock transcription factor (*HSF-1*) gene. We found that at acute thermal exposures, survivorship and mitochondrial performance follow a latitudinal gradient. Chronic thermal performance is more complex, however, with the mid-latitude population showing decreased performance compared to both the southern and northern populations at lower temperatures. Gene regulation during the heat shock response is similarly crucial to organismal performance, and a knockdown of HSF-1 indicates a complex network of gene interactions. Finally, we found that a slower rate of thermal exposure similar to conditions in the intertidal allows organisms to more highly upregulate important HSPs, conferring protection and minimizing harmful effects of acute thermal stress. These findings allow us to better understand the mechanisms underlying thermal tolerance, determine why certain populations or species outperform others, and predict organismal responses to changing climatic conditions in the future.

GENERAL INTRODUCTION

Climate change and the oceans

Understanding the effects of rising ocean temperatures on organisms and ecosystems has become an increasingly important area of study as the global climate continues to change (IPCC; Parmesan 2006;). Temperature is an abiotic stressor that plays a major role in determining the biogeographic distribution of organisms; as temperatures increase, organisms are pushed closer and closer to their thermal limits (Bozinovic et al., 2011; Deutsch et al., 2015; Sunday et al., 2012). These limits typically align with maximal habitat temperatures; near the equator many organisms live closer to maximum tolerable physiological limits (Deutsch et al., 2008; Somero, 2010; Sunday et al., 2011). Studies of thermal tolerance and adaptation often aim to understand the mechanistic basis for these limits as well as their capacity to change with warming climatic conditions (Bozinovic et al., 2011; Deutsch et al., 2015; Pörtner et al., 2006). In some cases, organisms show evidence of poleward movement (Helmuth et al., 2006; Parmesan, 2006; Perry et al., 2005; Poloczanska et al., 2013; Sagarin et al., 1999). However, many organisms are not able to move, necessitating their adaptation to new thermal regimes for survival.

Adaptive physiology and thermal stress

Because thermal stress can affect multiple levels of biological organization, from whole-animal fitness to individual cells, organisms (particularly ectotherms) have evolved ways to cope with their thermal regimes. These coping mechanisms include the heat shock response, which is a coordinated upregulation of heat shock protein genes (*HSP*s) that protect essential protein functions within a cell (Feder and Hofmann, 1999; Morimoto, 1998; Sørensen et al., 2003). The

heat shock response itself is ubiquitous in many taxa (Feder and Hofmann, 1999; Lindquist, 1986). However, there are numerous aspects of the response that are currently being studied and still not fully understood: the complex network of genes involved in the regulation (Mahat et al., 2016; Richter et al., 2010; Roncarati and Scarlato, 2017) and response (Sørensen et al., 2005; Stillman and Tagmount, 2009; Zhang et al., 2012), the timing and magnitude of induction (Buckley et al., 2001; Tomanek, 2008; Tomanek and Somero, 2000), and the evolutionary patterns of gene expression (Chen et al., 2018; Gleason and Burton, 2015; Healy et al., 2010; Narum et al., 2013).

Another aspect of the response to thermal stress occurs in the mitochondria. The effects of temperature on mitochondrial performance are well characterized and include changes to mitochondrial amount (Dhillon and Schulte, 2011; Egginton and Johnston, 1984), oxidative capacity (Chung et al., 2017; Guderley, 2004), membrane composition and fluidity (Grim et al., 2010; Kraffe et al., 2007), and enzyme activity (Guderley, 2004; McClelland et al., 2006; Orczewska et al., 2010). Although there is a widely held belief that organelles and less-organizationally complex systems have higher thermal tolerance (Pörtner, 2002), there is evidence in some systems that mitochondrial functions fail at temperatures below whole organism tolerance limits (Christen et al., 2018; Iftikar and Hickey, 2013; O'Brien et al., 2018). The degree to which mitochondria are responsible for setting organismal thermal limits remains to be determined.

Thermal stress and the rocky intertidal

The rocky intertidal provides a fascinating environment for the study of local adaptation. Organisms inhabiting the intertidal are exposed to a number of abiotic stressors, including wide

fluctuations in temperature. These temperature extremes are a main driver of organismal zonation and latitudinal distribution (Helmuth et al., 2006; Somero, 2002; Stillman, 2002). Intertidal organisms living in highly variable environments often demonstrate earlier induction of the heat shock response (induction below their maximal habitat temperatures vs. induction at or above maxima) or higher constitutive gene expression than congeners from more stable environments, allowing them to cope with daily temperature extremes (Dong et al., 2008; Stillman and Tagmount, 2009; Tomanek, 2008). However, because many of these organisms in highly variable intertidal areas are living near their thermal limits already, their heat shock response may be less equipped to adapt to further temperature increases, making them more susceptible to the adverse effects of climate warming (Somero, 2010; Tomanek, 2008). Therefore, the capacity for intertidal species to adapt (particularly for populations living in unstable thermal regimes) has been an area of ongoing study.

Tigriopus californicus as a study system for local adaptation

Tigriopus californicus is a copepod that inhabits high splash pools in the rocky intertidal zone. It is found along the west coast of North America, from Baja California to Alaska (Dethier, 1980; Ganz and Burton, 1995). Because of its isolated habitat, it has a low capacity for gene flow and therefore high genetic isolation between populations (Barreto et al., 2018). There is extensive evidence for local adaptation of thermal tolerance in *T. californicus*, with populations in the southern distribution of its range showing higher survivorship following thermal stress than populations in the north (Kelly et al., 2013; Pereira et al., 2017; Willett, 2010). Furthermore, populations have different strategies for coping with heat stress, including changes in gene expression (Schoville et al., 2012) and gene regulation (Tangwancharoen et al., 2018). The high

degree of genetic isolation, local adaptation, and variable thermal tolerance make *T. californicus* an excellent system for studies of thermal adaptation and physiology.

Outline of the dissertation

The goal of this dissertation is to elucidate the mechanisms underlying heat tolerance in *Tigriopus californicus.* We have examined thermal tolerance at multiple biological levels in an attempt to come to a more complete understanding of the response to thermal stress in this system. Additionally, we have compared responses in three populations within the latitudinal range of *T. californicus* in order to understand the influence of a latitudinal thermal gradient and to determine how widely applicable these data may be to the whole system (and perhaps to other marine ectotherms as well). Chapter 1 examines the thermal tolerance of the three populations at both an acute and chronic time scale in conjunction with mitochondrial performance measures across a range of temperatures. Chapter 2 seeks to understand the importance of heat shock transcription factor on the heat shock response using a combination of RNA interference and RNA-sequencing. Finally, Chapter 3 assesses how the rate of thermal exposure affects life history and gene expression in each of the three populations in order to better understand the mechanisms of the heat shock response in a more natural thermal stress exposure regime. Taken together, these studies can help us synthesize a more complete picture of the thermal response in *T. californicus.*

REFERENCES

Barreto, F. S., Watson, E. T., Lima, T. G., Willett, C. S., Edmands, S., Li, W., and Burton, R. S. (2018). Genomic signatures of mitonuclear coevolution across populations of *Tigriopus californicus*. *Nat. Ecol. Evol.* 2, 1250–1257. doi:10.1038/s41559-018-0588-1.

Bozinovic, F., Calosi, P., and Spicer, J. I. (2011). Physiological correlates of geographic range in animals. *Annu. Rev. Ecol. Evol. Syst.* 42, 155–179. doi:10.1146/annurev-ecolsys-102710-145055.

Buckley, B. a, Owen, M. E., and Hofmann, G. E. (2001). Adjusting the thermostat: the threshold induction temperature for the heatshock response in intertidal mussels (genus Mytilus) changes as a function of thermal history. *J. Exp. Biol.* 204, 3571–3579.

Chen, B., Feder, M. E., and Kang, L. (2018). Evolution of heat-shock protein expression underlying adaptive responses to environmental stress. *Mol. Ecol.* 27, 3040–3054. doi:10.1111/mec.14769.

Christen, F., Desrosiers, V., Dupont-Cyr, B. A., Vandenberg, G. W., Le François, N. R., Tardif, J. C., Dufresne, F., Lamarre, S. G., and Blier, P. U. (2018). Thermal tolerance and thermal sensitivity of heart mitochondria: Mitochondrial integrity and ROS production. *Free Radic. Biol. Med.* 116, 11–18. doi:10.1016/j.freeradbiomed.2017.12.037.

Chung, D. J., Bryant, H. J., and Schulte, P. M. (2017). Thermal acclimation and subspecies-specific effects on heart and brain mitochondrial performance in a eurythermal teleost (*Fundulus heteroclitus*). *J. Exp. Biol.* 220, 1459–1471. doi:10.1242/jeb.151217.

Dethier, M. N. (1980). Tidepools as refuges: Predation and the limits of the harpacticoid copepod *Tigriopus californicus* (Baker). *J. Exp. Mar. Bio. Ecol.* 42, 99–111. doi:10.1016/0022-0981(80)90169-0.

Deutsch, C. A., Tewksbury, J. J., Huey, R. B., Sheldon, K. S., Ghalambor, C. K., Haak, D. C., and Martin, P. R. (2008). Impacts of climate warming on terrestrial ectotherms across latitude. *Proc. Natl. Acad. Sci.* 105, 6668–6672. doi:10.1073/pnas.0709472105.

Deutsch, C., Ferrel, A., Seibel, B., Pörtner, H.-O., and Huey, R. B. (2015). Climate change tightens a metabolic constraint on marine habitats. *Science (80-.).* 348, 1132–1136.

Dhillon, R. S., and Schulte, P. M. (2011). Intraspecific variation in the thermal plasticity of mitochondria in killifish. *J. Exp. Biol.* 214, 3639–3648. doi:10.1242/jeb.057737.

Dong, Y., Miller, L. P., Sanders, J. O. N. G., and Somero, G. N. (2008). Heat-shock protein 70 (Hsp70) expression in four limpets of the genus *Lottia:* Interspecific variation in constitutive and inducible synthesis correlates with *in situ* exposure to heat stress. 70, 173–181. doi:10.2307/25470698.

Egginton, S., and Johnston, I. A. (1984). Effects of acclimation temperature on routine metabolism muscle mitochondrial volume density and capillary supply in the elver (*Anguilla anguilla* L.). *J. Therm. Biol.* 9, 165–170. doi:10.1016/0306-4565(84)90016-0.

Feder, M. E., and Hofmann, G. E. (1999). Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annu. Rev. Physiol.* 61, 243–282. doi:10.1146/annurev.physiol.61.1.243.

Ganz, H. H., and Burton, R. S. (1995). Genetic differentiation and reproductive incompatibility among Baja California populations of the copepod *Tigriopus californicus*. *Mar. Biol.* 123, 821–827. doi:10.1007/BF00349126.

Gleason, L. U., and Burton, R. S. (2015). RNA-seq reveals regional differences in transcriptome response to heat stress in the marine snail *Chlorostoma funebralis*. *Mol. Ecol.* 24, 610–627. doi:10.1111/mec.13047.

Grim, J. M., Miles, D. R. B., and Crockett, E. L. (2010). Temperature acclimation alters oxidative capacities and composition of membrane lipids without influencing activities of enzymatic antioxidants or susceptibility to lipid peroxidation in fish muscle. *J. Exp. Biol.* 213, 445–452. doi:10.1242/jeb.036939.

Guderley, H. (2004). Metabolic responses to low temperature in fish muscle. *Biol. Rev. Camb. Philos. Soc.* 79, 409–427. doi:10.1017/S1464793103006328.

Healy, T. M., Tymchuk, W. E., Osborne, E. J., and Schulte, P. M. (2010). Heat shock response of killifish (*Fundulus heteroclitus*): candidate gene and heterologous microarray approaches. *Physiol. Genomics* 41, 171–184. doi:10.1152/physiolgenomics.00209.2009.

Helmuth, B., Mieszkowska, N., Moore, P., and Hawkins, S. J. (2006). Living on the edge of two changing worlds: Forecasting the responses of rocky intertidal ecosystems to climate change. *Annu. Rev. Ecol. Evol. Syst.* 37, 373–404. doi:10.1146/annurev.ecolsys.37.091305.110149.

Iftikar, F. I., and Hickey, A. J. R. (2013). Do mitochondria limit hot fish hearts? understanding the role of mitochondrial function with heat stress in *Notolabrus celidotus*. *PLoS One* 8. doi:10.1371/journal.pone.0064120.

Kelly, M. W., Grosberg, R. K., and Sanford, E. (2013). Trade-offs, geography, and limits to thermal adaptation in a tide pool copepod. *Am. Nat.* 181, 846–854. doi:10.1086/670336.

Kraffe, E., Marty, Y., and Guderley, H. (2007). Changes in mitochondrial oxidative capacities during thermal acclimation of rainbow trout *Oncorhynchus mykiss:* roles of membrane proteins, phospholipids and their fatty acid compositions. *J. Exp. Biol.* 210, 149–165. doi:10.1242/jeb.02628.

Lindquist, S. (1986). The heat-shock response. *Annu. Rev. Biochem.* 55, 1151–1191. doi:10.1146/annurev.bi.55.070186.005443.

Mahat, D. B., Salamanca, H. H., Duarte, F. M., Danko, C. G., and Lis, J. T. (2016). Mammalian heat shock response and mechanisms underlying its genome-wide transcriptional regulation. *Mol. Cell* 62, 63–78. doi:10.1016/j.molcel.2016.02.025.

McClelland, G. B., Craig, P. M., Dhekney, K., and Dipardo, S. (2006). Temperature- and exercise-induced gene expression and metabolic enzyme changes in skeletal muscle of adult zebrafish *(Danio rerio)*. *J. Physiol.* 577, 739–751. doi:10.1113/jphysiol.2006.119032.

Morimoto, R. I. (1998). Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. *Genes Dev.* 12, 3788–3796.

Narum, S. R., Campbell, N. R., Meyer, K. A., Miller, M. R., and Hardy, R. W. (2013). Thermal adaptation and acclimation of ectotherms from differing aquatic climates. *Mol. Ecol.* 22, 3090–3097. doi:10.1111/mec.12240.

O'Brien, K. M., Rix, A. S., Egginton, S., Farrell, A. P., Crockett, E. L., Schlauch, K., Woolsey, R., Hoffman, M., and Merriman, S. (2018). Cardiac mitochondrial metabolism may contribute to differences in thermal tolerance of red- and white-blooded Antarctic notothenioid fishes. *J. Exp. Biol.*, jeb.177816. doi:10.1242/jeb.177816.

Orczewska, J. I., Hartleben, G., and O'Brien, K. M. (2010). The molecular basis of aerobic metabolic remodeling differs between oxidative muscle and liver of threespine sticklebacks in response to cold acclimation. *AJP Regul. Integr. Comp. Physiol.* 299, R352–R364. doi:10.1152/ajpregu.00189.2010.

Parmesan, C. (2006). Ecological and evolutionary responses to recent climate change. *Annu. Rev. Ecol. Evol. Syst.* 37, 637–669. doi:10.1146/annurev.ecolsys.37.091305.110100.

Pereira, R. J., Sasaki, M. C., and Burton, R. S. (2017). Adaptation to a latitudinal thermal gradient within a widespread copepod species: The contributions of genetic divergence and phenotypic plasticity. *Proc. R. Soc. B Biol. Sci.* 284. doi:10.1098/rspb.2017.0236.

Perry, A. L., Low, P. J., Ellis, J. R., and Reynolds, J. D. (2005). Climate change and distribution in marine fishes. *Science (80-.).* 308, 1912–1915.

Poloczanska, E. S., Brown, C. J., Sydeman, W. J., Kiessling, W., Schoeman, D. S., Moore, P. J., Brander, K., Bruno, J. F., Buckley, L. B., Burrows, M. T., Duarte, C. M., Halpern, B. S., Holding, J., Kappel, C. V., O'Connor, M. I., Pandolfi, J. M., Parmesan, C., Schwing, F., Thompson, S. A., and Richardson, A. J. (2013). Global imprint of climate change on marine life. *Nat. Clim. Chang.* 3, 919–925. doi:10.1038/nclimate1958.

Pörtner, H. O. (2002). Climate variations and the physiological basis of temperature dependent biogeography: systemic to molecular hierarchy of thermal tolerance in animals. *Comp. Biochem. Physiol. - A Mol. Integr. Physiol.* 132, 739–761. doi:10.1109/CITS.2015.7297730.

Pörtner, H. O., Bennett, A. F., Bozinovic, F., Clarke, A., Lardies, M. A., Lucassen, M., Pelster, B., Schiemer, F., and Stillman, J. H. (2006). Trade-offs in thermal adaptation: The need for a molecular to ecological integration. *Physiol. Biochem. Zool.* 79, 295–313. doi:10.1086/499986.

Richter, K., Haslbeck, M., and Buchner, J. (2010). The heat shock response: Life on the verge of death. *Mol. Cell* 40, 253–266. doi:10.1016/j.molcel.2010.10.006.

Roncarati, D., and Scarlato, V. (2017). Regulation of heat-shock genes in bacteria: from signal sensing to gene expression output. *FEMS Microbiol. Rev.* 41, 549–574. doi:10.1093/femsre/fux015.

Sagarin, R. D., Barry, J. P., Gilman, S. E., Baxter, C. H., and Nifio-southern, E. (1999). Climate-related change in an intertidal community over short and long time scales. *Ecol. Monogr.* 69, 465–490.

Schoville, S. D., Barreto, F. S., Moy, G. W., Wolff, A., and Burton, R. S. (2012). Investigating the molecular basis of local adaptation to thermal stress: population differences in gene expression across the transcriptome of the copepod *Tigriopus californicus*. *BMC Evol. Biol.* 12, 170. doi:10.1186/1471-2148-12-170.

Somero, G. N. (2002). Thermal physiology and vertical zonation of intertidal animals: Optima, limits, and costs of living. *Integr. Comp. Biol.* 42, 780–789. doi:10.1093/icb/42.4.780.

Somero, G. N. (2010). The physiology of climate change: how potentials for acclimatization and genetic adaptation will determine “winners” and “losers.” *J. Exp. Biol.* 213, 912–920. doi:10.1242/jeb.037473.

Sørensen, J. G., Kristensen, T. N., and Loeschcke, V. (2003). The evolutionary and ecological role of heat shock proteins. *Ecol. Lett.* 6, 1025–1037. doi:10.1046/j.1461-0248.2003.00528.x.

Sørensen, J. G., Nielsen, M. M., Kruhøffer, M., Justesen, J., and Loeschcke, V. (2005). Full genome gene expression analysis of the heat stress response in *Drosophila melanogaster*. *Cell Stress Chaperones* 10, 312–328. doi:10.1379/CSC-128R1.1.

Stillman, J. H. (2002). Causes and consequences of thermal tolerance limits in rocky intertidal porcelain crabs, genus *Petrolisthes*. *Integr. Comp. Biol.* 42, 790–796. doi:10.1093/icb/42.4.790.

Stillman, J. H., and Tagmount, A. (2009). Seasonal and latitudinal acclimatization of cardiac transcriptome responses to thermal stress in porcelain crabs, *Petrolisthes cinctipes*. *Mol. Ecol.* 18, 4206–4226. doi:10.1111/j.1365-294X.2009.04354.x.

Sunday, J. M., Bates, A. E., and Dulvy, N. K. (2011). Global analysis of thermal tolerance and latitude in ectotherms. *Proc. R. Soc. B Biol. Sci.* 278, 1823–1830. doi:10.1098/rspb.2010.1295.

Sunday, J. M., Bates, A. E., and Dulvy, N. K. (2012). Thermal tolerance and the global redistribution of animals. *Nat. Clim. Chang.* 2, 686–690. doi:10.1038/nclimate1539.

Tangwancharoen, S., Moy, G. W., and Burton, R. S. (2018). Multiple modes of adaptation: regulatory and structural evolution in a small heat shock protein gene. *Mol. Biol. Evol.* 35, 2110–2119. doi:10.1093/molbev/msy138.

Tomanek, L. (2008). The importance of physiological limits in determining biogeographical range shifts due to global climate change: The heat-shock response. *Physiol. Biochem. Zool.* 81, 709–717. doi:10.1086/590163.

Tomanek, L., and Somero, G. N. (2000). Time course and magnitude of synthesis of heat-shock proteins in congeneric marine snails (Genus *Tegula*) from different tidal heights. *Physiol. Biochem. Zool.* 73, 249–256. doi:Doi 10.1086/316740.

Willett, C. S. (2010). Potential fitness trade-offs for thermal tolerance in the intertidal copepod *Tigriopus californicus*. *Evolution (N. Y).* 64, 2521–2534. doi:10.1111/j.1558-5646.2010.01008.x.

Zhang, G., Fang, X., Guo, X., Li, L., Luo, R., Xu, F., et al. (2012). The oyster genome reveals stress adaptation and complexity of shell formation. *Nature* 490, 49–54. doi:10.1038/nature11413.

CHAPTER ONE

Variation in thermal tolerance and its relationship to mitochondrial function across populations of *Tigriopus californicus*

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ABSTRACT

Variation in thermal tolerance plays a key role in determining the biogeographic distribution of organisms. Consequently, identifying the mechanistic basis for thermal tolerance is necessary for understanding not only current species range limits, but also the capacity for range limits to shift in response to climate change. Although variation in mitochondrial function likely contributes to variation in thermal tolerance, the extent to which mitochondrial function underlies local thermal adaptation is not fully understood. In the current study, we examine variation in thermal tolerance and mitochondrial function among three populations of the intertidal copepod *Tigriopus californicus* found across a latitudinal thermal gradient along the coast of California, USA. We tested (1) acute thermal tolerance using survivorship and knockdown assays, (2) chronic thermal tolerance using survivorship of nauplii and developmental rate, and (3) mitochondrial performance at a range of temperatures using ATP synthesis fueled by complexes I, II, and I&II, as well as respiration of permeabilized fibers. We find evidence for latitudinal thermal adaptation: the southernmost San Diego population outperforms the northernmost Santa Cruz in measures of survivorship, knockdown temperature, and ATP synthesis rates during acute thermal exposures. However, under a chronic thermal regime, survivorship and developmental rate are more similar in the southernmost and northernmost population than in the mid-range population (Abalone Cove). Though this pattern is unexpected, it aligns well with population-specific rates of ATP synthesis at these chronic temperatures. Combined with the tight correlation of ATP synthesis decline and knockdown temperature, these data suggest a role for mitochondria in setting thermal range limits and indicate that divergence in mitochondrial function is likely a component of adaptation across latitudinal thermal gradients.

INTRODUCTION

Environmental temperature is one of the most influential abiotic factors in shaping the performance and survival of organisms (Hochachka and Somero, 2002). Upper and lower thermal limits of populations and species typically increase from the poles to the equator (Sunday et al., 2011), and in general maximum habitat temperatures at equatorward range limits closely match maximum tolerated temperatures (Sunday et al., 2012). Temperatures that limit organismal performance also often align with habitat temperatures that occur over prolonged periods (i.e., seasons) (Johnston and Dunn, 1987; Pörtner, 2010). Together these observations suggest that variation in temperature and upper thermal tolerance play key roles in determining the biogeographic distribution of organisms (Deutsch et al., 2015; Pörtner, 2010; Sunday et al., 2011, 2012). Thus, identifying the mechanistic basis for differences in upper thermal limits among species and populations is necessary to understand not only current species range limits, but also the capacity for range limits to shift as a result of local genetic adaptation and phenotypic plasticity in response to natural or anthropogenic climate change (Bay et al., 2017; Healy et al., 2018; Pörtner et al., 2006; Pritchard and Di Rienzo, 2010; Seebacher et al., 2015; Sunday et al., 2012).

Mitochondria play a role in a diversity of cellular functions (e.g., calcium signaling, cell growth and differentiation, cell cycle control and cell death), and their central role in energy metabolism suggests mitochondria may be intimately associated with thermal performance and tolerance in ectotherms. For instance, loss of mitochondrial ATP synthesis capacity in fish hearts is thought to occur at temperatures immediately below tolerance limits during acute exposures to high temperatures (Christen et al., 2018; Iftikar and Hickey, 2013; O'Brien et al., 2018). In contrast, in some species loss of whole organism tolerance occurs at temperatures below those

resulting in decreased mitochondrial oxidative capacity (e.g., see Dahlhoff et al., 1991; Dahlhoff and Somero, 1993). In any case, relative changes in mitochondrial oxidative phosphorylation and proton leak at sub-lethal temperatures may underlie thermal limits for whole-organism aerobic capacity and variation in aerobic capacity across temperatures (e.g., Pörtner, 2001). In addition, mitochondrial functions are known to respond to temperature both as a result of phenotypic plasticity and local genetic adaptation through changes in mitochondrial amount (Dhillon and Schulte, 2011; Egginton and Johnston, 1984; O'Brien, 2011; Orczewska et al., 2010), oxidative capacity (Chung et al., 2017a; Chung and Schulte, 2015; Grim et al., 2010; Guderley, 2004; Kraffe et al., 2007; Seebacher et al., 2010), oxygen affinity (Chung et al., 2017b), membrane composition (Chung et al., 2018b; Grim et al., 2010; Hazel, 1995; Kraffe et al., 2007), and enzyme activities and amounts (Guderley, 2004; LeMoine et al., 2008; McClelland et al., 2006; Orczewska et al., 2010; St-Pierre et al., 1998), which clearly suggest that modulation of mitochondrial functions is an important component of cellular responses to temperature change. Thus, although variation in mitochondrial functions likely contributes to the mechanistic basis for variation in thermal tolerance, neither the role of mitochondrial mechanisms across different timescales of thermal exposure (i.e., acute versus chronic), nor the extent to which variation in mitochondrial functions underlies local thermal adaptation across environmental temperature gradients are fully understood.

