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Exploration of novel hybrid thermal phenotypes and genetic variation for thermal tolerance in the marine copepod Tigriopus californicus

Author

Blackwell, Reginald

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Exploration of novel hybrid thermal phenotypes and genetic variation for thermal tolerance in the marine copepod *Tigriopus californicus*

A Dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

in

Marine Biology

by

Reginald Caeaesar Blackwell Jr.

Committee in charge:

Ronald Burton, Chair
Andrew Allen
Eric Allen
Bianca Brahamsha
Lin Chao

2021

The dissertation of Reginald Caeaesar Blackwell Jr. is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

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VITA

Education

2011	A.S., Biological Science, Butte College
2011	A.A., Social and Behavioral Studies, Butte College
2014	B.S., Biology, Humboldt State University
2018	M.S., Oceanography, U.C. San Diego ,
2021	Ph.D., Marine Biology, U.C. San Diego

ABSTRACT OF THE DISSERTATION

Exploration of novel hybrid thermal phenotypes and genetic variation for thermal tolerance in the marine copepod *Tigriopus californicus*

by

Reginald Caeaesar Blackwell Jr.

Doctor of Philosophy in Marine Biology

University of California San Diego, 2021

Professor Ronald Burton, Chair

Thermal stress will become a more frequent occurrence as global temperatures continue to rise due to human induced climate change. Unlike many fortunate humans, animals do not have air conditioners to escape the harmful effects of higher temperatures. To persist organisms will move to suitable conditions increasing the likely hood of hybridization events or adapt by some sort of evolutionary mechanism. This dissertation contains research on the variation for thermal limit in the high intertidal copepod *Tigriopus californicus*. In Chapter 1, I investigated the genetic variation for thermal limit in a subtropical population from San Roque, Baja California, Mexico. In Chapter 2, I created interpopulation hybrids to explore the production of transgressive thermal phenotypes. In Chapter 3, I investigated the gene expression response to

thermal stress in transgressive segregants of increased maximum thermal limit in crosses between Catalina Island and Abalone Cove, California, USA. In Chapter 4, I genotyped hybrids using the RNAseq data from Chapter 3 and population specific SNPs to determine the genetic composition of positive transgressive phenotypes.

General Introduction

Thermal tolerance is a determinant in the spatial distribution and persistence of many taxa (Wiens and Donoghue 2004; Sunday et al. 2011; Lancaster and Humphreys 2020). Temperature is a key element of climate and strong selective pressure for organisms that is rapidly increasing globally (IPCC 2018). Since 1880, global temperature has increased by 2.0 ℃ with 19 of the 20 warmest years occurring after 2001 (GISSTEMP Team, 2020). As the pace of global temperature rise increases, it may overtake the evolutionary capacity of populations and species. In addition to rising temperature, the intensity and duration of heat waves is increasing the probability of mortality in terrestrial and marine ecosystems (Williams et al. 2016; Buckley et al. 2016). To counter the harmful effects of increasing temperatures some organisms shift their distribution toward the poles and higher altitudes where conditions are more suitable (Helmuth et al. 2006). Organisms unable to shift their distribution will have to adapt using the standing genetic variation or receive complementary genotypes from immigration that may produce novel genotypes (Rieseberg et al. 1999; Visser 2008). Understanding the genetic variation in populations for response to environmental stress is increasingly becoming important.

Increasing frequency of extreme temperatures events can increase mortality in populations. In the case of limited migration capacity, organism can respond by adjusting capacity of their physiology (Visser 2008). However, animals living close to their thermal maxima are unlikely to evolve to the predicted increase in temperatures (Deutsch et al. 2008). If organisms are unable to increase their thermal limit extinction risks are inevitable with increasing temperatures.

In some species the general trend of higher latitudinal shifts is averaging 16.9 km decade and 11.0 meters per decade shift to higher elevations, this movement of species is consistent with average change in temperature (tracking temperature change) (Chen 2011). Tracking SNPs in *Drosophila sp*. populations from Australia showed southern high latitude populations becoming more genetically similar to northern populations indicating migration of genetic material from one population to the other over a 20 year period (Umina et al. 2005). Species with large latitudinal ranges may experience movement of alleles across the range as conditions shift and alleles from a more tolerant population introgress into a more sensitive population. These incidents of introgression, the result of hybridization, can have unpredictable consequences on the stability of species. Hybridization can lead to reduced fitness of populations or an increase in fitness that occurs much quicker than classic evolutionary models (Grant and Grant 2019).

Tigriopus californicus is a unique system to study thermal limit questions within and between populations. This species lives in the splash zone of the intertidal mostly disconnected from the ocean putting it in conditions more similar to terrestrial organism. Populations are locally adapted to the environmental conditions and exhibit increasing temperature tolerance with decreasing latitude across its range from Baja California, Mexico to the southern tip of Alaska, United States (Edmands 2001; Willett 2010; Kelly et al. 2012; Schoville et al. 2012; Peterson et al. 2013; Pereira et al. 2017; Healy et al. 2019). Populations of *Tigriopus* californicus are genetically stable and highly genetically differentiated indicating low immigration between populations (Burton et al.1999; Pereira et al. 2016; Barreto et al. 2018). Regionally close populations display similar environmental tolerances (i.e., thermal tolerance) with decreasing similarity with increasing distance between populations (Lima and Willett 2017; Healy et al. 2019). Even with such great divergence between populations, lab created hybrids are still

possible with populations from California and further north allowing study of thermal tolerance variation within a population and the effects of hybridization between populations and the resulting thermal tolerance phenotypes (Ganz and Burton 1995; Peterson et al. 2013).

The goal of this dissertation is to explore the population variation for thermal tolerance in a population near the southern range limit in which variation is expected to be limited. The other goal of this study is to examine the occurrence of novel thermal phenotypes created by hybridization of ecologically similar and ecologically dissimilar populations of *T. californicus*. In Chapter 1, the objective was to determine the amount of genetic variation for the thermal limit in a southern range edge population of *Tigriopus californicus.* Populations near the end of a species a range