In the current study, we examine variation in thermal tolerance and mitochondrial function among three populations of the intertidal copepod *Tigriopus californicus* found across a latitudinal thermal gradient along the coast of California, USA (Figure 1-1). *T. californicus* inhabit high-intertidal splash pools on the west coast of North America from Baja California, Mexico, to Alaska, USA, and are an ideal candidate species to study local genetic adaptation of

both thermal tolerance and mitochondrial functions. Gene flow among populations of *T. californicus* is remarkably low even over short distances (Burton, 1998; Peterson et al., 2013; Willett and Ladner, 2009), and consequently there are high levels of genetic divergence among populations both in genes encoded in the mitochondrial genome and in genes in the nuclear genome that encode products that function in the mitochondria (e.g., 9.5 to 26.5% sequence divergence in the mitochondrial genome among populations; Barreto et al., 2018; Pereira et al., 2016; Peterson et al., 2013). Furthermore, there is substantial evidence for local adaptation of upper thermal tolerance among populations of *T. californicus,* with more southern populations generally able to tolerate higher temperatures than more northern populations (Hong and Shurin, 2015; Kelly et al., 2011; Leong et al., 2018; Pereira et al., 2017; Tangwancharoen and Burton, 2014; Willett, 2010; Willett and Son, 2018).

Differences in mitochondrial genotype and function have been linked to variation in thermal performance in species of *Drosophila* (Hoekstra et al., 2013; Pichaud et al., 2012); however, previously published studies in *T. californicus* have not directly addressed this potential mechanistic relationship. It has been observed that disruption of mitochondrial functions in inter-population F_2 hybrids of *T. californicus* does not result in decreased thermal tolerance (Pereira et al., 2014; Willett, 2012), despite lower mitochondrial ATP synthesis capacities at 20 °C (Ellison and Burton, 2008). However, ATP synthesis capacity has not been measured during exposures to high temperatures in *T. californicus,* and thus it is possible that mitochondrial dysfunction may play a role in determining variation in upper thermal tolerance in this species in general. To investigate the mechanistic relationships between upper thermal tolerance and mitochondrial function in *T. californicus* and how these relationships may contribute to latitudinal thermal adaptation across timescales (i.e., acute versus chronic or within-

generation versus across-generations), here we assess (1) intraspecific variation in upper thermal tolerance and performance among populations using survivorship of heat stresses from 34 to 37 °C, knockdown temperatures, developmental survivorship and developmental rate at 20 and 25 °C, (2) inter-population variation in mitochondrial ATP synthesis capacity and thermal sensitivity of ATP synthesis rates during acute thermal exposure, and (3) variation in mitochondrial oxidative and leak respiration rates among populations at both intermediate and high temperatures.

MATERIALS AND METHODS

Copepod collection and culture

Tigriopus californicus were collected from high rocky tide pools at three locations in California, USA (Figure 1-1): "SD" from Ocean Beach, San Diego County (32° 45' N, 117° 15' W), "AB" from Abalone Cove, Los Angeles County (33° 44' N, 118° 22' W), and "SCN" from Santa Cruz County (36° 56' N, 122° 02' W). These populations are found across a latitudinal gradient in temperature along ~775 km of the coast of California with about a 6 °C difference in sea surface temperature (Figure 1-1), and ~4 °C difference in mean annual air temperatures (e.g., Pereira et al., 2017) from SD to SCN.

Copepods were kept in multiple 400-mL beakers containing 250-mL filtered seawater (0.4 µm, 35 ppt). Cultures were fed ad libitum with a combination of ground TetraVeggie algae wafers and powdered Spirulina. Prior to use in any thermal exposure assays, cultures were maintained in the laboratory for at least one month (one generation) at 20 °C with a 12-h light/dark cycle.

Survivorship of acute heat stress across temperatures

Tolerance of a one-hour heat stress at different temperatures was measured using methods similar to those previously described for *T. californicus* (Kelly et al., 2011; Leong et al., 2018; Pereira et al., 2017; Tangwancharoen and Burton, 2014; Willett, 2010; Willett and Son, 2018). In brief, groups of ten adult copepods from each population were removed from stock cultures by glass pipette, and transferred to separate 15-mL Falcon™ tubes (Thermo Fisher Scientific, Waltham, MA) containing 10-mL of 20 °C filtered seawater. After 10 min, the tubes were submerged in a pre-heated water bath (Julabo USA Inc., Allentown, MA) at one of four temperatures: 34, 35, 36 or 37 °C. Following 1-h of heat stress, tubes were moved to a beaker containing 20 °C water for an additional hour to allow the temperature inside the tubes to gradually decrease back to holding conditions. Copepods were then transferred to a fresh 10-cm petri dish containing 20 °C filtered seawater. Tolerance of the acute high temperature exposures was assessed after three days as the proportion of surviving individuals in each group of ten (n = 6 per population and temperature).

Knockdown temperature

Maximum tolerated temperature during a ramping heat stress was assessed by knockdown temperature (i.e., the temperature at which movement and responsiveness cease; e.g., Gilchrist and Huey, 1999; Hoffmann et al., 2003). For each trial, eight adult copepods from each population were individually pipetted from stock cultures into 0.2 mL strip tubes (without caps), and carry-over water was replaced with 100 µL of 20 °C filtered seawater. After approximately a 10-min recovery, the strip tubes were placed in an Applied Biosystems SimpliAmp™ Thermal Cycler (Thermo Fisher Scientific, Waltham, MA). Locations of individuals from each population

were randomized in the thermal cycler for each trial. The heat stress regime utilized the AutoDelta function of the thermal cycler with the following protocol: 20 °C for 5 min, +0.1 °C every 20 s from 20 to 32 °C, and +0.1 °C every 60 s from 32 to 45 °C. The thermal cycler lid was left open during the temperature ramp and copepods were monitored continuously from above. Once a copepod stopped responding to gentle tapping of their tube, loss of responsiveness was assessed by cycling 40 μL of the water in the tube with a micropipette taking care not to touch the copepod directly. In general, this manipulation results in the observation of active swimming behavior in *T. californicus*; however, at high temperatures this response is no longer observed and copepods gradually sink to the bottom of the tubes passively. If a copepod actively responded to the water movement, responsiveness tests were paused and monitoring continued. If no active response was observed, the responsiveness test was repeated up to three times (total time for end-point determination for an individual was ~6 s). The first temperature at which a copepod did not respond to three successive tests was recorded as the individual's knockdown temperature (n = 16 per population over two trials). After this end point was determined, individuals were transferred by glass pipette to recovery 10-cm petri dishes containing 20 °C filtered seawater (one per population); survivorship 1 d after the assay was >90%.

Developmental survival and rate

Gravid females with mature (red) egg sacs were removed from stock cultures by glass pipette and immobilized on filter paper (n = 24 per population). Egg sacs were dissected from the females with a needle and placed in 6-well plates containing 20 °C filtered seawater (one egg sac per well). Eggs were allowed to hatch overnight at 20 °C, and in the morning the nauplii (i.e., offspring) from each sac were counted and split between two 6-well plates (one well per plate

per egg sac). The two plates were incubated for 21 d at 20 or 25 °C (one plate per temperature). Spirulina was added to each well twice per week as a food source, and salinity was adjusted using addition of deionized water to compensate for evaporation weekly. On days 7, 14 and 21, surviving individuals and the number of nauplii that had metamorphosed into copepodids were counted in each well. The number of wells in which adult males were observed was also recorded. Presence of adult males, rather than females, was used, because the final molt to adult male *T. californicus* results in visually conspicuous antennae that are used in mating behaviors (Burton, 1985; Tsuboko-Ishii and Burton, 2017). In contrast, young adult females are visually difficult to distinguish from juvenile females and males until later stages of maturity (Burton, 1985), and thus are sub-optimal indicators of developmental stage.

For measurement of developmental rate at 20 and 25 °C, mature egg sacs were dissected and split post-hatch as described above (n =18, 16 and 18 for SD, AB and SCN, respectively), but in this case split egg sacs were pooled into 10-cm petri dishes (one dish per population and temperature) and developmental stage was monitored daily. Rate of development was tracked by the day of appearance of copepodids in the dish and was scored for each individual (Tangwancharoen and Burton, 2014). Regardless of developmental temperature, the survivorship of offspring for all populations was 78.6-94.0%, and all surviving individuals metamorphosed by 14 d post clutch split.

ATP synthesis rate

ATP synthesis assays were conducted using similar methods to those of Ellison and Burton (2006). Mitochondria were isolated from groups of 30 adult copepods per assay (n = 6 per population). Preliminary tests indicated that this number of copepods was sufficient to show

linear ATP production over time for at least 1 h across the range of temperatures tested in this study (20 to 40 °C). Copepods were rinsed with 200 µL ice cold homogenization buffer (400 mM sucrose, 100 mM KCl, 6 mM EGTA, 3 mM EDTA, 70 mM HEPES, 1% w/v BSA, pH 7.6) (Moyes et al., 1985), and homogenized on ice in 800 µL homogenization buffer with a teflon on glass homogenizer. The homogenate was transferred to 1.5-mL microcentrifuge tubes (Eppendorf, Hamburg, Germany) and centrifuged at 1,000 g for 5 min at 4 °C. The supernatant was transferred to new microcentrifuge tubes and was centrifuged at 11,000 g for 10 min at 4 °C. The supernatant resulting from this second centrifugation was then removed and the pellet was resuspended in 255 µL assay buffer (560 mM sucrose, 100 mM KCl, 10 mM KH_2PO_4, 70 mM HEPES, pH 7.6; modified from Moyes et al., 1985).

Mitochondrial isolations were split into ten 25-µL aliquots in PCR tubes for ATP synthesis assays at nine incubation temperatures (one tube per temperature: 20, 25, 30, 35, 36, 37, 38, 39 and 40 °C, and one tube for measurement of initial ATP concentrations in the assays). The remaining 5 µL of isolate was used for protein content determination. To initiate synthesis assays, 5 µL of a substrate cocktail was added to each tube, and then the tubes were incubated at the desired temperature for 10 min in an Applied Biosystems SimpliAmp™ Thermal Cycler (Thermo Fisher Scientific, Waltham, MA). The substrate cocktail depended on the electron transport system (ETS) complexes that were used to drive electron transport and subsequent ATP synthesis. In our study, we measured ATP synthesis rate as a result of electron donation to complex I (CI; final substrate concentrations in assay: 5 mM pyruvate, 2 mM malate and 1 mM ADP), complex II (CII; final substrate concentrations in assay: 10 mM succinate, 0.5 µM rotenone, and 1 mM ADP), and complex I and complex II in combination (CI&II; final substrate concentrations in assay: 5 mM pyruvate, 2 mM malate, 10 mM succinate and 1 mM ADP). After

the incubation period, 25 µL of each assay was added to an equal volume of CellTiter-Glo (Promega, Madison, WI), which both halts ATP synthesis and allows quantification of ATP concentration. Tubes used to measure initial ATP concentrations in the assays also had 5 µL of substrate cocktail, but CellTiter-Glo was added immediately following substrate addition. Therefore, these tubes accounted for any signal that was not a result of ATP synthesis during the assays. After a 10-min incubation at room temperature, the samples and controls were mixed by shaking, read on a luminometer and compared with a set of ATP standards (5nM - 10 µM prepared in assay buffer; 25 µL of each standard was mixed with 25 µL of CellTiter-Glo as described above for samples and controls). ATP synthesis rates were calculated after control values were subtracted from sample values, and rates were then normalized for protein content in the corresponding mitochondrial isolation using NanoOrange Protein Quantitation Kit assays (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. Note that, while our buffer contained compounds (HEPES, sucrose, and potassium chloride) that have the potential to interfere with this assay at high concentrations (10 mM, 10 mM, and 20 mM, respectively), the final concentrations present after dilution were below these maxima for HEPES and potassium chloride (1.37 mM and 1.96 mM, respectively), and sucrose concentration was approximately the maximum recommended according to the manufacturer's instructions (10.98 mM).

High-resolution respirometry of cell-permeabilized copepods

Respirometry was performed using a Clark-type electrode system (Oxygraph Plus System, Hansatech Instruments Ltd., England). The electrode was calibrated at either 20 or 35 °C using air-saturated assay buffer (see "ATP synthesis rate" section above). Groups of 40 adult

copepods were crudely homogenized using blue polypropylene pestles (Thomas Scientific, Swedesboro, NJ) in a 1.5-mL microcentrifuge tube to which 500 µL cold (~0 °C) BIOPS was added (2.77 mM CaK_2EGTA, 7.23 mM K_2EGTA, 5.77 mM ATP, 6.56 mM $MgCl_2$, 20 mM taurine, 15 mM Phosphocreatine, 20 mM imidazole, 0.5 mM dithiothreitol, 50 mM K-MES, pH 7.1; Lemieux et al., 2011). Homogenized animals were then permeabilized by incubation in BIOPS containing 81.25 µg mL^{-1} saponin (concentration determined from Pichaud et al., 2012, and preliminary trials) for 30 min at 4 °C prior to use in respirometry assays. After incubation, BIOPS was removed and 500 µL of assay buffer was added. The permeabilized copepods in assay buffer were then transferred into the respirometry chamber. The ETS was activated with CI substrates (5 mM pyruvate and 5 mM malate) and 2.5 mM ADP, after which state III respiration rate was measured (i.e., the maximal respiration rate under phosphorylating conditions; Scheffler, 2008). 10 µM cytochrome c was then added to assess membrane integrity and in general resulted in no change in respiration rate. Next, 2 µg mL^{-1} oligomycin was added to measure state IV_{ol} respiration rate. State IV is the rate of respiration in the absence of phosphorylation of ADP, which is the minimum rate required to counter proton leak and maintain proton motive force across the inner mitochondrial membrane; state IV_{ol} achieves this artificially with the addition of oligomycin, which inhibits complex V (Scheffler, 2008). Concentrations of substrates and inhibitors were modified from Pesta and Gnaiger (2012) based on preliminary trials.

Statistical analyses

All statistical analyses were performed in R v3.4.0 (R Core Team, 2017) using generalized linear models (GLM) and ANOVA followed by post-hoc tests with a threshold for

statistical significance of $\alpha = 0.05$. Differences in survival of 1-h acute heat stress were tested with population and exposure temperature as factors in a logistic GLM with a binomial error distribution. Pairwise comparisons among temperatures within populations, and among populations within temperatures were conducted by t tests (paired or unpaired as appropriate) with a Bonferroni correction to determine significance. Data for knockdown temperatures and clutch sizes were analyzed by ANOVA with population as a factor followed by Tukey post-hoc tests. The same procedure was utilized to analyze mitochondrial respiration data, but population was added as an additional explanatory factor. Variation in time to metamorphosis (i.e., developmental rate) was assessed among treatments (populations x temperature) by Kruskal-Wallis ANOVA followed by Nemenyi tests. Significance of all pairwise comparisons was determined after a Bonferroni correction of alpha. Differences in developmental survival were calculated for each egg sac (25-20 °C; see Table S1 for group means, metamorphosis and adult proportions), and were analyzed by mixed-effect linear models with fixed effects of population and day and a random effect of egg sac. Post-hoc comparisons were then performed with Tukey tests. Mixed-effect models were also used to assess variation in ATP synthesis rates with population and temperature as fixed effects and mitochondrial isolation as a random effect. Post-hoc pairwise comparisons for ATP synthesis rate were conducted similarly to those for survival of 1-h heat stress, but with use of the Benjamini-Hochberg method to correct for multiple comparisons (Benjamini and Hochberg, 1995). In all cases, interactions between factors were included in the fitted linear models.

RESULTS

Thermal tolerance and performance

Patterns of variation in tolerance of acute heat stress among populations of *T. californicus* were consistent regardless of the methods used to assess tolerance. Survivorship of 1 h heat stress was affected by a significant interaction between population and exposure temperature ($p = 5.8 \times 10^{-5}$; Figure 1-2A). Post-hoc comparisons detected significant declines in survivorship between 35 and 36 °C in all populations ($p \leq 1.4 \times 10^{-5}$). However, the survival proportion of SCN was lower than that of SD at 35 °C ($p = 6.9 \times 10^{-4}$) and was lower than the survival proportions of both SD and AB at 36 and 37 °C ($p \leq 9.1 \times 10^{-8}$). This pattern of decreased upper thermal tolerance in the northern SCN population was also observed through variation in knockdown temperature among populations ($p = 6.1 \times 10^{-11}$; Figure 1-2B). For both measurements of acute upper thermal tolerance, there were slight trends for lower mean tolerance in AB copepods than in SD copepods, but these trends were not supported statistically after correction for multiple comparisons (1 h heat stress: $p \geq 9.5 \times 10^{-3}$ compared to corrected $\alpha = 1.7 \times 10^{-3}$; knockdown temperature: $p = 0.63$).

We observed significant variation in the number of eggs per clutch among populations ($p = 1.0 \times 10^{-12}$; Figure 3A) with smaller egg sacs from AB females than those from either SD or SCN females ($p < 1.0 \times 10^{-4}$). Differences in survival of offspring from split egg clutches over three weeks of development and early adulthood at 20 and 25 °C were significantly affected by an interaction between population and time ($p = 2.4 \times 10^{-3}$; Figure 3B). In general, development at 25 °C had either no effect or negative effects on survival compared to development at 20 °C in all three populations. There was no variation in the relative effects of development at 25 °C on survival with time in either SD or SCN ($p \geq 0.97$), and there were trends for greater negative effects of 25 °C on survival in SCN than in SD on all days, but these trends were not significant in post-hoc tests ($p \geq 0.23$). In contrast, the difference in survival between 25 and 20 °C in AB

became more negative over the three weeks with a significant decrease in relative survival at 21 d compared to 7 or 14 d ($p \leq 1.7 \times 10^{-2}$). Additionally, on day 21 the difference in survival between 25 and 20 °C was significantly lower in AB than in SD ($p < 1.0 \times 10^{-3}$). Not surprisingly, there were also significant effects of temperature on developmental rate in all three populations with more rapid development at 25 °C than at 20 °C ($p < 2.8 \times 10^{-11}$ for all populations; Figure 3C), although the decrease in median time to metamorphosis varied among populations (7, 8 and 9 days post clutch split at 20 °C to 4, 5 and 7 days post clutch split at 25 °C for SCN, SD and AB, respectively). There were also significant effects of population on time to metamorphosis at both temperatures: at 20 °C SCN developed faster than either SD or AB ($p < 8.9 \times 10^{-11}$ for both), and at 25 °C all three populations were significantly different from each other with fastest development in SCN, slowest development in AB and intermediate developmental rate in SD ($p \leq 5.8 \times 10^{-3}$). Overall, these results suggest modest negative effects of 25 °C on development in *T. californicus*, which are generally more pronounced in AB than in the other two populations in our study. Furthermore, AB demonstrates lower performance (i.e., smaller clutch sizes or slower developmental rate) than SD or SCN even at 20 °C.

ATP synthesis rate and mitochondrial respiration

Regardless of the substrates and complexes used (CI, CII or CI&II) to donate electrons to the ETS and drive ATP synthesis, synthesis rate was affected by significant interactions between population and temperature ($p \leq 5.1 \times 10^{-3}$). If electrons were donated to both CI and CII (Figure 1-4A), ATP synthesis rate increased from 20 to 30 °C in all three populations. Synthesis rate then plateaued from 30 to 35 °C in SCN, and from 30 to 36 °C in SD and AB. Above these temperatures ATP synthesis declined rapidly, and at temperatures greater than 36 °C rates in

SCN were either lower than those in AB ($p \leq 8.2 \times 10^{-3}$; 37 and 40 °C), or lower than those in AB and SD ($p \leq 2.5 \times 10^{-2}$; 38 and 39 °C). SCN ATP synthesis rate was also significantly higher than AB ATP synthesis rate at 25 °C ($p = 3.8 \times 10^{-2}$). If only CI substrates were used to drive ATP synthesis (Figure 1-4B), there were increases in synthesis rate with temperature from 20 to 35 °C in all populations. At temperatures above 35 °C, synthesis rate rapidly declined with temperature in SD and SCN, whereas in AB rates plateaued from 35 to ~37 °C before declining at higher temperatures. As a result, there were no significant differences between the populations above 35 °C ($p \geq 0.07$). In contrast, CI-driven ATP synthesis was faster in SCN than in AB from 20 to 35 °C ($p \leq 3.1 \times 10^{-2}$), and similar trends were observed between SD and AB, although these trends were not detected as significant in post-hoc tests ($0.051 < p < 0.108$ for all). If only CII was used to drive electron transport (Figure 1-4C), patterns of change in ATP synthesis rate with temperature in all populations were similar to those described above for CI&II-fueled ATP synthesis. However, only one population effect on CII-driven synthesis rate was detected by post-hoc tests with a higher rate in AB than in SCN at 40 °C ($p = 2.5 \times 10^{-2}$). Taken together, these results suggest that population- or temperature-mediated differences in CI&II ATP synthesis rate in *T. californicus* likely reflect contributions of population and temperature effects on synthesis rate when CI or CII are fueled separately, although under saturating conditions the independent CI and CII rates are not additive when the ETS is provided with both CI and CII substrates in combination. Additionally, our results indicate that AB synthesis rates, particularly when fueled through CI, are compromised relative to at least SCN rates from 20 to ~35 °C, and that ATP synthesis capacity suffers high-temperature collapse at lower temperatures in SCN than in either SD or AB.

In general, high-resolution respirometry experiments found that mitochondrial oxygen consumption rates fueled by CI substrates were variable within populations and temperatures in our study, and few differences were resolved statistically. State III respiration rate (Figure 1-5A) was not significantly affected by population ($p = 0.47$), temperature ($p = 0.57$) or an interaction between population and temperature ($p = 0.21$), and state IV_{ol} respiration rate (Figure 1-5B) and respiratory control ratio (RCR = state III / state IV_{ol}; Figure 1-5C) were also unaffected by population ($p \geq 0.19$) or an interaction between population and temperature ($p \geq 0.25$). There were significant main effects of temperature on state IV_{ol} oxygen consumption rate and RCR ($p \leq 3.6 \times 10^{-2}$), but these effects were not resolved by post-hoc tests (within population $p \geq 0.11$). The main effects of temperature that were detected may be a consequence of slight trends for increases in state IV_{ol} respiration rate and decreases in RCR from 20 to 35 °C in all populations. Of these trends, the most notable is the decline in mean RCR in SCN at 35 °C (post-hoc $p = 0.11$), which may be consistent with the rapid declines in ATP synthesis rate at temperatures above 35 °C in SCN described above.

DISCUSSION

The results of the current study demonstrate intraspecific variation in both thermal tolerance and ATP synthesis capacity among allopatric *Tigriopus californicus* populations that are found across a latitudinal thermal gradient. Differences in tolerance of acute high temperatures among populations are generally consistent with variation in habitat temperature, whereas differences in chronic temperature effects among populations are less clearly correlated with environmental temperatures. Populations have temperature-dependent differences in ATP synthesis capacity, as revealed by variation in ATP synthesis rates in isolated mitochondria

across temperatures. Furthermore, inter-population differences in loss of synthesis capacity during acute exposure to high temperatures parallel differences in acute upper thermal tolerance, and variation in ATP synthesis rate among populations at 25 °C is potentially consistent with differences in effects of chronic exposure to 25 °C on development as well. These results suggest that there are evolved differences in ATP synthesis capacity among populations of *T. californicus*, and that these differences are likely involved in local thermal adaptation with latitude in this species, particularly over acute timescales of thermal exposure.

Variation in thermal tolerance and mitochondrial function across timescales

Several studies have demonstrated intraspecific differences in upper thermal tolerance with latitude in *T. californicus* that are consistent with local thermal adaptation from Mexico to Alaska (Kelly et al., 2011; Pereira et al., 2017; Willett, 2010). These studies employed experimental tests similar to our 1-h heat stress protocol, and our results generally corroborate previous findings for SD, AB and SCN. Here, we also establish an alternative experimental method to examine variation in acute upper thermal tolerance in *T. californicus* through the use of knockdown temperatures, and we demonstrate that post-hoc tests with data from either of these thermal tolerance methods resolve similar inter-population patterns for variation in upper thermal limits (Figure 1-2). Our knockdown temperature assay is essentially similar to a critical thermal maximum assay (e.g., Beitinger et al., 2000), and provides several advantages such as reducing the number of animals required to measure upper thermal tolerance and allowing repeated tests of variation in upper thermal tolerance among individuals (e.g., Morgan et al., 2018). Therefore, this protocol is likely to facilitate future experiments investigating the genetic

basis of variation in upper thermal limits in *T. californicus* and of local thermal adaptation more generally.

There is a growing body of evidence suggesting that collapse of ATP synthesis capacity is mechanistically involved in setting upper thermal limits during acute exposure to high temperature, particularly in fish hearts (Christen et al., 2018; Iftikar and Hickey, 2013; O'Brien et al., 2018). Cardiovascular failure due to arrhythmias is potentially a weak physiological link underlying upper thermal tolerance in fish (e.g., Farrell, 2009), and it is now thought that insufficient energy supply due to decreased mitochondrial ATP synthesis may underlie this heart failure (e.g., Iftikar and Hickey, 2013). Our results suggest that loss of ATP synthesis capacity may also contribute to setting acute upper thermal limits in *T. californicus* (Figure 1-4), a species that does not rely on a heart to transport oxygen throughout the body. Rapid declines in CI&II-fueled ATP synthesis rate are observed at high temperatures in all three populations in our study, but these declines occur ~1 °C lower in SCN than in AB or SD (~36 versus ~37 °C; Figure 1-4A). In comparison, mean knockdown temperatures for the three populations are 36.5, 38.4 and 38.6 °C for SCN, AB and SD, respectively. Although the differences between the temperatures that result in high temperature knockdown or initial declines in ATP synthesis capacity vary somewhat among populations, declines in ATP synthesis rate occur at temperatures that are only slightly lower than knockdown temperatures in all cases. Furthermore, if our data are used to estimate CI&II-linked ATP synthesis rate for each population at knockdown temperatures, rates at upper thermal limits are similar regardless of population (0.89, 1.12 and 1.15 nmol min^{-1} mg mito. $protein^{-1}$ for SD, AB and SCN, respectively).

Here, we also demonstrate inter-population differences in the chronic effects of high temperature on survival and developmental rate in *T. californicus*. In the AB population,

development at 25 °C resulted in a decrease in survival over time when compared to development at 20 °C, and this led to a significant difference in the effects of 25 °C on developmental survival in AB compared to SD after three weeks (Figure 3B). Indeed, there was no evidence for a negative effect of 25 °C on survival of development in SD copepods, and similarly there was no significant effect of 25 °C relative to 20 °C in SCN. Somewhat contradicting these results for SD and SCN, Edmands and Deimler (2004) found negative effects of 25 °C development relative to 15 °C development in these populations, but consistent with our study, these authors found no difference between SD and SCN. Regardless, our data suggest that prolonged exposure to 25 °C has greater negative effects on AB than on the other two populations in our study.

In all populations, development at 25 °C, compared to 20 °C, caused an increase in developmental rate (i.e., decrease in time to metamorphosis) that was consistent with temperature coefficients (Q_{10}) of ~2 as would be expected (Hochachka and Somero, 2002); however, the increase in rate was smaller in AB than in either SD or SCN (Figure 1-3C). It is possible that this is simply a consequence of exponential effects of temperature on physiological rates and a lower 20 °C rate in AB. Alternatively, there was evidence for reduced ATP synthesis capacity in AB compared to SD or SCN when substrates were provided to both CI and CII (Figure 1-4A) or to CI alone (Figure 1-4C), particularly at 25 °C, which could result in trade-offs between development and other physiological costs when AB copepods undergo development at 25 °C. Energetic compromises in life history traits in AB are also consistent with smaller egg clutches at 20 °C than in the other populations and reduced ATP synthesis rates in AB copepods (at least compared to SCN copepods). The relationship between ATP synthesis rate, particularly under saturating substrate conditions, and energy demand at 20 and 25 °C among populations is

complex, as both ATP supply and demand are thermally sensitive and demand may vary among populations even at 20 °C. However, our results may indicate that intraspecific variation in ATP synthesis capacity is related to thermal effects over prolonged timescales as well as the acute effects discussed above.

Role of variable mitochondrial function in latitudinal thermal adaptation

Upper thermal limits in aquatic ectotherms decrease approximately linearly from the equator to the poles (Sunday et al., 2011) closely matching habitat temperatures (Sunday et al., 2012). Because *T. californicus* inhabit splash pools in the extreme upper intertidal, air temperatures are potentially better predictors of habitat temperature than sea surface temperatures for this species (e.g., Pereira et al., 2017), and differences in mean knockdown temperature among our populations (38.6, 38.4 and 36.5 °C for SD, AB and SCN, respectively; Figure 1-2) parallel differences in mean annual air temperature between the populations (Pereira et al., 2017), although the differences in knockdown temperatures are smaller than those for estimated habitat temperatures. In part due to these similarities, variation in acute upper thermal tolerance is thought to be an important consequence of local thermal adaptation in this species (Kelly et al., 2011; Pereira et al., 2017; Willett, 2010). Thus, by extension, our data suggest that differences in the thermal sensitivity of ATP synthesis capacity (Figure 1-4) likely contribute to local adaptation across this latitudinal gradient. However, it is important to note that our study examined this trait in only three populations of *T. californicus* that are found across a relatively small proportion of the latitudinal range of the species (Mexico to Alaska), and therefore the extent to which our results can be generalized to larger latitudinal ranges remains unclear and merits future experimental consideration.

The predominant signatures of selection that are typically detected for genes in the mitochondrial genome are those of purifying selection (Palozzi et al., 2018; Stewart et al., 2008), which is perhaps not surprising given the central roles many of these genes play in mitochondrial protein or RNA complexes. However, mitochondrial genes are often involved in adaptive responses (Ballard and Whitlock, 2004), and signatures of directional selection have been detected for some genes encoded in the mitochondrial genome in *T. californicus,* particularly CI genes (*nad3*, *nad5*, and *nad6*; Barreto et al., 2018). Furthermore, nuclear genes encoding products involved in mitochondrial functions have elevated nucleotide substitution rates compared to other nuclear genes in this species (Barreto et al., 2018; Willett and Burton, 2004). Given our results for latitudinal variation in the temperatures that result in loss of ATP synthesis capacity among populations, it is possible that at least some of this genetic variation contributes to the differences in thermal sensitivity of ATP synthesis rate among populations.

In contrast to variation in upper thermal tolerance at acute timescales of exposure, differences in chronic effects of high temperature on survival or developmental rate were not clearly associated with latitudinal variation in temperature in the current study. Variation in life history traits (i.e., egg clutch size, developmental survival and developmental rate) and changes in these traits due to elevated temperature were consistent with reduced survival and developmental rate in AB compared to SD and SCN (Figure 1-3). Although our data suggest that variation in ATP synthesis capacity may contribute to these differences (discussed above), these patterns are not obviously related to differences in habitat temperatures among SD, AB and SCN. Thus, our data for these three populations do not support a role of mitochondria in latitudinal adaptation associated with prolonged thermal exposures (i.e., months or seasons) in *T. californicus*.

Variation in life history traits with latitude, and therefore environmental temperature, is common in ectotherms (e.g., Chung et al., 2018a), and *T. californicus* is not an exception to this trend (Hong and Shurin, 2015). Latitudinal changes in these traits in *T. californicus* are smooth and gradual, such that relatively little variation is expected along the Californian coast in general (Hong and Shurin, 2015), which is consistent with our results in SD and SCN, which span less than half of California's latitudinal range. Therefore, it is possible that comparisons of more geographically distant populations than those used in the current study would reveal variation in chronic temperature effects on ATP synthesis capacity that parallel differences in habitat temperatures.

Taken together, the data presented in the current study suggest a role for mitochondrial functions, particularly ATP synthesis capacity, in determining the limits of tolerance of both short- and long-term exposures to elevated temperatures. Variation in acute upper thermal tolerance in *T. californicus* is consistent with local thermal adaptation (Kelly et al., 2011; Pereira et al., 2017; Willett, 2010; the current study), and therefore our results suggest that divergence in ATP synthesis capacity is a component of adaptation across latitudinal thermal gradients as well. Given that the acute effects of temperature in our study more closely paralleled habitat temperatures than chronic effects of temperature, our data also suggest that extreme temperature events impose important selection pressures that likely drive local thermal adaptation, as has been suggested previously (Siegle et al., 2018; Somero, 2010). Determining both the extent to which thermal sensitivity of ATP synthesis capacity influences the effects of these rare events on organisms and the mitochondrial mechanisms that underlie this sensitivity will be critical steps in accurately predicting the impacts of future environmental change on ectotherms.

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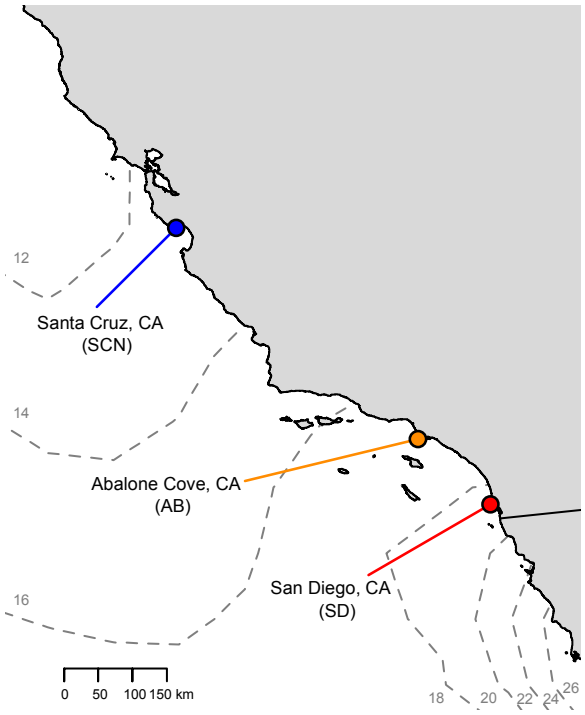

Figure 1-1. Locations of *T. californicus* collections sites along the coast of California (grey = land; white = Pacific Ocean). Sites are indicated by colored circles (red = San Diego [SD]; orange = Abalone Cove [AB]; blue = Santa Cruz [SCN]). Dashed grey lines outline contours of average sea surface temperatures from the first week of July 2017 with temperature in degrees Celsius indicated by small grey numbers (obtained from http://www.cpc.ncep.noaa.gov/products/GIS/GIS_DATA/sst_oiv2/index.php).

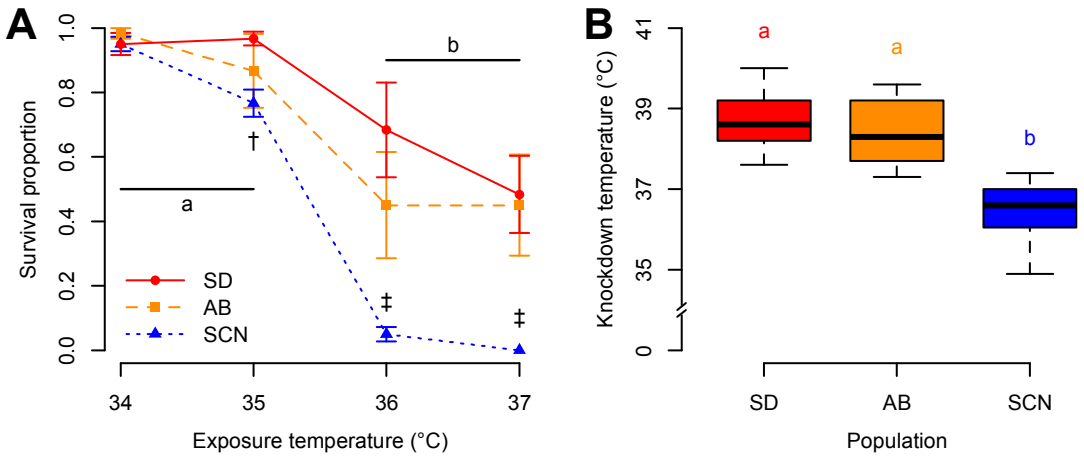

Figure 1-2. Acute upper thermal tolerance assessed by survivorship of 1 h heat stress (A) and knockdown temperature (B) in San Diego (SD; red, circles, solid lines), Abalone Cove (AB; orange, squares, dashed lines) and Santa Cruz (SCN; blue, triangles, dotted lines) copepods. A: data are presented as mean ± SEM; shared letters indicate temperatures that are not significantly different within populations; dagger symbols indicate differences among populations within a temperature († = SCN different from SD, ‡ = SCN different from SD and AB). B: data are presented as box plots; shared letters indicate populations that do not differ significantly.

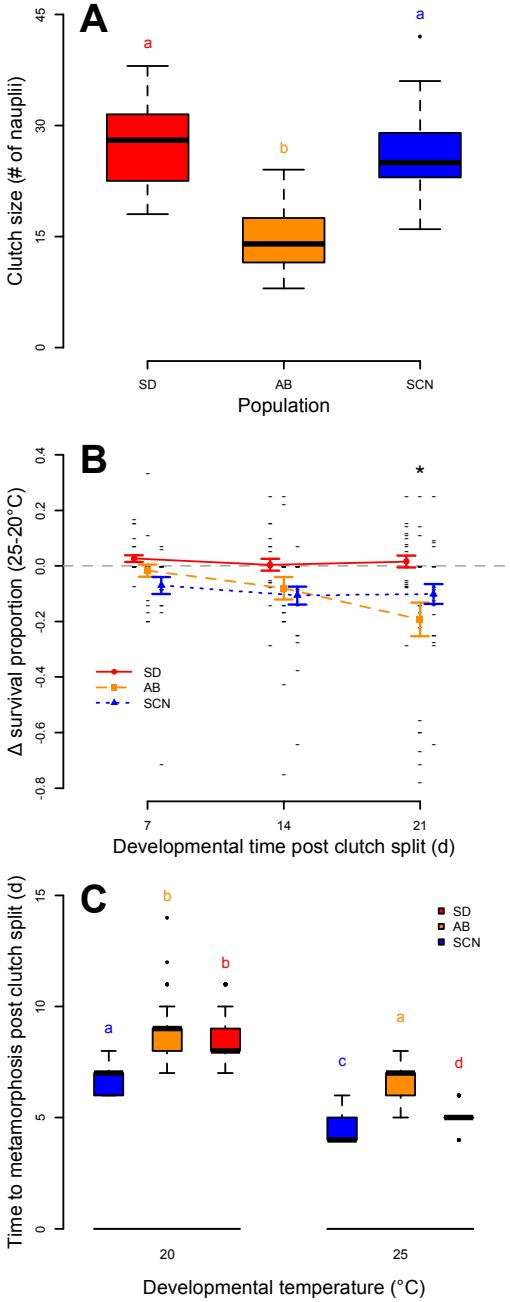

Figure 1-3. Egg clutch size (A), survival of development at 25 °C compared 20 °C (B), and time to metamorphosis at 20 and 25 °C (C) in San Diego (SD; red, circles, solid lines), Abalone Cove (AB; orange, squares, dashed lines) and Santa Cruz (SCN; blue, triangles, dotted lines) copepods. A,C: data are presented as box plots; shared letters indicate groups that are not significantly different. B: data are presented as mean ± SEM; small black dashes display difference for individual clutches; asterisk indicates a significantly lower difference in survival between 25 and 20 °C at 21 d than at 7 or 14 d for AB and a significant difference between SD and AB at 21 d.

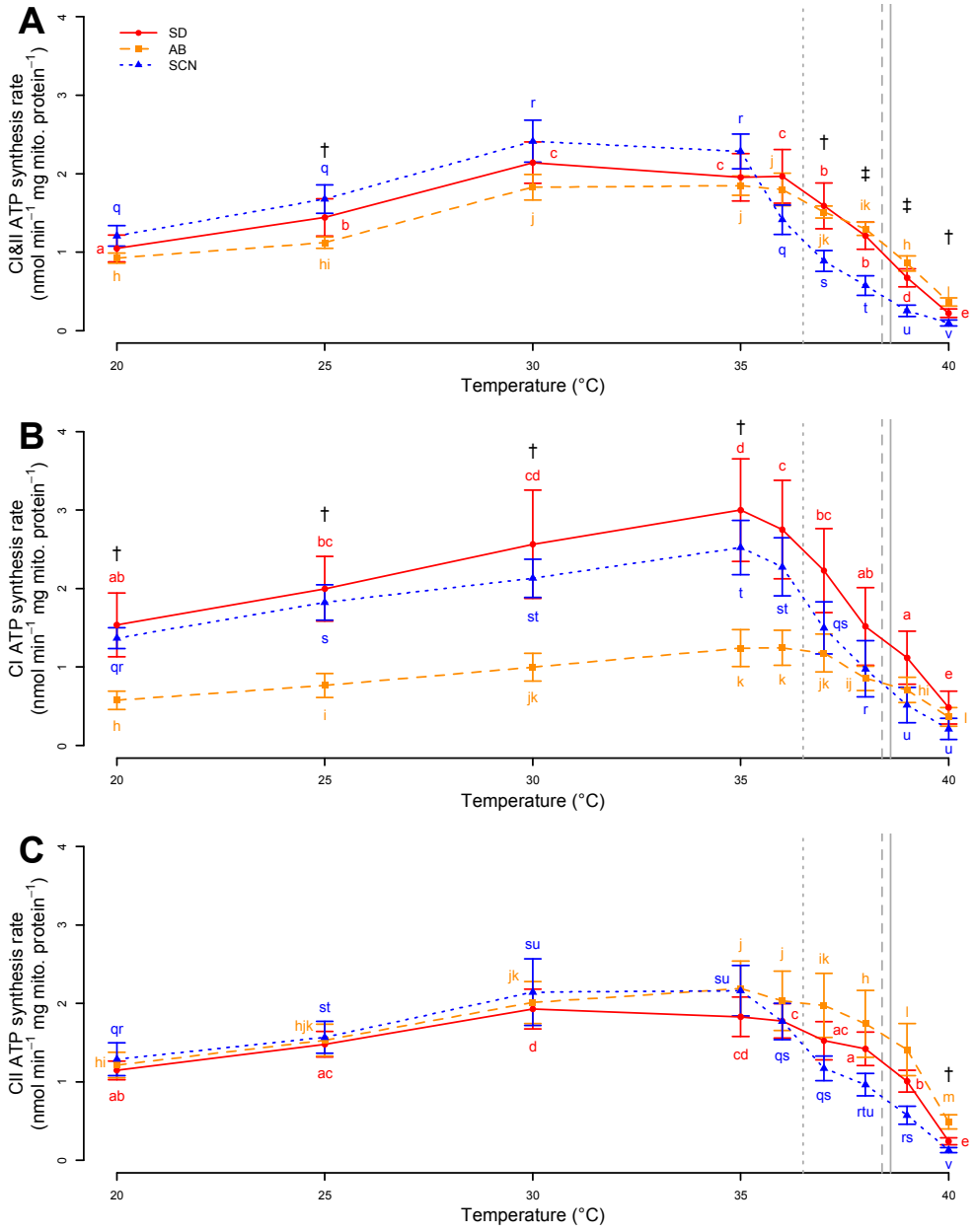

Figure 1-4. CI&II- (A), CI- (B) and CII-fueled (C) ATP synthesis rate from 20 to 40 °C in San Diego (SD; red, circles, solid lines), Abalone Cove (AB; orange, squares, dashed lines) and Santa Cruz (SCN; blue, triangles, dotted lines) copepods. Data are presented as mean ± SEM. Letters indicate the results of post-hoc tests within populations. Daggers indicate temperatures at which there is a difference between SCN and AB. Double daggers indicate temperatures at which there is a difference between SCN and both AB and SD. Vertical grey lines display mean knockdown temperatures for each population (SD – solid; AB – dashed; SCN – dotted).

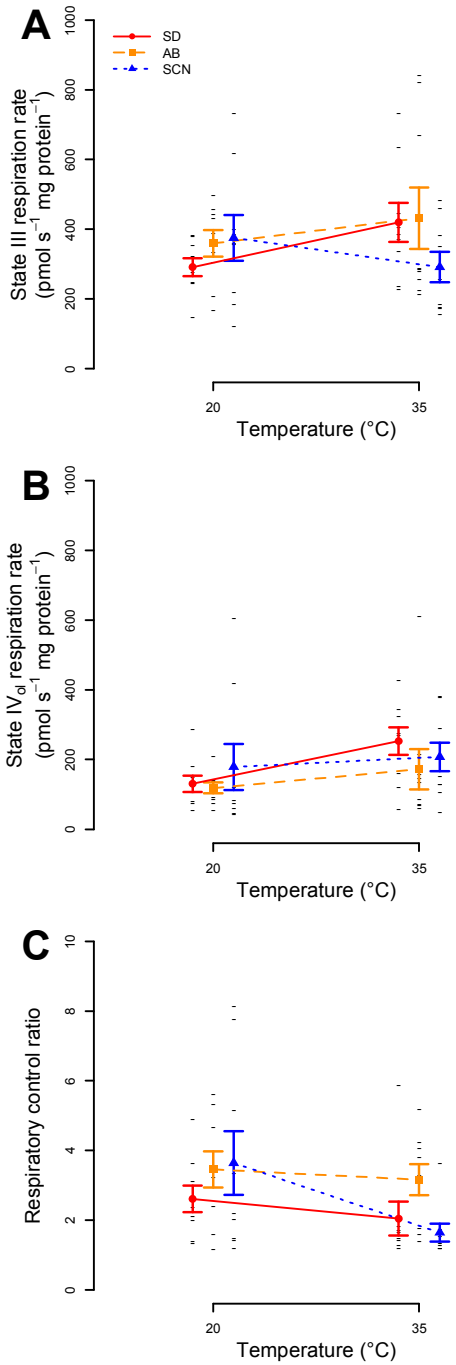

Figure 1-5. State III (A) and state IV_{ol} (B) mitochondrial respiration, and respiratory control ratios (RCRs; C) at 20 and 35 °C in San Diego (SD; red, circles, solid lines), Abalone Cove (AB; orange, squares, dashed lines) and Santa Cruz (SCN; blue, triangles, dotted lines) copepods. Data are presented as mean ± SEM, and small black dashes display values for each replicate. Significant effects of temperature for state IV_{ol} and RCR were detected by ANOVA, but post-hoc tests did not resolve any statistical differences among groups for all three traits.

APPENDIX

Table A1-1. Proportion of nauplii that survived or metamorphosed (mean ± SEM), and proportion of clutches with adult males throughout development at 20 or 25 °C.

	Time (d)	San Diego (SD)		Abalone Cove (AB)		Santa Cruz (SCN)	
		20 °C	25 °C	20 °C	25 °C	20 °C	25 °C
Survival proportion	7	0.97 ± 0.01	1.00 ± 0.00	0.96 ± 0.03	0.95 ± 0.02	0.97 ± 0.01	0.90 ± 0.03
	14	0.96 ± 0.01	0.96 ± 0.02	0.96 ± 0.03	0.88 ± 0.04	0.95 ± 0.02	0.84 ± 0.03
	21	0.92 ± 0.03	0.94 ± 0.03	0.89 ± 0.04	0.69 ± 0.07	0.92 ± 0.02	0.82 ± 0.03
Metamorphosis proportion	7	0.86 ± 0.06	0.96 ± 0.02	0.90 ± 0.05	0.99 ± 0.01	0.95 ± 0.02	0.99 ± 0.01
	14	1.00 ± 0.00	0.99 ± 0.01	0.98 ± 0.02	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
	21	1.00 ± 0.00	1.00 ± 0.00	0.99 ± 0.01	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
Proportion of clutches with adult males	7	0.00	0.00	0.00	0.00	0.00	0.00
	14	0.00	0.58	0.00	0.38	0.00	0.25
	21	0.67	1.00	0.38	0.83	0.88	0.96

REFERENCES

Ballard, J. W. O., and Whitlock, M. C. (2004). The incomplete natural history of mitochondria. *Mol. Ecol.* 13, 729–744. doi:10.1046/j.1365-294X.2003.02063.x.

Barreto, F. S., Watson, E. T., Lima, T. G., Willett, C. S., Edmands, S., Li, W., and Burton, R. S. (2018). Genomic signatures of mitonuclear coevolution across populations of *Tigriopus californicus*. *Nat. Ecol. Evol.* 2, 1250–1257. doi:10.1038/s41559-018-0588-1.

Bay, R. A., Rose, N., Barrett, R., Bernatchez, L., Ghalambor, C. K., Lasky, J. R., Brem, R. B., Palumbi, S. R., and Ralph P. (2017). Predicting responses to contemporary environmental change using evolutionary response architectures. *Am. Nat.* 189, 463–473. doi:10.1086/691233.

Beitinger, T., Bennett, W., and McCauley, R. (2000). Temperature tolerances of North American freshwater fishes exposed to dynamic changes in temperature. *Environ. Biol. Fishes* 58, 237–275. doi:10.1023/A:1007676325825.

Benjamini, Y., and Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat. Soc. Ser. B* 57, 289–300.

Burton, R. S. (1985). Mating system of the intertidal copepod *Tigriopus californicus*. *Mar. Biol.* 86, 247–252. doi:10.1007/BF00397511.

Burton, R. S. (1998). Intraspecific phylogeography across the Point Conception biogeographic boundary. *Evolution (N. Y).* 52, 734–745.

Christen, F., Desrosiers, V., Dupont-Cyr, B. A., Vandenberg, G. W., Le François, N. R., Tardif, J. C., Dufresne, F., Lamarre, S. G., and Blier, P. U. (2018). Thermal tolerance and thermal sensitivity of heart mitochondria: Mitochondrial integrity and ROS production. *Free Radic. Biol. Med.* 116, 11–18. doi:10.1016/j.freeradbiomed.2017.12.037.

Chung, D. J., Bryant, H. J., and Schulte, P. M. (2017a). Thermal acclimation and subspecies-specific effects on heart and brain mitochondrial performance in a eurythermal teleost (*Fundulus heteroclitus*). *J. Exp. Biol.* 220, 1459–1471. doi:10.1242/jeb.151217.

Chung, D. J., Healy, T. M., McKenzie, J. L., Chicco, A. J., Sparagna, G. C., and Schulte, P. M. (2018a). Mitochondria, temperature, and the pace of life. *Integr. Comp. Biol.*, 1–13. doi:10.1093/icb/icy013.

Chung, D. J., Morrison, P. R., Bryant, H. J., Jung, E., Brauner, C. J., and Schulte, P. M. (2017b). Intraspecific variation and plasticity in mitochondrial oxygen binding affinity as a response to environmental temperature. *Sci. Rep.* 7, 1–10. doi:10.1038/s41598-017-16598-6.

Chung, D. J., and Schulte, P. M. (2015). Mechanisms and costs of mitochondrial thermal acclimation in a eurythermal killifish *(Fundulus heteroclitus)*. *J. Exp. Biol.* 218, 1621–1631. doi:10.1242/jeb.120444.

Chung, D. J., Sparagna, G. C., Chicco, A. J., and Schulte, P. M. (2018b). Patterns of

mitochondrial membrane remodeling parallel functional adaptations to thermal stress. *J. Exp. Biol.* 221, jeb174458. doi:10.1242/jeb.174458.

Dahlhoff, E., Brien, J. O., Somero, G. N., and Vetter, R. D. (1991). Temperature effects on mitochondria from hydrothermal vent invertebrates: evidence for adaptation to elevated and variable habitat temperatures. *Physiol. Zool.* 64, 1490–1508.

Dahlhoff, E., and Somero, G. N. (1993). Effects of temperature on mitochondria from abalone (genus: *Haliotis*): Adaptive plasticity and its limits. *J. Exp. Biol.* 185, 151–168.

Deutsch, C., Ferrel, A., Seibel, B., Pörtner, H.-O., and Huey, R. B. (2015). Climate change tightens a metabolic constraint on marine habitats. *Science (80-.).* 348, 1132–1136.

Dhillon, R. S., and Schulte, P. M. (2011). Intraspecific variation in the thermal plasticity of mitochondria in killifish. *J. Exp. Biol.* 214, 3639–3648. doi:10.1242/jeb.057737.

Edmands, S., and Deimler, J. K. (2004). Local adaptation, intrinsic coadaptation and the effects of environmental stress on interpopulation hybrids in the copepod *Tigriopus californicus*. *J. Exp. Mar. Bio. Ecol.* 303, 183–196. doi:10.1016/j.jembe.2003.11.012.

Egginton, S., and Johnston, I. A. (1984). Effects of acclimation temperature on routine metabolism muscle mitochondrial volume density and capillary supply in the elver (*Anguilla anguilla* L.). *J. Therm. Biol.* 9, 165–170. doi:10.1016/0306-4565(84)90016-0.

Ellison, C. K., and Burton, R. S. (2006). Disruption of mitochondrial function in interpopulation hybrids of *Tigriopus californicus*. *Evolution (N. Y).* 60, 1382–1391. doi:10.1111/j.0014-3820.2006.tb01217.x.

Ellison, C. K., and Burton, R. S. (2008). Interpopulation hybrid breakdown maps to the mitochondrial genome. *Evolution (N. Y).* 62, 631–638.

Farrell, A. P. (2009). Environment, antecedents and climate change: lessons from the study of temperature physiology and river migration of salmonids. *J. Exp. Biol.* 212, 3771–3780. doi:10.1242/jeb.023671.

Gilchrist, G. W., and Huey, R. B. (1999). The direct response of *Drosophila melanogaster* to selection on knockdown temperature. *Heredity (Edinb).* 83, 15–29. doi:10.1038/sj.hdy.6885330.

Grim, J. M., Miles, D. R. B., and Crockett, E. L. (2010). Temperature acclimation alters oxidative capacities and composition of membrane lipids without influencing activities of enzymatic antioxidants or susceptibility to lipid peroxidation in fish muscle. *J. Exp. Biol.* 213, 445–452. doi:10.1242/jeb.036939.

Guderley, H. (2004). Metabolic responses to low temperature in fish muscle. *Biol. Rev. Camb. Philos. Soc.* 79, 409–427. doi:10.1017/S1464793103006328.

Hazel, J. R. (1995). Thermal adaptation in biological-membranes - is homeoviscous adaptation the explanation. *Annu. Rev. Physiol.* 57, 19–42. doi:Doi

10.1146/Annurev.Ph.57.030195.000315.

Healy, T. M., Brennan, R. S., Whitehead, A., and Schulte, P. M. (2018). Tolerance traits related to climate change resilience are independent and polygenic. *Glob. Chang. Biol.* doi:10.1111/gcb.14386.

Hochachka, P. W., and Somero, G. N. (2002). *Biochemical Adaptation: Mechanism and Process in Physiological Evolution*. Oxford University Press.

Hoekstra, L. A., Siddiq, M. A., and Montooth, K. L. (2013). Pleiotropic effects of a mitochondrial-nuclear incompatibility depend upon the accelerating effect of temperature in *Drosophila*. *Genetics* 195, 1129–1139. doi:10.1534/genetics.113.154914.

Hoffmann, A. A., Sørensen, J. G., and Loeschcke, V. (2003). Adaptation of *Drosophila* to temperature extremes: Bringing together quantitative and molecular approaches. *J. Therm. Biol.* 28, 175–216. doi:10.1016/S0306-4565(02)00057-8.

Hong, B. C., and Shurin, J. B. (2015). Latitudinal variation in the response of tidepool copepods to mean and daily range in temperature. *Ecology* 96, 2348–2359. doi:10.1890/14-1695.1.

Iftikar, F. I., and Hickey, A. J. R. (2013). Do mitochondria limit hot fish hearts? understanding the role of mitochondrial function with heat stress in *Notolabrus celidotus*. *PLoS One* 8. doi:10.1371/journal.pone.0064120.

Johnston, I., and Dunn, J. (1987). Temperature acclimation and metabolism in ectotherms with particular reference to teleost fish. *Symp. Soc. Exp. Biol.* 41, 67–93.

Kelly, M. W., Sanford, E., and Grosberg, R. K. (2011). Limited potential for adaptation to climate change in a broadly distributed marine crustacean. *Proc. R. Soc. B Biol. Sci.* 279, 349–356. doi:10.1098/rspb.2011.0542.

Kraffe, E., Marty, Y., and Guderley, H. (2007). Changes in mitochondrial oxidative capacities during thermal acclimation of rainbow trout *Oncorhynchus mykiss:* roles of membrane proteins, phospholipids and their fatty acid compositions. *J. Exp. Biol.* 210, 149–165. doi:10.1242/jeb.02628.

Lemieux, H., Semsroth, S., Antretter, H., Höfer, D., and Gnaiger, E. (2011). Mitochondrial respiratory control and early defects of oxidative phosphorylation in the failing human heart. *Int. J. Biochem. Cell Biol.* 43, 1729–1738. doi:10.1016/j.biocel.2011.08.008.

LeMoine, C. M. R., Genge, C. E., and Moyes, C. D. (2008). Role of the PGC-1 family in the metabolic adaptation of goldfish to diet and temperature. *J. Exp. Biol.* 211, 1448–1455. doi:10.1242/jeb.014951.

Leong, W., Sun, P. Y., and Edmands, S. (2018). Latitudinal clines in temperature and salinity tolerance in tidepool copepods. *J. Hered.* 109, 71–77. doi:10.1093/jhered/esx061.

McClelland, G. B., Craig, P. M., Dhekney, K., and Dipardo, S. (2006). Temperature- and exercise-induced gene expression and metabolic enzyme changes in skeletal muscle of adult

zebrafish *(Danio rerio)*. *J. Physiol.* 577, 739–751. doi:10.1113/jphysiol.2006.119032.

Morgan, R., Finnøen, M. H., and Jutfelt, F. (2018). CTmax is repeatable and doesn't reduce growth in zebrafish. *Sci. Rep.* 8, 1–8. doi:10.1038/s41598-018-25593-4.

Moyes, C. D., Moon, T. W., and Ballantyne, J. S. (1985). Glutamate catabolism in mitochondria from *Mya arenaria* mantle: Effects of pH on the role of glutamate dehydrogenase. *J. Exp. Zool.* 236, 293–301. doi:10.1002/jez.1402360306.

O'Brien, K. M. (2011). Mitochondrial biogenesis in cold-bodied fishes. *J. Exp. Biol.* 214, 275–285. doi:10.1242/jeb.046854.

O'Brien, K. M., Rix, A. S., Egginton, S., Farrell, A. P., Crockett, E. L., Schlauch, K., Woolsey, R., Hoffman, M., and Merriman, S. (2018). Cardiac mitochondrial metabolism may contribute to differences in thermal tolerance of red- and white-blooded Antarctic notothenioid fishes. *J. Exp. Biol.*, jeb.177816. doi:10.1242/jeb.177816.

Orczewska, J. I., Hartleben, G., and O'Brien, K. M. (2010). The molecular basis of aerobic metabolic remodeling differs between oxidative muscle and liver of threespine sticklebacks in response to cold acclimation. *AJP Regul. Integr. Comp. Physiol.* 299, R352–R364. doi:10.1152/ajpregu.00189.2010.

Palozzi, J. M., Jeedigunta, S. P., and Hurd, T. R. (2018). Mitochondrial DNA purifying selection in mammals and invertebrates. *J. Mol. Biol.* 430, 4834–4848. doi:10.1016/j.jmb.2018.10.019.

Pereira, R. J., Barreto, F. S., and Burton, R. S. (2014). Ecological novelty by hybridization: Experimental evidence for increased thermal tolerance by transgressive segregation in *Tigriopus californicus*. *Evolution (N. Y).* 68, 204–215. doi:10.1111/evo.12254.

Pereira, R. J., Barreto, F. S., Pierce, N. T., Carneiro, M., and Burton, R. S. (2016). Transcriptome-wide patterns of divergence during allopatric evolution. *Mol. Ecol.* 25, 1478–1493. doi:10.1111/mec.13579.

Pereira, R. J., Sasaki, M. C., and Burton, R. S. (2017). Adaptation to a latitudinal thermal gradient within a widespread copepod species: The contributions of genetic divergence and phenotypic plasticity. *Proc. R. Soc. B Biol. Sci.* 284. doi:10.1098/rspb.2017.0236.

Pesta, D., and Gnaiger, E. (2012). "High-Resolution Respirometry: OXPHOS Protocols for Human Cells and Permeabilized Fibers from Small Biopsies of Human Muscle.," in *Mitochondrial Bioenergetics. Methods in Molecular Biology (Methods and Protocols), vol 810*, eds. C. Palmeira and M. A (Humana Press).

Peterson, D. L., Kubow, K. B., Connolly, M. J., Kaplan, L. R., Wetkowski, M. M., Leong, W., Phillips, B. C., and Edmands, S. (2013). Reproductive and phylogenetic divergence of tidepool copepod populations across a narrow geographical boundary in Baja California. *J. Biogeogr.* 40, 1664–1675. doi:10.1111/jbi.12107.

Pichaud, N., Ballard, J. W. O., Tanguay, R. M., and Blier, P. U. (2011). Thermal sensitivity of

mitochondrial functions in permeabilized muscle fibers from two populations of *Drosophila simulans* with divergent mitotypes. *Am. J. Physiol. Integr. Comp. Physiol.* 301, R48–R59. doi:10.1152/ajpregu.00542.2010.

Pichaud, N., Ballard, J. W. O., Tanguay, R. M., and Blier, P. U. (2012). Naturally occurring mitochondrial DNA haplotypes exhibit metabolic differences: Insight into functional properties of mitochondria. *Evolution (N. Y).* 66, 3189–3197. doi:10.1111/j.1558-5646.2012.01683.x.

Pörtner, H.-O. (2010). Oxygen- and capacity-limitation of thermal tolerance: a matrix for integrating climate-related stressor effects in marine ecosystems. *J. Exp. Biol.* 213, 881–893. doi:10.1242/jeb.037523.

Pörtner, H. (2001). Climate change and temperature-dependent biogeography: Oxygen limitation of thermal tolerance in animals. *Naturwissenschaften* 88, 137–146. doi:10.1007/s001140100216.

Pörtner, H. O., Bennett, A. F., Bozinovic, F., Clarke, A., Lardies, M. A., Lucassen, M., Pelster, B., Schiemer, F., and Stillman, J. H. (2006). Trade-offs in thermal adaptation: The need for a molecular to ecological integration. *Physiol. Biochem. Zool.* 79, 295–313. doi:10.1086/499986.

Pritchard, J. K., and Di Rienzo, A. (2010). Adaptation - Not by sweeps alone. *Nat. Rev. Genet.* 11, 665–667. doi:10.1038/nrg2880.

Scheffler, I. E. (2008). *Mitochondria*. Second. Hoboken, New Jersey: John Wiley & Sons, Inc.

Seebacher, F., Brand, M. D., Else, P. L., Guderley, H., Hulbert, A. J., and Moyes, C. D. (2010). Plasticity of oxidative metabolism in variable climates: Molecular mechanisms. *Physiol. Biochem. Zool.* 83, 721–732. doi:10.1086/649964.

Seebacher, F., White, C. R., and Franklin, C. E. (2015). Physiological plasticity increases resilience of ectothermic animals to climate change. *Nat. Clim. Chang.* 5, 61–66. doi:10.1038/nclimate2457.

Siegle, M. R., Taylor, E. B., and O'Connor, M. I. (2018). Prior heat accumulation reduces survival during subsequent experimental heat waves. *J. Exp. Mar. Bio. Ecol.* 501, 109–117. doi:10.1016/j.jembe.2018.01.012.

Somero, G. N. (2010). The physiology of climate change: how potentials for acclimatization and genetic adaptation will determine "winners" and "losers." *J. Exp. Biol.* 213, 912–920. doi:10.1242/jeb.037473.

St-Pierre, J., Charest, P. M., and Guderley, H. (1998). Relative contribution of quantitative and qualitative changes in mitochondria to metabolic compensation during seasonal acclimatisation of rainbow trout *Oncorhynchus mykiss*. *J. Exp. Biol.* 201, 2961–2970.

Stewart, J. B., Freyer, C., Elson, J. L., and Larsson, N. G. (2008). Purifying selection of mtDNA and its implications for understanding evolution and mitochondrial disease. *Nat. Rev. Genet.*

9, 657–662. doi:10.1038/nrg2396.

Sunday, J. M., Bates, A. E., and Dulvy, N. K. (2011). Global analysis of thermal tolerance and latitude in ectotherms. *Proc. R. Soc. B Biol. Sci.* 278, 1823–1830. doi:10.1098/rspb.2010.1295.

Sunday, J. M., Bates, A. E., and Dulvy, N. K. (2012). Thermal tolerance and the global redistribution of animals. *Nat. Clim. Chang.* 2, 686–690. doi:10.1038/nclimate1539.

Tangwancharoen, S., and Burton, R. S. (2014). Early life stages are not always the most sensitive: Heat stress responses in the copepod *Tigriopus californicus*. *Mar. Ecol. Prog. Ser.* 517, 75–83. doi:10.3354/meps11013.

Tsuboko-Ishii, S., and Burton, R. S. (2017). Sex-specific rejection in mate-guarding pair formation in the intertidal copepod, *Tigriopus californicus*. *PLoS One* 12, 1–16. doi:10.1371/journal.pone.0183758.

Willett, C. S. (2010). Potential fitness trade-offs for thermal tolerance in the intertidal copepod *Tigriopus californicus*. *Evolution (N. Y).* 64, 2521–2534. doi:10.1111/j.1558-5646.2010.01008.x.

Willett, C. S. (2012). Hybrid breakdown weakens under thermal stress in population crosses of the copepod *Tigriopus californicus*. *J. Hered.* 103, 103–114. doi:10.1093/jhered/esr109.

Willett, C. S., and Burton, R. S. (2004). Evolution of interacting proteins in the mitochondrial electron transport system in a marine copepod. *Mol. Biol. Evol.* 21, 443–453. doi:10.1093/molbev/msh031.

Willett, C. S., and Ladner, J. T. (2009). Investigations of fine-scale phylogeography in *Tigriopus californicus* reveal historical patterns of population divergence. *BMC Evol. Biol.* 9, 1–20. doi:10.1186/1471-2148-9-139.

Willett, C. S., and Son, C. (2018). The evolution of the thermal niche across locally adapted populations of the copepod *Tigriopus californicus*. *Bull. South. Calif. Acad. Sci.* 117, 150–156.

CHAPTER TWO

Consequences of *HSF* knockdown on gene expression during the heat shock response in *Tigriopus californicus*

Alice E. Harada and Ronald S. Burton

ABSTRACT

While the existence of a cellular heat shock response is nearly universal, the mechanisms responsible for differential thermal tolerance are not completely understood. Though many of the genes involved in cell protection and protein repair are regulated by the highly conserved heat shock transcription factor-1 (HSF-1), the network of transcriptional regulation of the heat shock response is not fully characterized. Here we investigate the role of HSF-1 in gene expression following thermal stress using RNA interference (RNAi) to knockdown HSF-1 in the intertidal copepod *Tigriopus californicus*. The majority of differentially expressed genes between the control and HSF-1 knockdown groups were upregulated, indicating that HSF-1 normally functions to repress their expression. These genes included a chitinase gene, lending further support to previous findings that chitin and cuticle formation are highly regulated following heat stress. We also found evidence of HSF's interaction with other transcription factors as observed in other systems. Additionally, we saw evidence of decreased transcription of heat shock protein genes following knockdown, supporting the role of HSF-1 in the heat shock response. Though we performed a genome scan for genes associated with canonical heat shock elements (HSEs), we did not see evidence that those genes were more highly represented in our results, indicating that requirements for binding and interaction of HSF-1 with a given gene are more complex. Further study of some of the pathways implicated by the findings presented here, particularly in comparisons among populations of *T. californicus,* may help us understand the role and importance of HSF-1 in not only the heat shock response, but also more broadly in thermal tolerance.

INTRODUCTION

As the climate warms and organisms are pushed to their thermal limits, understanding the mechanistic basis underlying thermal tolerance is of increasing interest. Thermal stress elicits a near universal cellular "heat shock response" which has been characterized in a diversity of organisms over the past 40 years, ranging from slime molds (Loomis and Wheeler, 1980) to *Drosophila* (Ashburner and Bonner, 1979; Hoffmann et al., 2003) to humans (Kregel, 2002). Many of the genes involved are conserved across taxa (Feder and Hofmann, 1999; Lindquist, 1986), including several families of heat shock proteins (HSPs) involved in processing cellular proteins that have lost functional conformation due to elevated temperature; HSPs prevent aggregation of denatured proteins, re-fold proteins in correct conformations, or target misfolded proteins for degradation (Feder and Hofmann, 1999; Morimoto, 1998; Sørensen et al., 2003). However, despite generally contributing to thermal tolerance, there is some variation in the expression of the heat shock response in different systems (Feder and Hofmann, 1999; Morimoto, 1998). For example, some systems show a “frontloading” of heat shock proteins, whereby thermally tolerant organisms demonstrate constitutive expression (regardless of stress) of HSPs and other genes involved in the heat shock response (Barshis et al., 2013; Gleason and Burton, 2015). In contrast, many organisms tend to show a more pronounced upregulation of these genes in the face of stress (e.g., Lockwood et al., 2010; Schoville et al., 2012; Sørensen et al., 2005).

Differentially expressed genes involved in the heat shock response are often regulated by a highly conserved heat shock transcription factor-1 (HSF-1) (Morimoto, 1998; Wu, 1995). In addition to its role in thermal response, HSF-1 has been shown to be important in a number of other processes across taxa; these include development (Åkerfelt et al., 2010; Anckar and

Sistonen, 2007; Jedlicka et al., 1997; Xiao et al., 1999), longevity (Åkerfelt et al., 2010; Seo et al., 2013; Steinkraus et al., 2008), and a range of stress responses (Åkerfelt et al., 2010; Brunquell et al., 2016; Furuhashi and Sakamoto, 2015; Jedlicka et al., 1997; Labbadia et al., 2017; Mazin et al., 2018; Morton and Lamitina, 2013; Sorger, 1991; Takii et al., 2017; Walker et al., 2003; Zhong et al., 1998). Following heat shock, HSF-1 monomers trimerize and accumulate in the nucleus (Morimoto, 1998; Westwoodt and Wu, 1993) where they then bind to heat shock elements (HSEs), which are short inverted repeat sequences in the promoter region of genes. The canonical HSE sequence motif that binds HSF-1 is highly conserved; substitutions within HSEs can decrease expression of the gene associated (Åkerfelt et al., 2010; Fernandes et al., 1994; Sorger, 1991; Tangwancharoen et al., 2018).

An important aspect of cellular response to thermal stress is the variety of genes involved. Though HSPs are clearly important, there can be anywhere from hundreds to thousands of genes with differential expression following a given thermal stress, and many of them are not HSPs (e.g., Brunquell et al., 2016; Lockwood et al., 2010; Schoville et al., 2012; Stillman and Tagmount, 2009). For example, several studies have found cuticle protein genes or collagen-associated genes to be involved in the heat shock response; Brunquell et al. (2016) found cuticle genes to be the top category of genes upregulated by HSF-1 independently of heat stress, while Schoville et al. (2012) found that cuticle genes were up- or downregulated by populations of *Tigriopus californicus* depending on their heat tolerance.

This study seeks to determine the suite of genes under regulation by HSF. Though the mechanism of HSF-1 action seems straightforward, previous studies have found that having a "perfect," canonical HSE does not guarantee binding by HSF (Guertin and Lis, 2010). While we are able to generate a list of genes associated with perfect HSEs, these genes are not necessarily

regulated by HSF-1 *in vivo*, so further investigation into HSF binding and gene activation within an animal is necessary to understand the role of transcription factors in the heat shock response. In this study we use *Tigriopus californicus*, an intertidal copepod with a well-characterized heat shock response (e.g., Pereira et al., 2017; Tangwancharoen et al., 2018). Although there are several types of HSF in many organisms, only HSF-1, the most conserved and active HSF in the heat shock response (Brunquell et al., 2016; Pirkkala et al., 2001; Sarge et al., 1993), has been found in the *Tigriopus* draft genome (Barreto et al., 2018). Gene knockdown via RNAi has previously been successfully employed to knock down expression of a specific heat shock protein in *T. californicus* (Barreto et al., 2015); here we use this approach for knockdown of HSF. Following validated knockdown and subsequent heat stress, we used RNA sequencing to identify genes that are differentially expressed following heat stress with and without knockdown of HSF.

METHODS

Copepod collection and maintenance

Copepods were collected from tidepools in San Diego, California (SD: 32°45'N, 117°15'W), and maintained in 400-ml beakers containing 200-ml of 0.2 μm-filtered seawater. Beakers were kept at 20 °C with a 12-h light/dark cycle. Copepods were fed powdered spirulina ad libitum and maintained under common garden conditions for at least one month prior to use in this study.

dsRNA synthesis

Because HSF-1 lacks introns (Tangwancharoen et al., 2018), genomic DNA was used as template for synthesis of dsRNA. Genomic DNA was extracted by incubating individual copepods in 15 µl lysis buffer (Willett and Burton, 2001) at 55 °C for 90min followed by 95 °C for 15min. Following lysis, PCR was used to amplify 300-600 bp regions of the HSF gene using primers with T7 promoter sequences (TAATACGACTCACTATAGGG) added on the 5' end (see Table 2-1 for primer sequences). PCR reactions were performed in 25-µL reactions and contained 1X GoTaq Green Master Mix (Promega), 0.8 µM primers, and 1 µL DNA. Thermal cycling parameters were 95 °C for 3min, 34 cycles of 95 °C for 20s, 58 °C for 1min, and 72 °C for 1min, and finally 72 °C for 5min. Molecular weight of the PCR product was checked on a 1.5% agarose gel. Following the method of Barreto et al. (2015), we also produced a control dsRNA for use in some experiments by amplifying a 392-bp region of the pCR4-TOPO plasmid (Invitrogen).

Following DNA amplification, PCR product was purified using Sephadex G-50 columns (GE Healthcare) and 1 µg product was used as a template for transcription in the HiScribe T7 High Yield RNA Synthesis Kit (New England Bioscience) following the manufacturer's protocol, which included overnight incubation for dsRNA < 300bp and 2-h incubation for dsRNA >300bp. Transcription product was treated with Turbo DNase (37 °C, 1 h; Thermo Fisher Scientific) to remove DNA template and purified with an RNeasy column (Qiagen).

Electroporation

For dsRNA delivery, groups of 100 adult copepods were placed into 0.4-cm gap electroporation cuvettes (Bio-Rad) containing 140 µL artificial seawater and 60 µL target dsRNA (50 µg), control dsRNA, or DEPC-H_2O. Barreto et al. (2015) did not see significant

differences between control dsRNA and DEPC-H_2O electroporation. Here we used both methods and also did not see differences in control gene expression. Copepods were electroporated in a Gene Pulser Xcell (Bio-Rad) with square-wave pulses (1-100V and 10-50V) and subsequently transferred back to petri dishes in a 20 °C incubator and held for 2 days before use in any further experiments.

Heat shock

Copepods from either control dsRNA or target dsRNA groups that survived electroporation (typically around 50) were counted and split into two groups, with half serving as control animals and half placed into 15 mL conical tubes. Copepods were heat stressed by transferring tubes to a 35 °C water bath for 1-h, after which they were transferred back to 20 °C incubators. Subsets of each group were either (1) left at 20 °C (control) for survivorship assessment three days later (10 animals), or (2) allowed to recover for 1-h before homogenization for Western blot (5 animals) or RNA extraction in Trizol (remaining animals, at least 10).

RNA extraction and gene expression validation

RNA was extracted from groups of at least 10 animals (depending on the number remaining after electroporation) using TRI Reagent (Sigma) in accordance with manufacturer's instructions. cDNA was synthesized using the High Capacity RNA-to-cDNA kit (Applied Biosystems) and diluted to 3 ng μl^{-1}. Quantitative PCR was performed in 15 µl reactions containing 9 ng template, 1X iTaq Universal SYBR Green Supermix (Bio-Rad), and 0.35 µM of each primer (Table 2-1). Reactions were run on a Stratagene MX3000P (Agilent) with 95 °C

denaturation for 2 min, 40 cycles of 95 °C for 10 s and 59 °C for 20 s. Gene expression was assessed using the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak, 2008) and normalized with the *GAPDH* gene.

Western blot validation

Groups of 5 copepods were homogenized in a mixture of 20 µl 10% sodium dodecyl sulfate and 20 µl Laemmli sample buffer using polypropylene pestles (Thomas Scientific) in 1.5 ml microcentrifuge tubes. The homogenate was heated at 95 °C for 5 min and then frozen at -20 °C prior to use. Samples were run on a 7.5% SDS-PAGE gel, probed with an α-HSF polyclonal antibody. This antibody was produced in a manner similar to the HSPB1 antibody described in Tangwancharoen et al. (2018): *T. californicus HSF-1* cDNA was amplified with N-terminal 6xHis-tag, inserted into a pProEx Htb expression vector (Invitrogen) and transformed into *E. coli* BL21 (DE3) pLysE cells. Expression of recombinant HSF was induced using addition of isopropyl-ß-D-1-thiogalactopyranoside (IPTG) and purified using His60 Ni Superflow Resin columns (Clontech). Purified HSF-1 was sent to GenScript for antibody production in rabbit. After incubation with the primary antibody, the blots were probed with a goat anti-rabbit secondary antibody. Western blots were visualized on a ChemiDoc (Bio-Rad) and analyzed for relative band intensity using Image Lab 4.0.1 (Bio-Rad).

Heat shock element genome scan

In order to find genes with upstream heat shock elements (HSEs), we performed a genome scan using the ChIPpeakAnno package in R (Zhu, 2013; Zhu et al., 2010). Parameters were set to identify genes with canonical HSEs ("NGAANNTTCNNGAAN") within 2000 bp

upstream of their start site. Blast2Go was used to identify gene functional categories of annotated genes.

RNA sequencing

After confirming a potentially successful knockdown of HSF using primarily qPCR results (but in some cases corroboration by Western blot visualization), RNA samples were chosen for sequencing from four groups: control with heat stress, control with no heat stress, knockdown with heat stress, and knockdown with no heat stress (5 replicates each). RNA remaining after use in cDNA synthesis and subsequent qPCR reactions was quantified and sent to Novogene (Davis, CA) for sequencing on a 150 bp Illumina HiSeq paired-end platform. Novogene performed library prep (using New England Biosciences Next® Ultra™ RNA Library Prep Kit) and quality control (Agilent 2100 analysis).

Differential expression analysis

Sequences were trimmed for quality using CLC Genomics Workbench version 11.0.1 (Qiagen Bioinformatics). Trimming involved removal of low quality sequences (limit = 0.01), removal of ambiguous nucleotides (maximal allowed = 2), removal of Novogene adapter sequences, and finally removal of sequences less than 50 nucleotides in length. Reads were then mapped to the *Tigriopus californicus* genome (version 4; https://i5k.nal.usda.gov/Tigriopus_californicus) using CLC Genomics Workbench (minimum length fraction of read overlap = 0.5, minimum sequence similarity = 0.8). Uniquely mapped reads were used in the subsequent analysis. Analyses were performed using DESeq2 (Love et al., 2014). Upon examination of read counts, two of the 5 knockdown samples did not show

evidence of successful knockdown of *HSF-1*; therefore, analysis was limited to the 3 replicates that did show knockdown.

Read counts were automatically normalized and filtered by DESeq2 using the average expression of each gene to establish a filtering threshold (Love et al., 2014). The false discovery rate (FDR) for differential expression was set to *P*-adjusted less than 0.05 and ±LFC (logarithmic fold change) of 1.

Principal component analysis

The principal component analysis (PCA) was plotted using the plotPCA function of the DESeq2 package using the top 1,000 variable genes.

Heat map

Heat maps were made in R (https://cran.r-project.org) using hierarchical clustering on dissimilarities of all genes showing significant differential expression in either the control versus knockdown comparison or the heat stress versus non-heat stress comparison.

RESULTS

Survivorship to electroporation and heat shock

Survivorship of electroporated samples chosen for sequencing ranged from 44 to 86 animals out of 100. There was no significant difference in survivorship following electroporation between copepods electroporated with target dsRNA or control/diH_2O (Figure 2-1; Fisher's exact test, n = 500 for each control and treatment, $P = 0.2671$). Two days post-electroporation, survivorship to a 1-h heat shock at 35 °C significantly differed between groups electroporated

with target dsRNA and control (Figure 2-2; Fisher's exact test, n = 50 for treatment and 53 for control, $P = 0.0152$).

HSF-1 and *hspb1* expression for knockdown validation

Fold change of *HSF* transcripts in comparison to controls was -1.0 or lower for all 5 replicates used for RNA sequencing (Figure 2-3). Log_2 fold change for *hspb1* (a presumed HSF target) was assessed after heat stress for both control and knockdown groups and was significantly lower in knockdown than in control (Figure 2-4; Mann-Whitney test, $P = 0.0079$).

Protein expression

Protein expression was used in conjunction with qPCR to validate knockdown. For samples chosen for RNA sequencing, a decrease in HSF protein levels was observed in some cases, though not all (Figure 2-5). Boxes around bands depict those from the same gel. Therefore, band intensity is compared between those bands from the same gel (i.e., C1 vs. T1; C2 and C3 vs. T2 and T3; C4 and C5 vs. T4 and T5). T1 showed a slight increase from the control, with the control band set as the reference with a value of 1 and T1 having a relative quantity of 1.944. T2 and T3 both show decreased relative quantity compared to the reference, C2: C2 = 1, C3 = 0.826, T2 = 0.087, T3 = 0.144. Finally, T4 shows lower relative quantity than C4 but not C5, while T5 has lower quantity than both controls: C4 = 1, C5 = 0.364, T4 = 0.515, T5 = 0.210.

Heat shock element genome scan

Our scan identified 396 unique genes containing at least one HSE within 2000 bp upstream of their start sites. Of these genes, 18 genes had 2 or 3 perfect HSEs. These included *SACM1L,* a gene encoding for phosphatidylinositide phosphatase; *ALDH16A1*, an aldehyde dehydrogenase; *DNAJA1,* a heat shock protein; *eIF3a,* a component of the eukaryotic translation initiation factor (eIF-3) complex that helps initiate cell proliferation; *GLRX3,* an iron-sulfur cluster assembly factor; *Hr4,* a nuclear hormone receptor; *INTS10,* a component of a complex involved in snRNA transcription; *NDUFA9,* a subunit of Complex I in the electron transport chain; *NKAIN,* a sodium ion transport regulator; *osa,* which can be involved in chromatin regulation and development; *sip-1,* or *hsp beta-1,* a small heat shock protein previously shown to be involved thermal tolerance in *T. californicus* (Barreto et al., 2015; Tangwancharoen et al., 2018); and *ttv,* a protein involved in development and biosynthesis. For a full list of genes with HSEs, see Table A2-1.

Illumina sequencing

Sequencing on a 150 bp Illumina HiSeq paired-end platform resulted in between ~30 and 50 million reads per sample. See Table 2-2 for number of total raw reads and number of mapped reads per sample.

Differential expression analysis

Of the 5 samples of each treatment used for RNA sequencing, 3 showed evidence of a limited knockdown of *HSF.* Because we did not observe decreased read counts of *HSF* in 2 of the 5 samples, we eliminated them from subsequent analyses. All further differential expression analyses use 3 samples for each group: controls 2, 3, and 4 and knockdown samples 1, 3, and 5.

Note that, while we did not choose "corresponding" control and knockdown samples, all samples were independent and only potentially grouped based on the date the knockdown occurred. Read counts were normalized in DESeq2 (median of ratios method) for the subset data (three control and three treatment samples). A knockdown of 9% is evident in the non-heat stressed group (5221 ± 309 control and 4740 ± 88 knockdown) and 7% in the heat stressed group (5965 ± 489 control and 5491 ± 352 knockdown).

A principal component analysis (Fig. 6) shows general separation along Principal Component 1 between control and knockdown samples with the exception of one control and one knockdown sample. There is clear separation between heat stress and control samples along Principal Component 2. Due to the overlap of the two samples in the comparison of knockdown and control, the low degree of observed knockdown, and the small sample size, we performed two separate differential expression analyses in order to increase power. First, we analyzed differential expression based on RNAi treatment alone (control vs. knockdown). In this comparison, 32 genes were identified as differentially expressed between groups (heat map of differentially expressed genes, see Figure 2-7). Of these, only 1 had a validated canonical HSE within 2000 bp and that gene (TCALIF_02782) was un-annotated. The list of 32 differentially expressed genes in response to knockdown included transcription factors and genes involved in chitin and cuticle formation. For the full list of genes, see Table A2-2.

Next, we compared differential expression between heat stress and non-heat stress groups, with the PCA (Figure 2-6) showing separation of thermal stress samples along a horizontal axis. In response to heat stress, 335 genes were differentially expressed (shown in a heat map, see Figure 2-8). Of these, 19 had canonical HSEs, and these included heat shock proteins, ion channel and transport proteins, and a transcription factor. See Table A2-3 for the

full list of genes with differential expression in response to heat stress. No genes were differentially expressed in both analyses (i.e., using treatment as the main factor or using heat as the main factor). However, of the 19 genes with HSEs, 8 showed decreased expression upon comparison of read counts between heat-stressed control and heat-stressed knockdown groups (see Table A2-4 for list of differentially expressed genes with HSEs). Though not of sufficient magnitude difference to be picked up in the transcriptome-wide differential expression analysis, these changes in gene expression provide some evidence for an effect that may be attributable to the knockdown. The 8 genes include several heat shock proteins, including *hspb1* and *DNAJA1* (an Hsp40), both of which have 2 heat shock elements *STIP1*, which coordinates HSP70 function; *Slc34a2,* a sodium-dependent phosphate transfer protein; *lin-11,* a transcription factor; and *Chrna3,* an acetylcholine receptor subunit.

DISCUSSION

In this study, we used RNAi in *Tigriopus californicus* to knockdown HSF, a key transcription factor in the cellular response to thermal stress. In samples where HSF knockdown was validated via qPCR, we used RNAseq to investigate the role of HSF in the regulation of the entire transcriptome. Though the degree of knockdown was limited, we saw a corresponding decrease in survivorship to heat stress in copepods electroporated with target dsRNA. Additionally, we saw evidence for differential expression between knockdown and control groups, both independent of heat stress and in a dampened upregulation of genes responding to heat shock. These changes in gene expression provide possible candidates for genes involved in HSF regulation under both stressful and non-stressful conditions.

In addition to RNAseq analysis, we also performed a genome scan to identify genes with potential HSF binding sites (HSEs) within 2000bp of their start sites. While this list of genes provides a starting point for future analysis, we did not find genes with HSEs to be significantly over-represented in our differential expression analysis. This appears to support the idea, as other studies have found, that HSF does not necessarily need canonical HSEs for binding and may even bind more successfully to degenerate HSEs (Guertin et al., 2012). Li et al. (2016) found evidence that HSF-1 is more likely to bind to canonical HSEs following heat shock, while preferring degenerate HSEs during development. This distinction could perhaps explain why many genes that were differentially expressed in our knockdown did not have canonical HSEs. Similarly, canonical HSEs are not always sufficient for binding HSF even after heat shock; using chromatin immunoprecipitation followed by sequencing (ChIP-seq) analysis, Guertin and Lis (2010) found that many HSE motifs were not occupied by HSF. Furthermore, even when HSF is strongly bound to an HSE, it does not lead to gene activation in some cases, and may require downstream promoter features for induction (Guertin and Lis, 2010; Vihervaara et al., 2017).

Interestingly, the majority of genes showing differential expression between knockdown and control were upregulated in the knockdown, indicating that HSF may normally act to repress these genes under both stress and non-stress conditions. These included a chitinase gene, *CHIA*. The upregulation of a chitinase in response to *HSF* knockdown indicates that under normal conditions HSF acts to downregulate this gene, possibly preventing it from breaking down the chitin that makes up the cuticle. During heat stress, cuticle genes have been found to be upregulated in heat tolerant populations of *T. californicus* (Schoville et al., 2012), and in *C. elegans* cuticle structure genes were identified as the top functional category under control by HSF (Brunquell et al., 2016). Due to their upregulation during the heat shock response and the

repression of chitinase under normal conditions, cuticle gene activity is evidently important in *Tigriopus*, though the reason for its upregulation remains to be elucidated. Genes associated with aging have been linked to cuticle structure genes in *C. elegans* (Brunquell et al., 2016). Involvement in processes related to longevity may explain the high degree of regulation of cuticle genes observed in *T. californicus*.

Of the 32 genes that were differentially expressed between the knockdown and control groups, 8 were downregulated in the knockdown. These downregulated genes include several probable transcription factors: *TBX20, HLHmbeta, lin-11, fd96Ca,* and *sox14.* Their downregulation in the knockdown group suggests that they are normally upregulated by HSF. HSF has been shown to interact with other transcription factors in a range of organisms (e.g., Brunquell et al., 2016; Stephanou et al., 1999), including other forms of HSF in some organisms (Takii et al., 2017). These types of interactions with other transcription factors complicate the role of HSF-1 and may explain why we don't see as clear as response to knockdown as we might have expected.

Upon comparison of heat shock and non-heat shock, regardless of knockdown or control treatments, 334 genes were differentially expressed (compared to 356 in Schoville et al., 2012). Of these, 107 were upregulated following heat stress. Additionally, 19 genes of the 334 had associated canonical HSEs within 2kb of their transcription start sites. Though none of the genes showing differential expression following heat shock (comparing all heat shock samples to all non-heat shock samples) were genes that also showed differential expression following knockdown (comparing all knockdown samples to all control samples), we compared average normalized read counts between control-heat stressed and knockdown-heat stressed within the group of differentially expressed genes with HSEs and found 5 that showed decreased

upregulation with knockdown treatment. All 5 are involved in the heat shock response: *STIP1*, which acts as a co-chaperone for HSP90; *hsp70* and *hsp83; DNAJA1,* an HSP40; and *sip-1,* or *hspb1*, which has been identified as an important gene in the heat shock response of *T. californicus* (Barreto et al., 2015; Tangwancharoen et al., 2018). While the decreased expression of these genes was not strong enough to be detected by the DESeq2 analysis, most likely due to their high upregulation following heat stress regardless of knockdown, their decreased read counts in the knockdown samples suggests that HSF plays a role in their upregulation following heat shock. Furthermore, *DNAJA1* and *hspb1* both have two canonical HSEs in their promoters, which may increase their level of control from HSF. In support of this result, Li et al. (2016) similarly found that genes involved in heat shock were more likely to have tandem canonical HSEs that bound HSF-1.

Despite the limited magnitude of HSF knockdown achieved in these experiments, we identified some genes with differential expression following a decrease of HSF. More complete knockdown of HSF would likely expand this list. Brunquell et al. (2016) used RNAi feeding with larval *C. elegans*, which appears to be an effective method of RNAi delivery in that system; although electroporation of *T. californicus* adults has been optimized and validated (Barreto et al., 2015), it was previously performed with *hspb1*, which shows very strong upregulation (~100-fold) following thermal stress (Schoville et al., 2012); because upregulation of *HSF* following heat treatment is far less pronounced (~1.3-fold), it can be difficult to validate with this method of RNAi.

One clear takeaway from the results of our RNAseq is the complexity of HSF interactions. Despite generating a list of genes with perfect HSEs, we did not see evidence for differential expression based on knockdown alone in any of these genes. While we did see

decreased upregulation following heat shock in a handful of HSE-adjacent genes, the difference was relatively small. This underscores the unpredictability of transcription factor mechanisms. Guertin and Lis (2010) used ChIP-seq to identify HSF binding locations in *Drosophila.* They found that only a small proportion of potential HSE motifs were occupied by HSF, and that even binding of HSF to HSEs did not always lead to gene activation. From our results, it seems likely that HSF may also bind to non-canonical HSEs and activate adjacent genes. It is also possible that some of the other genes it acts on, such as the transcription factors we identified, have their own control cascades, further complicating the interactions. In the future, a ChIP-seq approach could help clarify these interactions.

Studying the complex interactions between HSF and the genes it controls can bring us closer to understanding the role of gene regulation in thermal tolerance, in addition to other processes related to stress, development, and longevity. The *HSF* gene in northern *T. californicus* populations has ten non-synonymous substitutions compared to the southern population allele. It is possible, as observed with *hspb1* in Tangwancharoen et al. (2018), that these substitutions confer some advantage to the southern populations that allow them to tolerate high temperatures. However, when we consider other possible functions controlled by HSF, those substitutions may result in advantages for heat sensitive populations that we cannot currently predict. Further investigation into these pathways may shed a light on these functional tradeoffs and the selective pressures that determine them.

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Table 2-1. Primer sequences for dsRNA generation (excluding T7 tails) and qPCR.

Gene	Type	Forward primer (5'-3')	Reverse primer (5'-3')	Product size	GenBank Accession no.
hsp beta-1	qPCR	CGATTTTCATCTGGGTCTCAA	TTGAAGAACTCCTCCGCTGT	175	JW506233
GAPDH	qPCR	GGAGGAGGGGATGATGTTTT	CAACCACGAGCAATACGAGA	226	JW506006
HSF	qPCR	AGCGACAAGAGGAGATGGAG	GGTGGCCTTGTTATTCGAGA	100	JW505571
HSF	dsRNA (1)	AACGGAACAGACCAC	GATCAAATTGCAAGG	300	JW505571
HSF	dsRNA (2)	GAACGAAGTCAGCGTCATCA	CTGGAGGTGTAAGGCGAGAG	537	JW505571
HSF	dsRNA (3)	TGAACGAAGTCAGCGTCATC	CTGGAGGTGTAAGGCGAGAG	538	JW505571
HSF	dsRNA (4)	GAACGAAGTCAGCGTCATCA	TGCTGAAGTTGCGAGAGAGA	417	JW505571
HSF	dsRNA (5)	CTCTCGCCTTACACCTCCAG	CGGGAATATTGGACATGGAC	499	JW505571
pCR4-TOPO	dsRNA	GTGCACGTCTGCTGTCAGAT	CCATAAAACCGCCCAGTCTA	392	N/A

Table 2-2. Number of total and mapped raw reads following Illumina HiSeq 150bp paired-end sequencing.

Sample	Thermal stress	Treatment	Mapped reads	Total reads
C1_HS	Heat stress	Control	30,343,660	37,450,348
C1_NH	Control	Control	25,777,114	31,935,534
C2_HS	Heat stress	Control	33,994,241	42,054,822
C2_NH	Control	Control	25,782,342	32,077,482
C3_HS	Heat stress	Control	30,547,347	37,091,920
C3_NH	Control	Control	28,851,834	35,080,076
C4_HS	Heat stress	Control	32,475,551	40,561,802
C4_NH	Control	Control	28,851,707	37,252,846
C5_HS	Heat stress	Control	29,493,290	37,482,498
C5_NH	Control	Control	28,325,119	35,950,830
T1_HS	Heat stress	Knockdown	35,409,565	44,584,750
T1_NH	Control	Knockdown	24,814,027	30,323,920
T2_HS	Heat stress	Knockdown	36,148,518	44,906,768
T2_NH	Control	Knockdown	33,486,508	40,943,344
T3_HS	Heat stress	Knockdown	34,153,124	41,909,338
T3_NH	Control	Knockdown	28,257,389	34,791,866
T4_HS	Heat stress	Knockdown	31,000,683	37,287,320
T4_NH	Control	Knockdown	31,439,092	38,851,956
T5_HS	Heat stress	Knockdown	33,363,818	42,283,090
T5_NH	Control	Knockdown	33,088,330	41,378,202

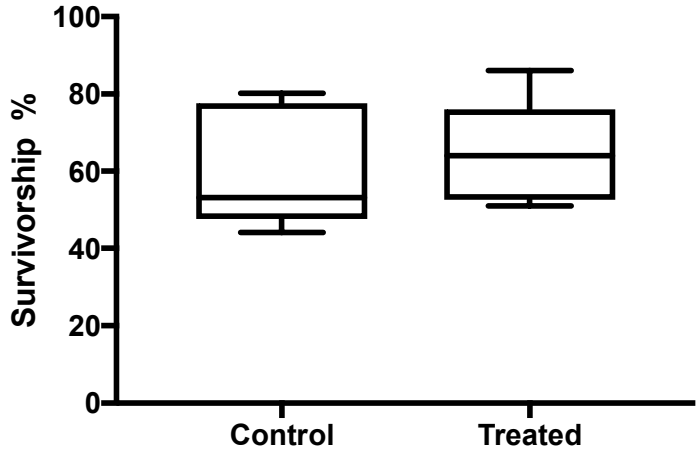

Figure 2-1. Percent survivorship of electroporation in animals treated with control vs. knockdown dsRNA. A two-tailed Fisher's exact test found no difference in survivorship based on treatment (n = 500 animals in each control and treatment, $P = 0.2671$). Survivorship is depicted as a percentage of animals surviving in 5 replicates of 100 animals each at two days post-electroporation (in the absence of thermal stress). Plots show minimum to maximum values (whiskers), 25th to 75th percentiles (boxes), and medians (middle lines).

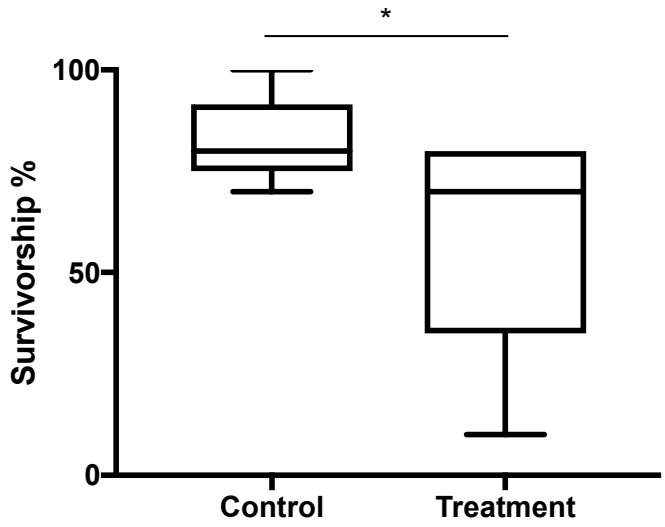

Figure 2-2. Percent survivorship following heat stress (1h at 35 °C) in control vs. HSF knockdown groups. A two-tailed Fisher's exact test found that survivorship significantly differed between treatment and control (n = 50 for treatment and 53 for control, $P = 0.0152$). Survivorship is depicted as a percentage of animals surviving in 5 replicates, with minimum to maximum values (whiskers), 25th to 75th percentiles (boxes), and medians (middle lines).

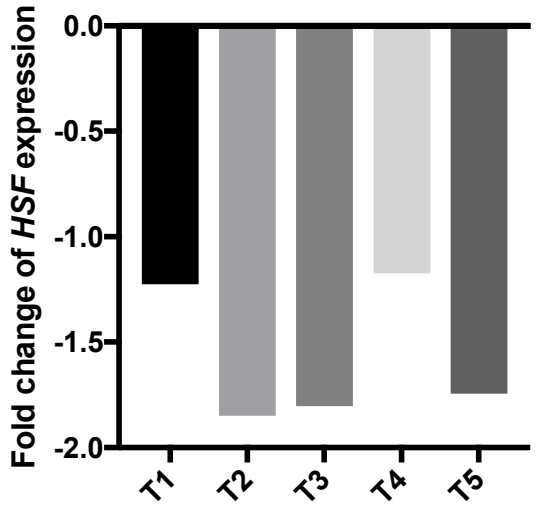

Figure 2-3. Fold change of *HSF* expression in knockdown groups relative to control groups following qPCR.

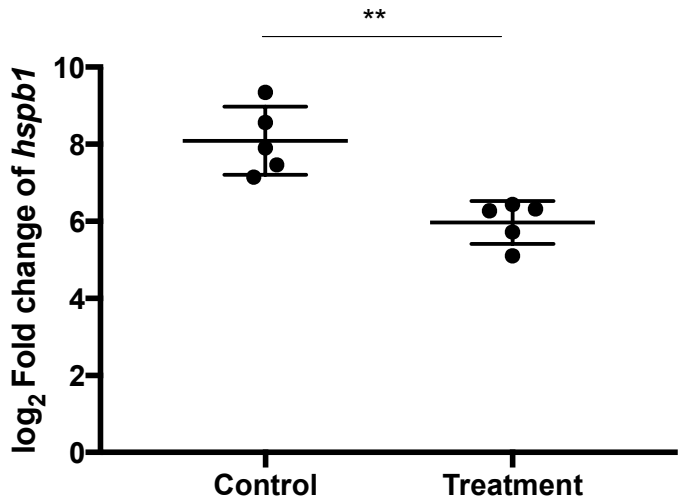

Figure 2-4. Log fold change of *hspb1* in control and knockdown groups following heat stress (1h at 35 °C) and recovery (1h at 20 °C). There was a significantly lower upregulation of *hspb1* in knockdown groups (P = 0.0079).

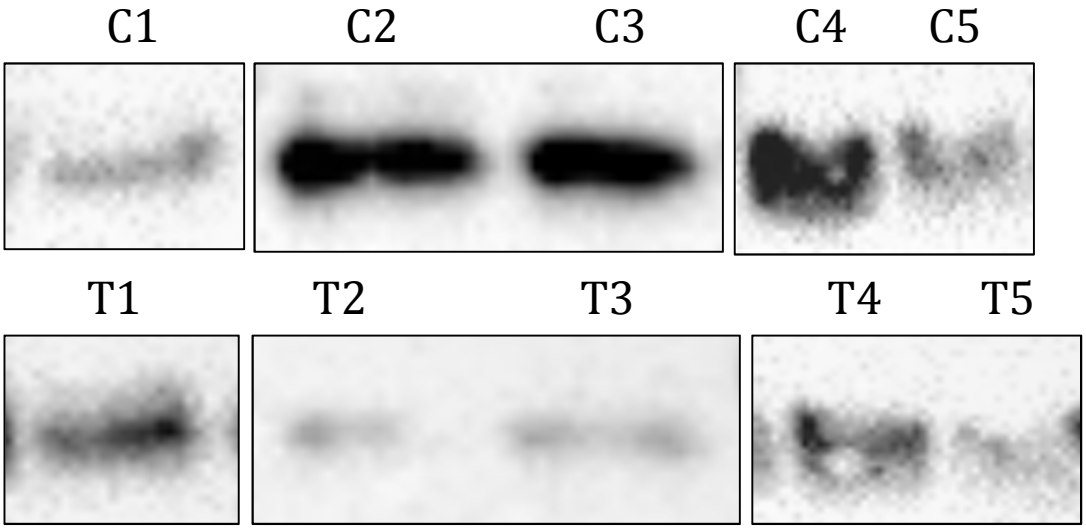

Figure 2-5. Western blots of HSF in control (C) vs. knockdown (T) samples. Bands shown come from different blots; those that are grouped in boxes originate from the same blot.

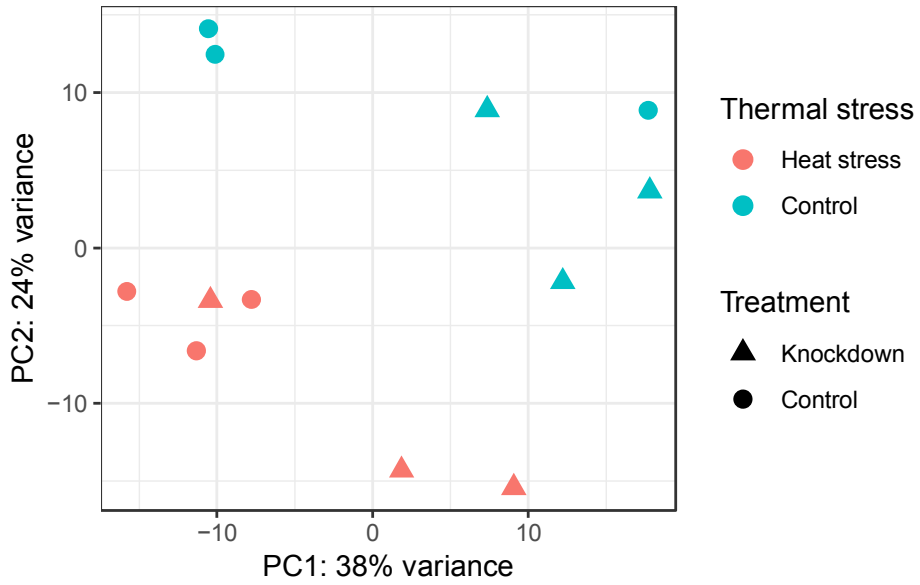

Figure 2-6. Principal component analysis (PCA) of thermal stress and knockdown vs. control treatments of the top 1000 differentially expressed genes from DESeq2 analysis.

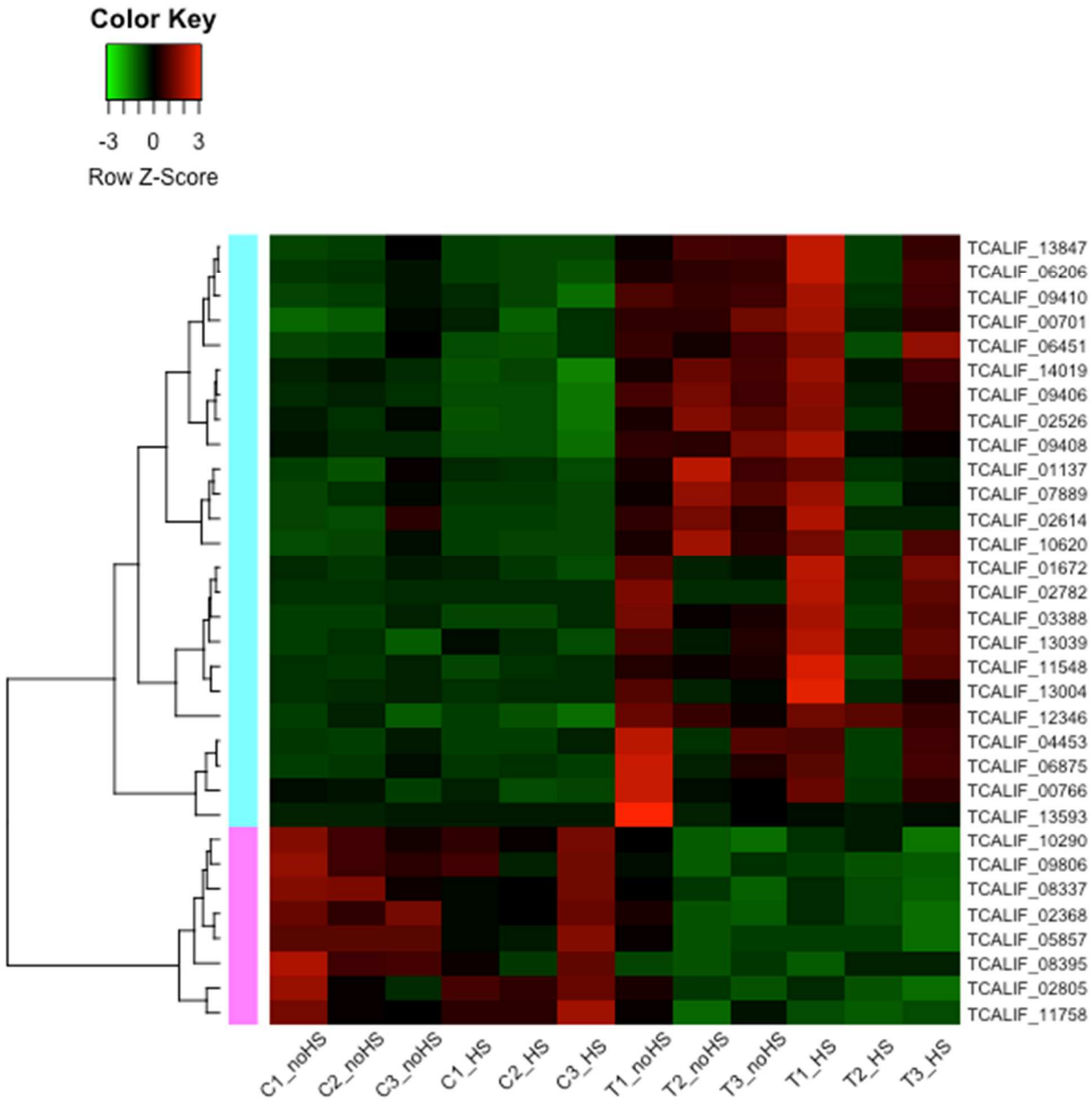

Figure 2-7. Differential expression of control vs. knockdown RNAseq samples. 32 genes were identified as differentially expressed between control and knockdown.

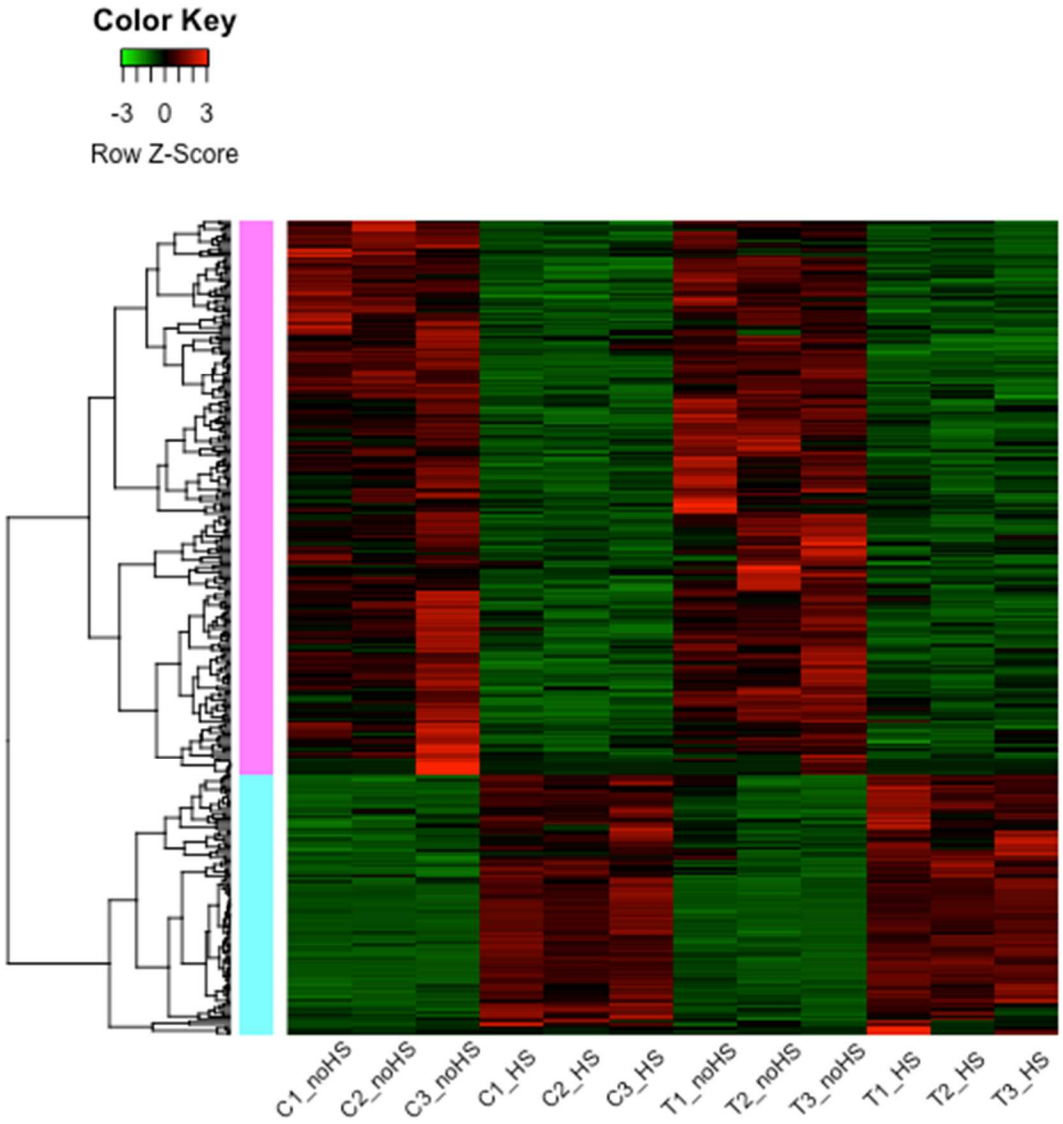

Figure 2-8. Differential expression between heat stress and non-heat stress samples. 334 genes showed differential expression. C-samples = no RNAi treatment; T-samples = RNAi treated

REFERENCES

Åkerfelt, M., Morimoto, R. I., and Sistonen, L. (2010). Heat shock factors: integrators of cell stress, development and lifespan. *Nat. Publ. Gr.* 11, 545–555. doi:10.1038/nrm2938.

Anckar, J., and Sistonen, L. (2007). "Heat shock factor 1 as a coordinator of stress and developmental pathways," in *Molecular Aspects of the Stress Response: Chaperones, Membranes, and Networks*, eds. P. Csermely and L. Vígh, 96–106.

Ashburner, M., and Bonner, J. J. (1979). The induction of gene activity in *Drosophila* by heat shock. *Cell* 17, 241–254.

Barreto, F. S., Schoville, S. D., and Burton, R. S. (2015). Reverse genetics in the tide pool: Knock-down of target gene expression via RNA interference in the copepod *Tigriopus californicus*. *Mol. Ecol. Resour.* 15, 868–879. doi:10.1111/1755-0998.12359.

Barreto, F. S., Watson, E. T., Lima, T. G., Willett, C. S., Edmands, S., Li, W., and Burton, R. S. (2018). Genomic signatures of mitonuclear coevolution across populations of *Tigriopus californicus*. *Nat. Ecol. Evol.* 2, 1250–1257. doi:10.1038/s41559-018-0588-1.

Barshis, D. J., Ladner, J. T., Oliver, T. A., Seneca, F. O., Traylor-Knowles, N., and Palumbi, S. R. (2013). Genomic basis for coral resilience to climate change. *Proc. Natl. Acad. Sci.* 110, 1387–1392. doi:10.1073/pnas.1210224110.

Brunquell, J., Morris, S., Lu, Y., Cheng, F., and Westerheide, S. D. (2016). The genome-wide role of HSF-1 in the regulation of gene expression in *Caenorhabditis elegans*. *BMC Genomics* 17, 1–18. doi:10.1186/s12864-016-2837-5.

Feder, M. E., and Hofmann, G. E. (1999). Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annu. Rev. Physiol.* 61, 243–282. doi:10.1146/annurev.physiol.61.1.243.

Fernandes, M., Xiao, H., and Lis, J. T. (1994). Fine structure analyses of the *Drosophila* and *Saccharomyces* heat shock factor - heat shock element interactions. 22, 167–173.

Furuhashi, T., and Sakamoto, K. (2015). Heat shock factor 1 prevents the reduction in thrashing due to heat shock in *Caenorhabditis elegans*. *Biochem. Biophys. Res. Commun.* 462, 190–194. doi:10.1016/j.bbrc.2015.04.086.

Gleason, L. U., and Burton, R. S. (2015). RNA-seq reveals regional differences in transcriptome response to heat stress in the marine snail *Chlorostoma funebralis*. *Mol. Ecol.* 24, 610–627. doi:10.1111/mec.13047.

Guertin, M. J., and Lis, J. T. (2010). Chromatin landscape dictates HSF binding to target DNA elements. *PLoS Genet.* 6. doi:10.1371/journal.pgen.1001114.

Guertin, M. J., Martins, A. L., Siepel, A., and Lis, J. T. (2012). Accurate prediction of inducible transcription factor binding intensities in vivo. *PLoS Genet.* 8.

doi:10.1371/journal.pgen.1002610.

Hoffmann, A. A., Sørensen, J. G., and Loeschcke, V. (2003). Adaptation of *Drosophila* to temperature extremes: Bringing together quantitative and molecular approaches. *J. Therm. Biol.* 28, 175–216. doi:10.1016/S0306-4565(02)00057-8.

Jedlicka, P., Mortin, M. A., and Wu, C. (1997). Multiple functions of *Drosophila* heat shock transcription factor in vivo. *EMBO J.* 16, 2452–2462. doi:10.1093/emboj/16.9.2452.

Kregel, K. C. (2002). Heat shock proteins: modifying factors in physiological stress responses and acquired thermotolerance. *J. Appl. Physiol.* 92, 2177–2186.

Labbadia, J., Brielmann, R. M., Neto, M. F., Lin, Y., Haynes, C. M., Morimoto, R. I. (2017). Mitochondrial stress restores the heat shock response and prevents proteostasis collapse during aging. *Cell Rep.* 21, 1481–1494. doi:10.1016/j.celrep.2017.10.038.

Li, J., Chauve, L., Phelps, G., Brielmann, R. M., and Morimoto, R. I. (2016). E2F coregulates an essential HSF developmental program that is distinct from the heat-shock response. *Genes Dev.* 30, 2062–2075. doi:10.1101/gad.283317.116.

Lindquist, S. (1986). The heat-shock response. *Annu. Rev. Biochem.* 55, 1151–1191. doi:10.1146/annurev.bi.55.070186.005443.

Lockwood, B. L., Sanders, J. G., and Somero, G. N. (2010). Transcriptomic responses to heat stress in invasive and native blue mussels (genus *Mytilus*): molecular correlates of invasive success. *J. Exp. Biol.* 213, 3548–3558. doi:10.1242/jeb.046094.

Loomis, W. F., and Wheeler, S. (1980). Heat shock response of *Dictyostelium*. *Dev. Biol.* 408, 399–408.

Love, M., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 15, 550.

Mazin, P. V., Shagimardanova, E., Kozlova, O., Cherkasov, A., Sutormin, R., Stepanova, V. V., Stupikov, A., Logacheva, M., Penin, A., Sogame, Y., Cornette, R., Tokumoto, S., Miyata, Y., Kikawada, T., Gelfand, M. S., and Gusey, O. (2018). Cooption of heat shock regulatory system for anhydrobiosis in the sleeping chironomid *Polypedilum vanderplanki*. *Proc. Natl. Acad. Sci.* 115, 201719493. doi:10.1073/pnas.1719493115.

Morimoto, R. I. (1998). Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. *Genes Dev.* 12, 3788–3796.

Morton, E. A., and Lamitina, T. (2013). *Caenorhabditis elegans* HSF-1 is an essential nuclear protein that forms stress granule-like structures following heat shock. *Aging Cell* 12, 112–120. doi:10.1111/acel.12024.

Pereira, R. J., Sasaki, M. C., and Burton, R. S. (2017). Adaptation to a latitudinal thermal gradient within a widespread copepod species: The contributions of genetic divergence and

phenotypic plasticity. *Proc. R. Soc. B Biol. Sci.* 284. doi:10.1098/rspb.2017.0236.

Pirkkala, L., Nykänen, P., and Sistonen, L. (2001). Roles of the heat shock transcription factors in regulation of the heat shock response and beyond. *FASEB J.* 15, 1118–1131. doi:10.1096/fj00-0294rev.

Sarge, K. D., Murphy, S. P., and Morimoto, R. I. (1993). Activation of heat shock gene transcription by heat shock factor 1 involves oligomerization, acquisition of DNA-binding activity, and nuclear localization and can occur in the absence of stress. *Mol. Cell. Biol.* 13, 1392–1407.

Schmittgen, T. D., and Livak, K. J. (2008). Analyzing real-time PCR data by the comparative CT method. *Nat. Protoc.* 3, 1101–1108. doi:10.1038/nprot.2008.73.

Schoville, S. D., Barreto, F. S., Moy, G. W., Wolff, A., and Burton, R. S. (2012). Investigating the molecular basis of local adaptation to thermal stress: population differences in gene expression across the transcriptome of the copepod *Tigriopus californicus*. *BMC Evol. Biol.* 12, 170. doi:10.1186/1471-2148-12-170.

Seo, K., Choi, E., Lee, D., Jeong, D., Jang, S. K., and Lee, S. (2013). Heat shock factor 1 mediates the longevity conferred by inhibition of TOR and insulin/IGF-1 signaling pathways in *C. elegans*. *Aging Cell* 12, 1073–1081. doi:10.1111/acel.12140.

Sørensen, J. G., Kristensen, T. N., and Loeschcke, V. (2003). The evolutionary and ecological role of heat shock proteins. *Ecol. Lett.* 6, 1025–1037. doi:10.1046/j.1461-0248.2003.00528.x.

Sørensen, J. G., Nielsen, M. M., Kruhøffer, M., Justesen, J., and Loeschcke, V. (2005). Full genome gene expression analysis of the heat stress response in *Drosophila melanogaster*. *Cell Stress Chaperones* 10, 312–328. doi:10.1379/CSC-128R1.1.

Sorger, P. K. (1991). Heat shock factor and the heat shock response. *Cell* 65, 363–366.

Steinkraus, K. A., Smith, E. D., Carr, D., Pendergrass, W. R., Sutphin, L., Kennedy, B. K., Kaeberlein, M. (2008). Dietary restriction suppresses proteotoxicity and enhances longevity by an *hsf-1*-dependent mechanism in *Caenorhabditis elegans*. 394–404. doi:10.1111/j.1474-9726.2008.00385.x.

Stephanou, A., Isenberg, D. A., Nakajima, K., and Latchman, D. S. (1999). Signal transducer and activator of transcription-1 and heat shock factor-1 interact and activate the transcription of the Hsp-70 and Hsp-90β gene promoters. *J. Biol. Chem.* 274, 1723–1728. doi:10.1074/jbc.274.3.1723.

Stillman, J. H., and Tagmount, A. (2009). Seasonal and latitudinal acclimatization of cardiac transcriptome responses to thermal stress in porcelain crabs, *Petrolisthes cinctipes*. *Mol. Ecol.* 18, 4206–4226. doi:10.1111/j.1365-294X.2009.04354.x.

Takii, R., Fujimoto, M., Matsuura, Y., Wu, F., Oshibe, N., Takaki, E., Katiyar, A., Akashi, H., Makino, T., Kawata, M., Nakai, A. (2017). HSF1 and HSF3 cooperatively regulate the heat

shock response in lizards. *PLoS One* 12, 1–20. doi:10.1371/journal.pone.0180776.

Tangwancharoen, S., Moy, G. W., and Burton, R. S. (2018). Multiple modes of adaptation: regulatory and structural evolution in a small heat shock protein gene. *Mol. Biol. Evol.* 35, 2110–2119. doi:10.1093/molbev/msy138.

Vihervaara, A., Mahat, D. B., Guertin, M. J., Chu, T., Danko, C. G., Lis, J. T., and Sistonen, L. (2017). Transcriptional response to stress is pre-wired by promoter and enhancer architecture. *Nat. Commun.* 8. doi:10.1038/s41467-017-00151-0.

Walker, G. A., Thompson, F. J., Brawley, A., and Scanlon, T. (2003). Heat shock factor functions at the convergence of the stress response and developmental pathways in *Caenorhabditis elegans*. *FASEB J.* 17, 1960–1962.

Westwoodt, J. T., and Wu, C. (1993). Activation of *Drosophila* heat shock factor: conformational change associated with a monomer-to-trimer transition. *Mol. Cell. Biol.* 13, 3481–3486.

Willett, C. S., and Burton, R. S. (2001). Viability of cytochrome c genotypes depends on cytoplasmic backgrounds in *Tigriopus californicus*. *Evolution (N. Y).* 55, 1592–1599.

Wu, C. (1995). Heat shock transcription factors: Structure and Regulation. *Annu. Rev. Cell Dev. Biol.* 11, 441–469.

Xiao, X., Zuo, X., Davis, A. A., McMillan, D. R., Curry, B. B., Richardson, J. A., and Benjamin, I. J. (1999). HSF1 is required for extra-embryonic development, postnatal growth and protection during inflammatory responses in mice. *EMBO J.* 17, 1750–1758. doi:10.1093/emboj/17.6.1750.

Zhong, M., Orosz, A., and Wu, C. (1998). Direct sensing of heat and oxidation by *Drosophila* heat shock transcription factor. *Mol. Cell* 2, 101–108. doi:10.1016/S1097-2765(00)80118-5.

Zhu, L. (2013). "Integrative analysis of ChIP-chip and ChIP-seq dataset," in *Methods in Molecular Biology (Methods and Protocols): Tiling Arrays (volume 1067)*, eds. T. Lee and A. Shui Luk (Totowa, NJ: Humana Press), 105–124.

Zhu, L., Gazin, C., Lawson, N., Pagès, H., Lin, S., Lapointe, D., and Green, M. (2010). ChIPpeakAnno: a Bioconductor package to annotate ChIP-seq and ChIP-chip data. *BMC Bioinformatics* 11, 237.

CHAPTER THREE

Ecologically relevant temperature ramping rates enhance the protective heat shock response in an intertidal ectotherm

Alice E. Harada and Ronald S. Burton

ABSTRACT

Thermal stress experiments are essential for understanding organisms' thermal limits and the physiological processes that contribute to establishing those limits. Experiments typically employ either an abrupt transfer to near-lethal temperatures or a gradually increasing thermal exposure. In the current study, we used three populations of the intertidal copepod *Tigriopus californicus* that are known to differ in upper thermal tolerance to investigate the effects of gradual versus abrupt thermal exposures on survivorship, developmental time, and heat shock protein gene expression. Developmental rate of nauplii was unaffected following the gradual exposure, whereas developmental time slowed by ~2 days (~20%) following an abrupt exposure. The gradual exposure also improved survivorship in comparison to the abrupt exposure. Furthermore, the heat shock protein genes *hsp70* and *hspb1* showed greater upregulation during the gradual thermal exposure compared to the abrupt exposure. Though the differences in response to each thermal regime varied in magnitude among the different populations, the types of responses were very similar (i.e. following the gradual exposure survivorship increased, developmental time showed no effect, and heat shock protein gene upregulation during the exposure increased). Therefore, the enhanced protective effect of the heat shock response during gradual exposures appears to be conserved within the species despite population-level differences in thermal tolerance. Thus, an ecologically relevant thermal exposure likely enables improved cellular protective mechanisms by allowing for an effective and timely heat shock response, which plays a role in mitigating the effects of thermal stress and thereby enhances tolerance to elevated temperatures.

INTRODUCTION

Effects of temperature occur at all levels of biological organization, from the molecular level to whole ecosystems, and these effects are thought to play a major role in establishing the biogeographic distributions of organisms, particularly ectotherms (Dahlhoff and Somero 1993; Somero 2005, 2012; Fangue et al. 2009; Sunday et al. 2012). Recently, it has been demonstrated that occasional exposure to extreme temperatures, or increased variation in temperature, may play a bigger role in organisms' adaptive responses to stress than rising average temperatures (Clusella-Trullas et al. 2011; Vasseur et al. 2014). Experiments examining thermal stress can provide insight into the physiological mechanisms underlying whole-organism thermal limits to acute temperature change, as well as long-term consequences of acute stress, such as changes in growth, reproduction, and mortality (Pörtner et al. 2006). These effects are typically viewed as tradeoffs: in order for organisms to survive a stressor, they must allocate energy away from non-essential processes (such as growth and reproduction) and toward physiological maintenance measures, such as the induction of the heat shock response (Angilletta et al. 2003).

Because of their extremely variable habitats, rocky intertidal organisms are commonly used in studies of thermal stress. These studies frequently employ gradual thermal ramping methods to mimic the warming of a tidepool (Denny et al. 2006; Tomanek and Zuzow 2010; Kelly et al. 2011; Paganini et al. 2014; Bjelde et al. 2015; Gleason and Burton 2015; Jimenez et al. 2016). Abrupt thermal exposures, sometimes referred to as plunging or static assays, are less commonly used in studies of intertidal organisms but can still provide valuable insights into physiological responses and are often used to observe specific processes (such as the heat shock response) or outcomes (such as survivorship) at temperatures near an organism's lethal limit (Dong et al. 2010; Willett 2010; Schoville et al. 2012; Fields et al. 2016; Giomi et al. 2016; Kelly

et al. 2017; Vergara-Amado et al. 2017). However, although there are studies and reviews comparing gradual and abrupt thermal exposures for terrestrial ectotherms (reviewed in Terblanche et al. 2011), this comparison has not, to our knowledge, been made in detail for an intertidal species.

In this study, we directly compare the effects of an abrupt versus gradual thermal exposure on life history traits and heat shock protein gene expression in the copepod *Tigriopus californicus,* a common inhabitant of high rocky tidepools along the west coast of North America (Dethier 1980; Ganz and Burton 1995). *T. californicus* populations are adapted to their local habitats, with southern populations showing higher heat tolerance than their northern conspecifics (Willett 2010; Kelly et al. 2011; Schoville et al. 2012; Pereira et al. 2014; Tangwancharoen and Burton 2014). To date, most studies of *T. californicus* have used abrupt exposure (i.e., rapid increase in temperature, typically for one hour) to study thermal stress responses. Gradual ramping exposures (i.e., slowly increasing temperature over the course of several hours or more) have occasionally been used in this species (e.g., Kelly et al. 2011), but there have been no direct comparisons of the two protocols. The habitat of *T. californicus* makes understanding the consequences of rates of temperature change particularly relevant: because it is found only in shallow splash pools in the high intertidal, it experiences more extreme and more rapid temperature fluctuations than species inhabiting the lower intertidal where wave action can provide quick respite from the heat.

Here we developed a gradually increasing thermal exposure that is similar to the conditions observed in the natural tidepool habitats of *T. californicus*. We chose three populations of *T. californicus* from California to compare: San Diego (SD) is the farthest south and most heat tolerant, Abalone Cove (AB, Los Angeles County) has a mid-level heat tolerance,

and Santa Cruz (SCN) is the most northern and least heat tolerant population (Willett 2010; Tangwancharoen and Burton 2014). We compared the effects of abrupt versus gradual exposures on developmental rate and survivorship among all populations. Furthermore, *T. californicus* has been shown to upregulate heat shock proteins (HSPs) in response to thermal stress, especially in thermally tolerant populations (Schoville et al. 2012; Lima and Willett 2017), so to clarify some of the mechanisms potentially underlying the life history effects we chose three key HSP genes involved in heat shock response of *T. californicus—hsp60, hsp70,* and *hspb1* (Arya et al. 2007; Schoville et al. 2012; Barreto et al. 2015)—and examined their expression following both types of exposures.

Based on previous research, the expected outcome of a comparison of slowly ramping thermal exposures versus abrupt exposures is unclear. One possible outcome is that gradual exposure to an extreme but sublethal temperature will result in more detrimental effects on life history traits and reduced thermal limits due to the relatively longer duration of thermal stress ("heat load") relative to an abrupt exposure (see Pörtner 2010). This possibility is supported by a number of studies (Terblanche et al. 2007; Chown et al. 2009; Mitchell and Hoffmann 2010) that found that slower ramping rates during gradual thermal exposures led to lower thermal limits and poorer tolerance. These negative effects of ramping may be amplified in the more thermally sensitive northern population, SCN (i.e. we might see a bigger difference in the lethal temperature between the abrupt and gradual exposures for SCN than for AB or SD). An alternative outcome, that a gradual thermal exposure can yield higher thermal tolerance due to enhanced HSP expression, has also found some support (Chidawanyika and Terblanche 2011; Bahar et al. 2013). Because of the much longer duration of thermal stress in the gradual exposure, it remains to be seen whether higher upregulation of heat shock proteins is enough to

mitigate adverse effects of thermal stress. The effect of HSP gene upregulation may again be especially apparent in the northern thermally sensitive population, which does not upregulate heat shock proteins as highly as the southern populations (Schoville et al. 2012).

MATERIALS AND METHODS

Culturing conditions

Copepods were collected from high rocky tidepools at three sites in California, USA: "SD" from Ocean Beach, San Diego County (32° 45' N, 117° 15' W), "AB" from Abalone Cove, Los Angeles County (33° 44' N, 118° 22' W), and "SCN" from Santa Cruz County (36° 56' N, 122° 02' W). Animals were held in 400-ml beakers in 250 ml of 0.4 µm filtered seawater (35 ppt) and fed ground TetraVeggie algae wafers. Prior to use in thermal stress or gene regulation experiments, populations were maintained under laboratory conditions at 20 °C with a 12-h light/dark cycle for at least one full generation (~1 month).

Development of gradually increasing thermal exposure

To determine an approximate temperature ramping rate for the gradual exposure, iButton temperature loggers (Maxim Integrated) were used to monitor field conditions within tidepools. Loggers were coated with Plasti Dip rubber coating (Performix) and secured with marine epoxy (Splash Zone) in tidepools containing *T. californicus.* Loggers were placed out of direct sunlight and submerged to monitor habitat utilized by the animals. Two loggers were deployed at the SD site to test variation among pools. Loggers measured temperature every 20 minutes for 7 days (Figure 3-1). Although the temperature loggers were placed in only one of the collection sites for a relatively short period of time, the goal of the deployment was to determine rates of

temperature change in copepod-inhabited pools on hot sunny days when maximum thermal stress might be experienced.

The rate of heating during the day in SD *T. californicus* tidepools averaged 0.033 °C per minute (ranging from 0.029 °C min^{-1} to 0.039 °C min-1. From these data, we developed a gradual thermal ramping exposure to approximate this rate of observed temperature increase: in a thermal cycler we raised the temperature 2 °C every 40 min (for an average increase of 0.05 °C min^{-1}). So that both abrupt and gradual thermal regimes exposed animals to the same maximum temperature for the same duration, the maximum temperature was held for 1-h in both treatments. For the abrupt thermal regime, we measured the rate of heating of 1 ml seawater in a microcentrifuge tube and found that it took approximately 3 minutes to reach our maximum exposure temperature for a heating rate of 5 °C min^{-1}, approximately 100-fold faster than the gradual thermal exposure.

Survivorship assay

For each population, six replicate groups of ten adult animals were exposed to each (abrupt or gradual) thermal exposure. The abrupt exposure consisted of placing 1.5 ml microcentrofuge tubes containing 10 copepods in 1 ml seawater into a circulating water bath set to 36 °C. Animals were left in the water bath for 1-h then placed back into 20 °C incubators. For the gradual thermal exposure, groups of 10 copepods were placed in 0.3 ml of seawater in 0.5 ml microcentrifuge tubes and a thermal cycler was used to increase temperature in 2 °C increments every 40 min (as described above) starting at 20 °C until 36 °C was reached. The peak temperature was held for 1-h, then temperature was decreased back to 20 °C in 8 °C increments every 40 min (Figure 3-2). Following treatment, all animals were placed into 6-well plates (10

animals per well) with fresh filtered seawater and food. Survivorship was counted 3 days post exposure (Tangwancharoen & Burton 2014). A generalized linear model was fit to the data using a binomial distribution, and significance was assessed by ANOVA.

Developmental rate

Gravid female copepods have external egg sacs that change in color from green to red as they develop. Red egg sacs were removed from gravid copepods and left to hatch overnight in separate wells of a 12-well plate in a 20 °C incubator. Individual broods of nauplii were split between two treatments: half were heat-treated and half were kept as controls (i.e., maintained at 20 °C). The number of broods used for the gradual exposure were SD: 7, AB: 9, and SCN: 9. The number of broods used for the abrupt exposure were SD: 13, AB: 5, and SCN: 8. Broods were exposed to the corresponding thermal exposures as described in the "Survivorship assay" section above. Following treatment, nauplii were fed and checked daily for metamorphosis into the first copepodid stage (CI). The date of the first appearance of CI copepodids was recorded for each group (Tangwancharoen and Burton 2014), and a two-way ANOVA followed by a Sidak's multiple comparisons test was used to assess variation in developmental rate among the treatments.

Heat shock protein (*HSP*) gene expression

Groups of 50 adult animals were heat stressed using the abrupt or gradual thermal exposures while control groups were was kept at 20 °C (3 groups per treatment). Because all animals from SCN died in the 36 °C abrupt exposure, we used 35 °C as the peak temperature for all gene expression studies in the SCN population and 36 °C for both SD and AB. Total RNA

was extracted immediately following the heat treatment (no recovery time) or from control groups. Additionally, we examined the time course of gene regulation during the gradual thermal exposure using three groups of 30 animals compared to a control. Groups were removed after reaching a moderate temperature (28 °C for SD and AB and 27 °C for SCN), a stressful temperature (34 °C for SD and AB and 33 °C for SCN), and finally after completing their 1-h exposure at the peak temperature (36 °C or 35 °C).

In both experiments, RNA was extracted using Trizol and cDNA was synthesized from 200 ng RNA using the High Capacity RNA-to-cDNA kit, which employs random octamers and oligo-dT primers (Applied Biosystems). cDNA was then diluted to 3 ng μl^{-1} (RNA equivalent) before adding to a 15 µl reaction containing 9 ng template, 1X iTaq Universal SYBR Green supermix (Bio-Rad), and 0.35µM of each primer (sequences and GenBank accession numbers listed in Table 3-1). Reactions were run on a Stratagene MX3000P (Agilent) system with a denaturation at 95 °C for 2 min, and 40 cycles of 95 °C for 10 s and 59 °C for 20 s, followed by a melting dissociation curve step. Relative expression of each gene was assessed using the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak 2008). Fold change estimates were normalized using the geometric average of *myosin* and *GAPDH* genes, which have been identified as suitable reference genes for *T. californicus* qPCR following thermal stress (Schoville et al. 2012; Barreto et al. 2015).

For each of three target HSP genes (*hsp60, hsp70,* and *hspb1*), log fold change (with 95% confidence interval) was calculated comparing either the gradual or abrupt thermal exposure to a control group. Significance was assessed using a randomized block ANOVA followed by Sidak's multiple comparisons test between treatments for each population. Similarly, for the analysis of gene regulation during the abrupt exposure, log fold change (with 95% confidence

interval) was calculated comparing three temperatures during the gradual ramp (27 °C for SCN and 28 °C for SD/AB; 33 and 34°C; and 35/36 °C) to a control group. Significance was assessed using a randomized block ANOVA followed by Tukey's multiple comparisons test between treatments for each population.

RESULTS

Survivorship

In all populations, survivorship was significantly higher following the gradual exposure than it was following the abrupt exposure (Figure 3-3). Two generalized linear models with binomial distribution were compared using a likelihood ratio test: one with thermal exposure type and population both as main effects, and one with an added interaction between the two terms. Since inclusion of the interaction term did not improve the fit of the model, the simpler model with no interaction term was chosen. An ANOVA showed a significant effect of thermal exposure type ($X^2_{1,30}$= 15.40, $P < 0.0001$) but not of population, though population had a nearly significant effect ($X^2_{2, 30} = 5.72$, $P = 0.0573$).

Developmental rate

The differential effects of the abrupt versus gradual thermal exposure compared to controls on developmental rate were striking. For the abrupt exposure (Figure 3-4A), a two-way ANOVA showed a significant effect of population ($F_{2, 46} = 3.546$, $P = 0.0370$) and exposure ($F_{1, 46} = 33.05$, $P <0.0001$) but no significant interaction ($F_{2, 46} = 1.424$, $P = 0.2511$). Post-hoc tests found a significantly slower developmental rate following heat stress compared to control in SD ($P = 0.0200$), AB ($P = 0.0113$), and SCN ($P = 0.0003$). The average delay in development was

about 2 days, slowing from 7 days in the control groups to about 9 days after the abrupt exposure. In contrast, there was no significant effect of exposure ($F_{1, 44} = 3.025$, $P = 0.0890$) or population ($F_{2, 44} = 0.2436$, $P = 0.7849$) on developmental rate in the animals that experienced the gradual thermal exposure (Figure 3-4B).

Heat shock protein gene expression

In comparisons of the relative expression of three HSP genes (*hsp60, hsp70,* and *hspb1*) following either an abrupt or a gradual thermal exposure, the gradual exposure led to higher upregulation than the abrupt exposure in most comparisons for *hsp70* and *hspb1* (Figure 3-5). There was a significant interaction between population and thermal exposure for all three genes (*hsp60*: $F_{2,6,3} = 11.22$, $P = 0.0094$; *hsp70*: $F_{2,6,3} = 11.69$, $P = 0.0085$; *hspb1*: $F_{2,6,3} = 25$, $P = 0.1926$). *Hsp60* did not show strong changes in expression in any of the populations or treatments, but was upregulated to a greater extent by the abrupt exposure than the gradual exposure in the SD population only (SD: $P = 0.0141$; AB: $P = 0.5511$; SCN: $P = 0.4367$). For *hsp70,* upregulation of gene expression was greater after the gradual thermal exposure than the abrupt exposure in AB ($P = 0.0101$) and SCN ($P = 0.0056$), but not in SD ($P = 0.7782$). The gradual exposure significantly increased upregulation of *hspb1* in comparison to the abrupt exposure in all three populations (SD and AB: $P < 0.0001$; SCN: $P = 0.0003$).

Hsp70 and *hspb1* also showed upregulation during a gradual thermal exposure when we measured expression at three temperature/time points during the gradual exposure (Figure 3-6). *Hsp70* showed a significant effect of temperature but not of population, nor did it show an interaction (temperature: $F_{2,12,3} = 63.21$, $P < 0.0001$; population: $F_{2,6,3} = 1.511$, $P = 0.2941$; interaction: $F_{4,12,3} = 0.4696$, $P = 0.7572$). *Hspb1* showed a significant effect of temperature,

population, and interaction (temperature: $F_{2,12,3} = 58.33$, $P < 0.0001$; population: $F_{2,6,3} = 19.39$, $P = 0.0024$; interaction: $F_{4,12,3} = 3.366$, $P = 0.0457$). In the post-hoc analysis, the same pattern was observed for both genes in all three populations: there was significant upregulation between the low and middle temperature (*hsp70,* SD*: P* = 0.0001; *hsp70,* AB: *P* = 0.0002; *hsp70,* SCN: *P* = 0.0013; *hspb1,* SD*: P* = 0.0137; *hspb1,* AB: *P* < 0.0001; *hspb1,* SCN: *P* = 0.0004), as well as between the low and high temperature (*hsp70,* SD*: P* = 0.0003; *hsp70,* AB: *P* = 0.0004; *hsp70,* SCN: *P* = 0.0003; *hspb1,* SD*: P* = 0.0341; *hspb1,* AB: *P* < 0.0001; *hspb1,* SCN: *P* = 0.0003), but not a significant difference between the middle and high temperature (*hsp70,* SD*: P* = 0.7974; *hsp70,* AB: *P* = 0.8483; *hsp70,* SCN: *P* = 0.6486; *hspb1,* SD*: P* = 0.8689; *hspb1,* AB: *P* = 0.3639; *hspb1,* SCN: *P* = 0.9964).

DISCUSSION

In this study, we directly compared effects of an abrupt thermal exposure versus a gradual ramping exposure on *T. californicus*. Animals that experienced the gradual exposure experienced high temperatures for substantially longer than those that experienced the abrupt temperature change, since the duration of exposure to the maximum temperature was 1-h in both regimes, but the relatively slow increase in temperature for the gradual exposure meant that copepods also experienced relatively high temperatures during the ramping phase. Despite this increased exposure to high temperature, the gradual exposure proved to be less stressful. Survivorship of animals that experienced the gradual exposure was much higher than those that experienced the abrupt treatment. Higher heat tolerance during a gradual thermal ramp, often measured via knockdown temperature, has been seen in some studies (e.g., Chidawanyika and Terblanche 2011; Bahar et al. 2013), but often slowly ramped groups show either lower tolerance

than abruptly stressed animals or no difference (Terblanche et al. 2007; Mitchell and Hoffmann 2010; Overgaard et al. 2012; Nguyen et al. 2014). In contrast to knockdown assays, we propose that measuring survivorship after thermal stress exposure is more informative because it integrates the compounding effects that heat exposure can have over time.

Remarkably, there was no effect of the gradual exposure on developmental rate, compared to a ~2 day (~20%) slower development of abruptly exposed copepods. Though the effects of thermal stress on developmental rate have been tested in many organisms, increased heat exposure often causes developmental rate to speed up (Bermudes and Ritar 2008; Roberts et al. 2012; Runcie et al. 2012), while slower developmental rate, as seen here following abrupt stress, is not common (Sgrò et al. 2010; Tangwancharoen and Burton 2014). To our knowledge there are no studies comparing the effects of gradual and abrupt thermal exposure on development, though there is evidence that cycling between control and stressful temperatures can significantly delay development or slow growth rates (Sgrò et al. 2010; Kingsolver and Woods 2016). One possibility that could contribute to these observed differences is the higher heat load experienced by the gradually exposed copepods. However, the life history data presented in the current study suggest that even when the time at high temperatures is longer, gradually ramping up to those temperatures over the course of hours at ecologically relevant rates of change makes them markedly less stressful for both larval and adult stages.

Although the gradual exposure had less impact on life history parameters than the abrupt exposure, gradually increasing temperature resulted in greater upregulation of heat shock protein genes, an observation consistent with previous studies (McMillan et al. 2005; Sobek et al. 2011; Sørensen et al. 2013). Though the heat shock response is often cited as evidence for increased cellular stress and therefore increased damage (Terblanche et al. 2011; Sørensen et al. 2013), the

opposite has previously been found in *T. californicus*: more thermally tolerant populations show higher upregulation of heat shock proteins (Schoville et al. 2012). This pattern fits with our observations of higher upregulation of selected heat shock proteins during and following the gradual exposure compared to the abrupt exposure and correspondingly higher survivorship and no change to developmental rate in gradually exposed copepods. There is evidently a mechanism for limiting damage that may be time-sensitive so that it cannot act quickly enough to be effective during an abrupt stress but will protect the animals when temperature increases gradually; our data suggest that the heat shock response is an excellent candidate process to underlie these effects. The high temperatures used in this study could potentially alter enough proteins into non-efficient conformation states that heat shock proteins cannot "keep up" during abrupt stress, whereas gradual exposure may allow HSPs to reach higher levels of upregulation during the critical period of high temperature stress (as the qPCR data suggests) such that the negative effects of unfolded proteins are mitigated.

A well-known phenomenon in studies of thermal stress is "hardening," characterized by a plastic response of animals that are able to better survive near-lethal temperatures following prior exposure to a stressful temperature (Bowler 2005; Hoffmann et al. 2013). Hardening commonly refers to a sublethal stress performed well in advance of the second more extreme stress, often 24 hours or more, followed by a recovery period (Loeschcke et al. 1994; Pasparakis et al. 2016). A long recovery period may not be required, as several studies have found evidence for rapid hardening, where exposure to a cold or warm temperature stress as soon as 2 hours prior to the next stress is enough to increase an organism's survival (Lee et al. 1987, 2006; Dahlgaard et al. 1998). Hardening, especially in the short term, can be linked to the upregulation of key heat shock proteins in a variety of ectotherms (Sconzo et al. 1986; Dahlgaard et al. 1998; Bahrndorff

et al. 2009; Benoit et al. 2011; Hu et al. 2014; Giomi et al. 2016). This appears to be an adaptive mechanism to protect against thermal stress in variable environments similar to what we see during the gradual exposure where maximal upregulation of heat shock proteins during ramping occurred prior to the peak temperature and remained highly upregulated. Thus, the benefits of gradual versus abrupt exposure that we observed (i.e., life history effects) may be a result of a similar frontloading or hardening-like effect despite appearing methodologically different (similarly proposed in Sgrò et al. 2010).

Though all organisms do not share the responses to gradual thermal exposure we observed in *T. californicus*, there are some valuable insights provided by this study that may be widely applicable. First, if the upregulation of key heat shock proteins is indeed responsible for limiting the negative consequences of heat stress, then varying the speed of the ramping rate could have significant effects at the organismal level. The ramping rate we employ (~0.05 °C min^{-1}) is one of the slower rates in the literature (Sgrò et al. 2010; Terblanche et al. 2011; Sørensen et al. 2013) but is consistent with rates of temperature change we observed in natural tidepools. Given that we observed high upregulation of heat shock protein genes well below lethal temperatures, the slow rate of temperature increase appears to have a protective effect. In fact, Sørensen et al. (2013) similarly found that a slower ramping (0.06 °C min^{-1} compared to 0.1 °C min^{-1}) caused higher upregulation of heat shock proteins in *Drosophila melanogaster*. Because the rate we used is closer to the warming rate measured in tidepools, our results may be more informative about the true thermal limits and responses of ectotherms than those of studies that use faster ramping (although faster ramping may be appropriate for intertidal species exposed to air at low tide). Second, we saw consistent developmental rate and HSP responses among the three populations tested following the gradual exposure. As stated previously, these

populations vary widely in response to acute heat stress (Willett 2010; Tangwancharoen and Burton 2014), and they continue to maintain population differences in survivorship in the present study. However, it is notable that we did not see a higher degree of mortality in the more thermally sensitive SCN population, but rather a higher degree of protection, with SCN showing the highest change in survivorship between the abrupt and gradual exposures. The similarity of the effects on each population's life history and gene expression suggests that our findings could apply widely to marine (if not also terrestrial) ectotherms.

The findings of this study indicate that the use of ecologically relevant thermal exposures is essential for a complete understanding of organismal response to thermal stress: all populations of *T. californicus* survived to higher temperatures in our study than had been observed previously. This necessitates a re-defining of the thermal limits of *T. californicus* populations and a reconsideration for how thermal tolerance assays are conducted in this species. Though abrupt exposure thermal assays can still be valuable for answering mechanistic questions regarding variation in thermal tolerance, gradual exposure assays may be able to place laboratory studies in a context more relevant to the organisms in their natural habitats, and to understand how organisms have evolved in response to local thermal regimes. Critically, our results emphasize that ecologically relevant thermal exposures may reveal that organisms can be more resilient than previous studies have suggested.

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Table 3-1. Primer sequences and GenBank accession numbers for *Tigriopus californicus* genes used in qPCR (Barreto et al. 2015).

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Product size	GenBank Accession no.
hsp60	GAGATGTTGATTGGCGTGGAC	CACATCTTGGACGAGTTTGGC	189	GBSZ01004972
hsp70	CTGGATTGATGCTCTTGTTCA	CTCTGTGCCGACCTTTTCC	184	JW519350
hsp beta-1	CGATTTTCATCTGGGTCTCAA	TTGAAGAACTCCTCCGCTGT	175	JW506233
myosin	GTGTCGCAAAAGCAAATGAC	GAACCTCAACCTCCTCCTCA	154	JW508385
GAPDH	GGAGGAGGGGATGATGTTTT	CAACCACGAGCAATACGAGA	226	JW506006

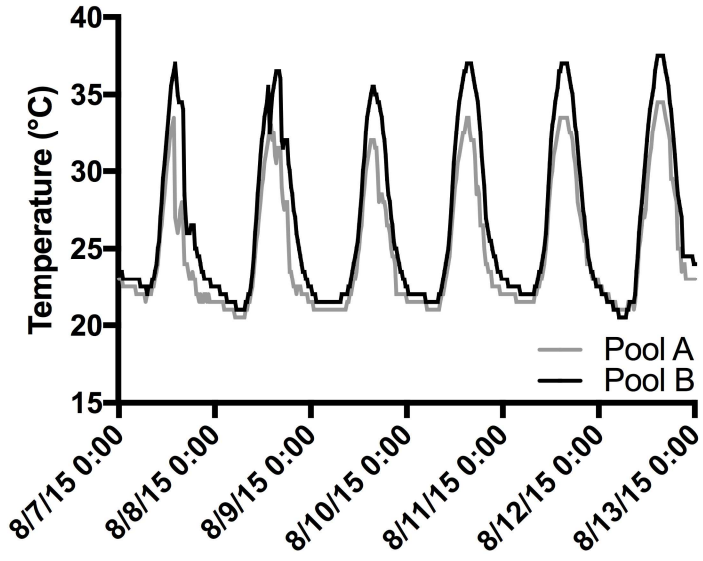

Figure 3-1. iButton Data Logger temperature measurements. iButton loggers were placed in two separate pools at the SD location. Temperature was recorded every 20 minutes from August 7 to August 13, 2015.

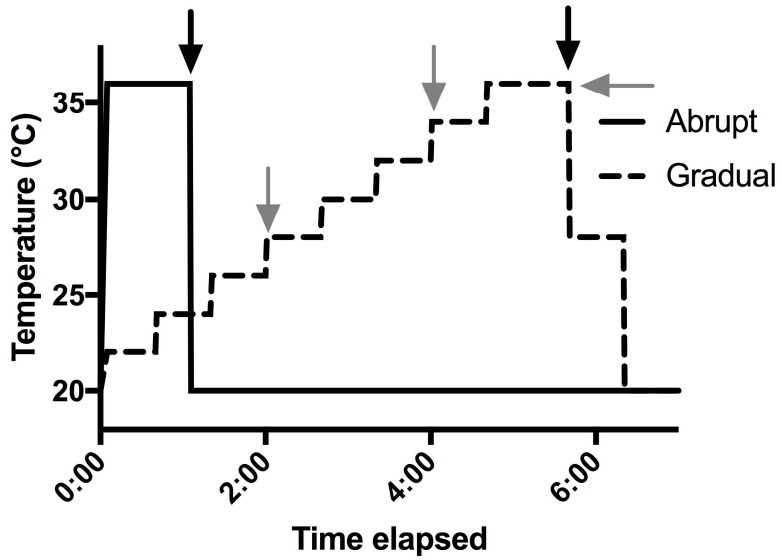

Figure 3-2. Abrupt vs. gradual thermal exposures. The abrupt exposure, represented by the solid line, places animals directly into a water bath set to the given temperature (in this case 36 °C). Due to the small volume of the tubes into which copepods are placed, water in the tube reaches the high temperature within a minute. The gradual exposure, represented by the dashed line, involves placing animals in microcentrifuge tubes into a thermal cycler, which raises the temperature by 2 °C every 40 min, and for 1-h at the peak temperature. They are then quickly ramped back down by 8 °C every 40 min. Black arrows indicate RNA extraction time points for comparison between abrupt and gradual exposure gene expression (Figure 3-5), and gray arrows indicate RNA extraction time points for examining gene regulation during gradual exposure (Figure 3-6).

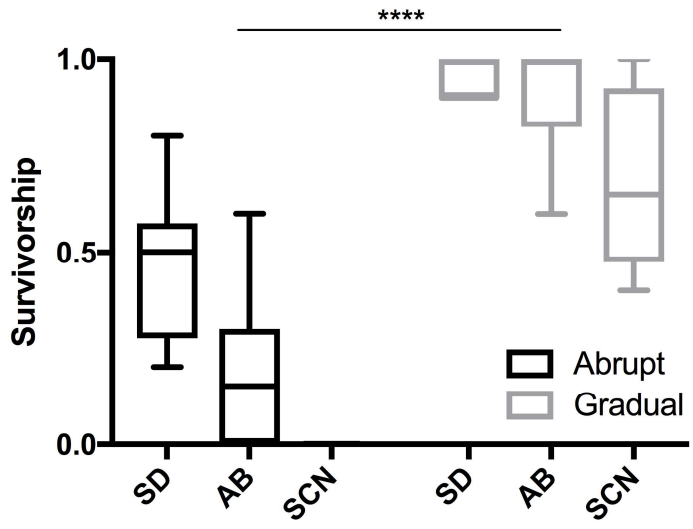

Figure 3-3. Survivorship following abrupt and gradual thermal exposure to 36 °C. Six groups of ten adult animals each were tested for each treatment of either a 1-h abrupt heat stress or a gradual stress. Whiskers of the box and whisker plots depict minimum to maximum values, boxes depict 25th to 75th percentiles, and the middle line denotes the median. An ANOVA run on a binomial generalized linear model showed a significant effect of temperature ($X^2_{1,30}$= 15.40, P < 0.0001) but not population ($X^2_{2,30}$ = 5.72, P = 0.0573).

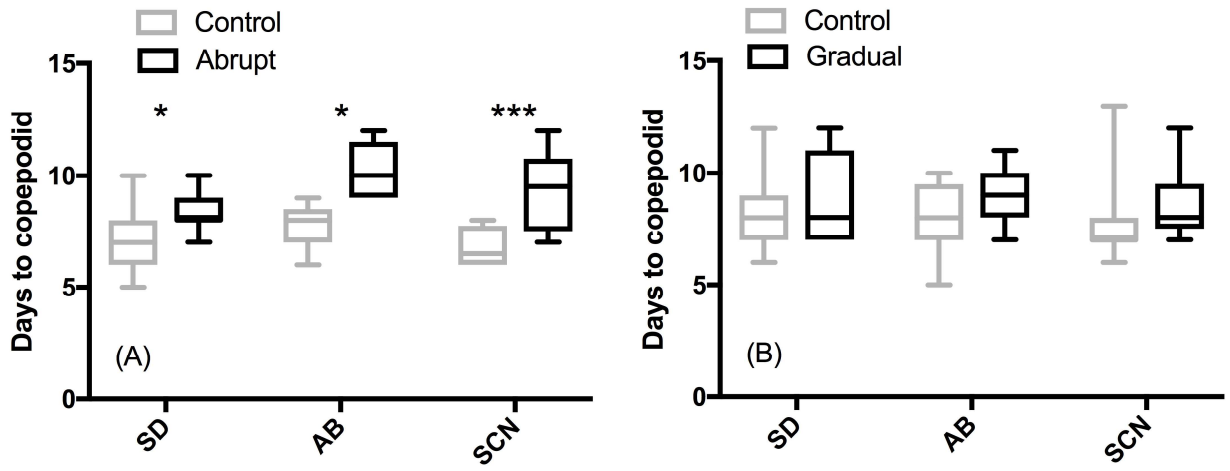

Figure 3-4. Developmental rate of nauplii following different heat stress protocols. Broods were split into control and thermal regime, either abrupt (A) or gradual (B). Days to reach first copepodid were counted. Whiskers of the box and whisker plots depict minimum to maximum values, boxes depict 25th to 75th percentiles, and the middle line denotes the median. For the abrupt stress, a two-way ANOVA showed a significant effect of population ($F_{2, 46} = 3.546$, $P = 0.0370$) and treatment ($F_{1, 46} = 33.05$, $P < 0.0001$). A Sidak's multiple comparisons test found a significantly slower developmental rate following the abrupt regime compared to control in SD ($P = 0.0200$), AB ($P = 0.0113$), and SCN ($P = 0.0003$). No significant difference in developmental rate was found between the gradual regime and control ($F_{1, 44} = 3.025$, $P = 0.0890$).

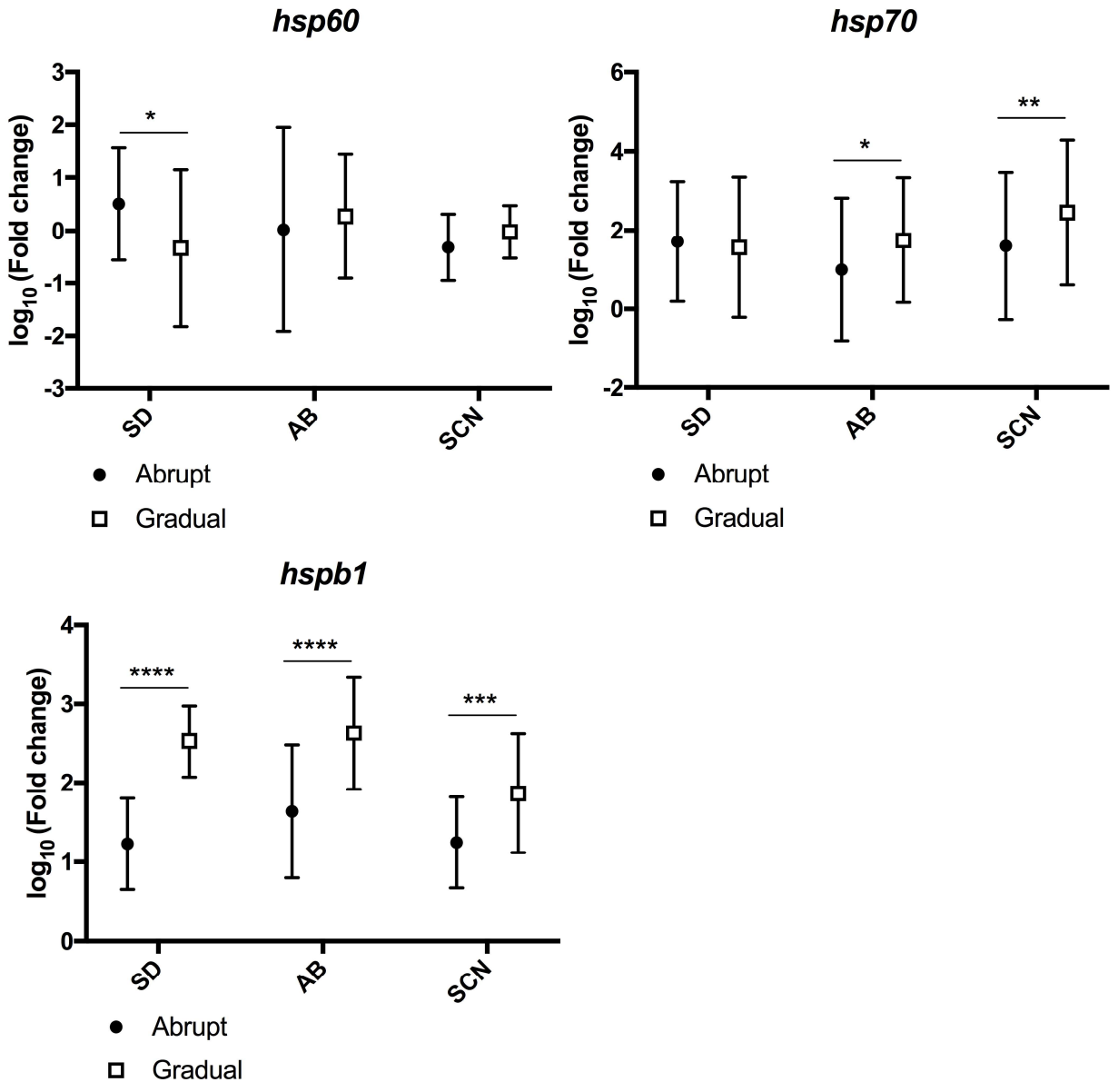

Figure 3-5. Gene expression following thermal stress. For each of three genes, log fold change (with 95% confidence interval) was calculated comparing either the abrupt or gradual regime to a control group. RNA was extracted immediately upon completion of the thermal exposures. Significance was assessed using a randomized block ANOVA followed by Sidak's multiple comparisons test; *hsp60* showed higher upregulation following the abrupt regime in SD according to the Sidak's test ($P = 0.0141$). For *hsp70,* the gradual regime caused higher upregulation in AB ($P = 0.0101$) and SCN ($P = 0.0056$). The gradual regime significantly increased upregulation of *hspb1* ($F_{2,1,3} = 609$, $P =< 0.0001$) in all three populations (SD and AB: $P < 0.0001$; SCN: $P = 0.0003$).

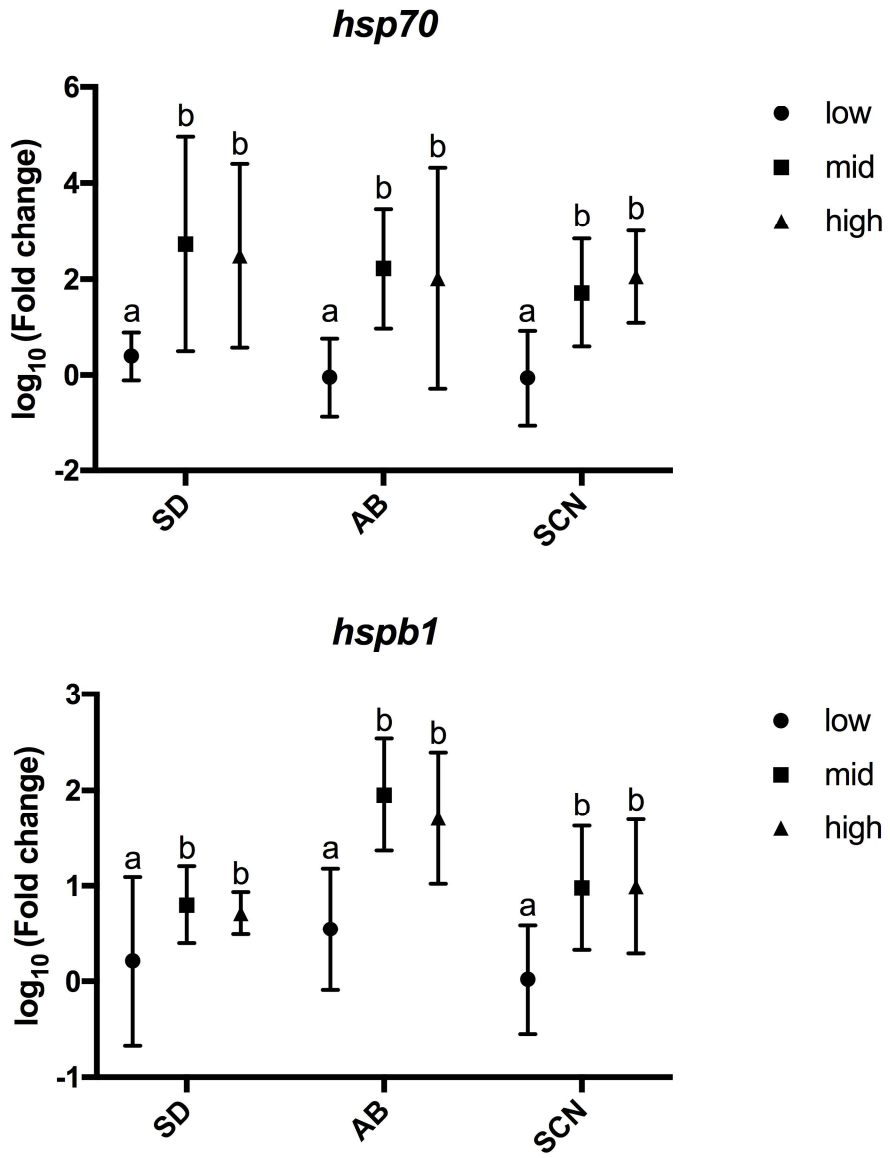

Figure 3-6. Gene expression during ramping stress. For each of three genes, log fold change (with 95% confidence interval) was calculated comparing three temperatures during the gradual regime (low: 27 °C for SCN and 28 °C for SD/AB; mid: 33 and 34°C; and high: 35/36 °C) to a control group. Significance was assessed using a randomized block ANOVA followed by Tukey's multiple comparisons test. For both genes, all three populations showed the same pattern: there was significantly higher upregulation from the low (27/28 °C) to the mid temperature (33/34 °C) and from the low to the high temperature (35/36 °C) but not between the mid and the high temperatures.

REFERENCES

Angilletta M.J., R.S. Wilson, C.A. Navas, and R.S. James. 2003. Tradeoffs and the evolution of thermal reaction norms. Trends Ecol Evol.

Arya R., M. Mallik, and S.C. Lakhotia. 2007. Heat shock genes - Integrating cell survival and death. J Biosci.

Bahar M.H., D. Hegedus, J. Soroka, C. Coutu, D. Bekkaoui, and L. Dosdall. 2013. Survival and Hsp70 gene expression in *Plutella xylostella* and its larval parasitoid *Diadegma insulare* varied between slowly ramping and abrupt extreme temperature regimes. PLoS One 8.

Bahrndorff S., J. Mariën, V. Loeschcke, and J. Ellers. 2009. Dynamics of heat-induced thermal stress resistance and hsp70 expression in the springtail, *Orchesella cincta*. Funct Ecol 23:233–239.

Barreto F.S., S.D. Schoville, and R.S. Burton. 2015. Reverse genetics in the tide pool: Knock-down of target gene expression via RNA interference in the copepod *Tigriopus californicus*. Mol Ecol Resour 15:868–879.

Benoit J.B., G. Lopez-Martinez, K.R. Patrick, Z.P. Phillips, T.B. Krause, and D.L. Denlinger. 2011. Drinking a hot blood meal elicits a protective heat shock response in mosquitoes. Proc Natl Acad Sci 108:8026–8029.

Bermudes M. and A.J. Ritar. 2008. Response of early stage spiny lobster *Jasus edwardsii* phyllosoma larvae to changes in temperature and photoperiod. Aquaculture 281:63–69.

Bjelde B.E., N.A. Miller, J.H. Stillman, and A.E. Todgham. 2015. The role of oxygen in determining upper thermal limits in *Lottia digitalis* under air exposure and submersion. Physiol Biochem Zool 88:483–493.

Bowler K. 2005. Acclimation, heat shock and hardening. J Therm Biol.

Chidawanyika F. and J.S. Terblanche. 2011. Rapid thermal responses and thermal tolerance in adult codling moth Cydia pomonella (Lepidoptera : Tortricidae). J Insect Physiol 57:108–117.

Chown S.L., K.R. Jumbam, J.G. Sørensen, and J.S. Terblanche. 2009. Phenotypic variance, plasticity and heritability estimates of critical thermal limits depend on methodological context. Funct Ecol 23:133–140.

Clusella-Trullas S., T.M. Blackburn, and S.L. Chown. 2011. Climatic predictors of temperature performance curve parameters in ectotherms imply complex responses to climate change. Am Nat 177:738–751.

Dahlgaard J., V. Loeschcke, P. Michalak, and J. Justesen. 1998. Induced thermotolerance and associated expression of the heat-shock protein Hsp70 in adult *Drosophila melanogaster*. Funct Ecol 12:786–793.

Dahlhoff E. and G.N. Somero. 1993. Effects of temperature on mitochondria from abalone (genus: *Haliotis*): Adaptive plasticity and its limits. J Exp Biol 185:151–168.

Denny M.W., L.P. Miller, and C.D.G. Harley. 2006. Thermal stress on intertidal limpets: long-term hindcasts and lethal limits. J Exp Biol 209:2420–2431.

Dethier M.N. 1980. Tidepools as refuges: Predation and the limits of the harpacticoid copepod *Tigriopus californicus* (Baker). J Exp Mar Bio Ecol 42:99–111.

Dong Y., T. Ji, X. Meng, S. Dong, and W. Sun. 2010. Difference in Thermotolerance Between Green and Red Color Variants of the Japanese Sea Cucumber , Apostichopus japonicus Selenka : Hsp70 and Heat-Hardening Effect 87–94.

Fangue N.A., J.G. Richards, and P.M. Schulte. 2009. Do mitochondrial properties explain intraspecific variation in thermal tolerance? J Exp Biol 212:514–522.

Fields P.A., E.M. Burmester, K.M. Cox, and K.R. Karch. 2016. Rapid proteomic responses to a near-lethal heat stress in the salt marsh mussel *Geukensia demissa*. J Exp Biol 219:2673–2686.

Ganz H.H. and R.S. Burton. 1995. Genetic differentiation and reproductive incompatibility among Baja California populations of the copepod *Tigriopus californicus*. Mar Biol 123:821–827.

Giomi F., C. Mandaglio, M. Ganmanee, G.-D. Han, Y.-W. Dong, G.A. Williams, and G. Sara. 2016. The importance of thermal history: costs and benefits of heat exposure in a tropical, rocky shore oyster. J Exp Biol 219:686–694.

Gleason L.U. and R.S. Burton. 2015. RNA-seq reveals regional differences in transcriptome response to heat stress in the marine snail *Chlorostoma funebralis*. Mol Ecol 24:610–627.

Hoffmann A.A., S.L. Chown, and S. Clusella-Trullas. 2013. Upper thermal limits in terrestrial ectotherms: How constrained are they? Funct Ecol 27:934–949.

Hu J. tao, B. Chen, and Z. hong Li. 2014. Thermal plasticity is related to the hardening response of heat shock protein expression in two *Bactrocera* fruit flies. J Insect Physiol 67:105–113.

Jimenez A.G., S. Alves, J. Dallmer, E. Njoo, S. Roa, and W.W. Dowd. 2016. Acclimation to elevated emersion temperature has no effect on susceptibility to acute, heat-induced lipid peroxidation in an intertidal mussel *(Mytilus californianus)*. Mar Biol 163:1–10.

Kelly M.W., M.S. Pankey, M.B. DeBiasse, and D.C. Plachetzki. 2017. Adaptation to heat stress reduces phenotypic and transcriptional plasticity in a marine copepod. Funct Ecol 31:398–406.

Kelly M.W., E. Sanford, and R.K. Grosberg. 2011. Limited potential for adaptation to climate change in a broadly distributed marine crustacean. Proc R Soc B Biol Sci 279:349–356.

Kingsolver J.G. and H.A. Woods. 2016. Beyond thermal performance curves: Modeling time-

dependent effects of thermal stress on ectotherm growth rates. Am Nat 187:283–294.

Lee R.E., C.P. Chen, and D.L. Denlinger. 1987. A rapid cold-hardening process in insects. Science 238:1415–7.

Lee R.E., K. Damodaran, S.X. Yi, and G.A. Lorigan. 2006. Rapid cold-hardening increases membrane fluidity and cold tolerance of insect cells. Cryobiology 52:459–463.

Lima T.G. and C.S. Willett. 2017. Locally adapted populations of a copepod can evolve different gene expression patterns under the same environmental pressures. Ecol Evol 1–14.

Loeschcke V., R.A. Krebs, and J.S.F. Barker. 1994. Genetic variation for resistance and acclimation to high temperature stress in *Drosophila buzzatii*. Biol J Linn Soc 52:83–92.

McMillan D.M., S.L. Fearnley, N.E. Rank, and E.P. Dahlhoff. 2005. Natural temperature variation affects larval survival, development and Hsp70 expression in a leaf beetle. Funct Ecol 19:844–852.

Mitchell K.A. and A.A. Hoffmann. 2010. Thermal ramping rate influences evolutionary potential and species differences for upper thermal limits in *Drosophila*. Funct Ecol 24:694–700.

Nguyen C., H. Bahar, G. Baker, and N.R. Andrew. 2014. Thermal tolerance limits of diamondback moth in ramping and plunging assays. PLoS One 9.

Overgaard J., T.N. Kristensen, and J.G. Sørensen. 2012. Validity of thermal ramping assays used to assess thermal tolerance in arthropods. PLoS One 7:1–7.

Paganini A.W., N.A. Miller, and J.H. Stillman. 2014. Temperature and acidification variability reduce physiological performance in the intertidal zone porcelain crab *Petrolisthes cinctipes*. J Exp Biol 217:3974–3980.

Pasparakis C., B.E. Davis, and A.E. Todgham. 2016. Role of sequential low-tide-period conditions on the thermal physiology of summer and winter laboratory-acclimated fingered limpets, *Lottia digitalis*. Mar Biol 163:1–17.

Pereira R.J., F.S. Barreto, and R.S. Burton. 2014. Ecological novelty by hybridization: Experimental evidence for increased thermal tolerance by transgressive segregation in *Tigriopus californicus*. Evolution (N Y) 68:204–215.

Pörtner H.-O. 2010. Oxygen- and capacity-limitation of thermal tolerance: a matrix for integrating climate-related stressor effects in marine ecosystems. J Exp Biol 213:881–893.

Pörtner, H. O., Bennett, A. F., Bozinovic, F., Clarke, A., Lardies, M. A., Lucassen, M., Pelster, B., Schiemer, F., and Stillman, J. H. 2006. Trade-offs in thermal adaptation: The need for a molecular to ecological integration. Physiol Biochem Zool 79:295–313.

Roberts S.D., C.D. Dixon, and L. Andreacchio. 2012. Temperature dependent larval duration and survival of the western king prawn, *Penaeus (Melicertus) latisulcatus* Kishinouye, from Spencer Gulf, South Australia. J Exp Mar Bio Ecol 411:14–22.

Runcie D.E., D.A. Garfield, C.C. Babbitt, J.A. Wygoda, S. Mukherjee, and G.A. Wray. 2012. Genetics of gene expression responses to temperature stress in a sea urchin gene network. Mol Ecol 21:4547–4562.

Schmittgen T.D. and K.J. Livak. 2008. Analyzing real-time PCR data by the comparative CT method. Nat Protoc 3:1101–1108.

Schoville S.D., F.S. Barreto, G.W. Moy, A. Wolff, and R.S. Burton. 2012. Investigating the molecular basis of local adaptation to thermal stress: population differences in gene expression across the transcriptome of the copepod *Tigriopus californicus*. BMC Evol Biol 12:170.

Sconzo G., M. Roccheri, M. La Rosa, D. Oliva, A. Abrignani, and G. Giudice. 1986. Acquisition of thermotolerance in sea urchin embryos correlates with the synthesis and age of the heat shock proteins. Cell Differ 19:173–177.

Sgrò C.M., J. Overgaard, T.N. Kristensen, K.A. Mitchell, F.E. Cockerell, and A.A. Hoffmann. 2010. A comprehensive assessment of geographic variation in heat tolerance and hardening capacity in populations of *Drosophila melanogaster* from Eastern Australia. J Evol Biol 23:2484–2493.

Sobek S., A. Rajamohan, D. Dillon, R.C. Cumming, and B.J. Sinclair. 2011. High temperature tolerance and thermal plasticity in emerald ash borer *Agrilus planipennis*. Agric For Entomol 13:333–340.

Somero G.N. 2005. Linking biogeography to physiology: Evolutionary and acclimatory adjustments of thermal limits. Front Zool 2:1.

Somero G.N. 2012. The Physiology of Global Change: Linking Patterns to Mechanisms. Ann Rev Mar Sci 4:39–61.

Sørensen J.G., V. Loeschcke, and T.N. Kristensen. 2013. Cellular damage as induced by high temperature is dependent on rate of temperature change - investigating consequences of ramping rates on molecular and organismal phenotypes in *Drosophila melanogaster*. J Exp Biol 216:809–814.

Sunday J.M., A.E. Bates, and N.K. Dulvy. 2012. Thermal tolerance and the global redistribution of animals. Nat Clim Chang 2:686–690.

Tangwancharoen S. and R.S. Burton. 2014. Early life stages are not always the most sensitive: Heat stress responses in the copepod *Tigriopus californicus*. Mar Ecol Prog Ser 517:75–83.

Terblanche J.S., J.A. Deere, S. Clusella-Trullas, C. Janion, and S.L. Chown. 2007. Critical thermal limits depend on methodological context. Proc R Soc B Biol Sci 274:2935–2943.

Terblanche J.S., A.A. Hoffmann, K.A. Mitchell, L. Rako, P.C. le Roux, and S.L. Chown. 2011. Ecologically relevant measures of tolerance to potentially lethal temperatures. J Exp Biol 214:3713–3725.

Tomanek L. and M.J. Zuzow. 2010. The proteomic response of the mussel congeners *Mytilus galloprovincialis* and *M. trossulus* to acute heat stress: implications for thermal tolerance limits and metabolic costs of thermal stress. J Exp Biol 213:3559–3574.

Vasseur D.A., J.P. Delong, B. Gilbert, H.S. Greig, C.D.G. Harley, K.S. Mccann, V. Savage, T.D. Tunney, and M.I.O. Connor. 2014. Increased temperature variation poses a greater risk to species than climate warming Increased temperature variation poses a greater risk to species than climate warming.

Vergara-Amado J., A.X. Silva, C. Manzi, R.F. Nespolo, and L. Cárdenas. 2017. Differential expression of stress candidate genes for thermal tolerance in the sea urchin *Loxechinus albus*. J Therm Biol 68:104–109.

Willett C.S. 2010. Potential fitness trade-offs for thermal tolerance in the intertidal copepod *Tigriopus californicus*. Evolution (N Y) 64:2521–2534.

CONCLUSION

The results of this dissertation can help us better understand the mechanistic basis of thermal tolerance in *Tigriopus californicus*. By comparing responses to thermal stress among three populations, we can also illuminate patterns of local adaptation. Together these can help us predict how organisms will respond to future changes in thermal regimes.

Mechanisms underlying acute versus chronic thermal tolerance

In Chapter 1, we examined the variation in thermal tolerance at acute and chronic timescales in three populations of *T. californicus* distributed across a latitudinal gradient. We found that knockdown temperature, survivorship, and mitochondrial performance (measured as ATP production) followed expected patterns of thermal tolerance at acute temperatures given each population's latitudinal distribution. Furthermore, temperatures at which ATP production began to decline were slightly lower than whole-animal knockdown temperatures, potentially indicating that the mitochondria have a role in determining acute thermal tolerance. However, the patterns of chronic thermal tolerance were not as clear, with the mid-latitude AB population showing lower ATP production and slower development than its northern and southern congeners. Though this result does not align with predicted responses to latitudinal distribution like the pattern of acute thermal tolerance, it emphasizes the role of mitochondria in organismal thermal performance and suggests divergence of function for different *T. californicus* populations.

Complexities of gene regulation during the heat shock response

Chapter 2 assessed the role of HSF-1 in the regulation of the heat shock response. Using RNAi followed by RNAseq, we analyzed differential expression following heat shock in the SD population of *T. californicus*. The majority of differentially expressed genes between the control and knockdown groups were upregulated, indicating that the normal function of HSF-1 is to repress these genes. This supports findings in other systems of HSF-1's role in various important processes in addition to the heat shock response. Though heat shock proteins (HSPs) were not detected in the differential expression analysis of the control versus knockdown groups, we saw evidence of decreased upregulation of important HSP genes following heat stress, indicating that the knockdown dampened the heat shock response in these genes. Finally, we performed a genome scan to identify genes with canonical HSEs; surprisingly few of these genes appeared in our differential expression analysis. This is consistent with studies in other systems that have found that perfect HSEs are not always necessary and usually not sufficient to guarantee binding and subsequent gene activation by HSF-1. These findings demonstrate the complexity of these mechanisms of gene regulation. Examining this response in a less thermally tolerant population of *T. californicus* could help determine the overall importance of HSF-1 in the heat shock response (as opposed to its role in non-stress functions) and whether its divergence can partially explain patterns of thermal tolerance.

Heat shock proteins are essential for surviving thermal stress

Finally, Chapter 3 focused on the effect of different thermal exposure rates on survivorship, developmental rate, and the heat shock response in three populations of *T. californicus*. Despite a shorter total thermal stress, an abrupt 1-h thermal exposure resulted in a

slower developmental rate (~2-d) and decreased survivorship in all three populations when compared to a gradual ramping thermal exposure. Greater upregulation of two important heat shock protein genes was also observed in the gradual exposure, indicating that the ability to upregulate these genes prior to reaching the maximal temperature is as an important protective mechanism.

Summary and future avenues of study

Taken together, the results of these chapters indicate the importance of examining thermal tolerance from multiple levels of biological organization. The dramatic differences in survivorship and developmental rate following a gradual versus abrupt thermal exposure appear to emphasize the importance of heat shock proteins in response to acute thermal stress. This importance is also corroborated by the decreased survivorship and dampened upregulation of heat shock protein genes following HSF-1 knockdown. Though we also see evidence for mitochondrial performance playing a role in thermal tolerance, the discrepancy between patterns of acute and chronic thermal tolerance in the AB population indicates a more complicated relationship between mitochondria and thermal limits. It is possible that while heat shock proteins are vital for acute thermal tolerance, mitochondrial performance plays a greater role over longer time scales of chronic exposure. Potential future directions for investigating thermal tolerance include characterizing mitochondrial performance across a wider latitudinal range and further analysis of the effects of heating rates on development, survivorship, and the heat shock response.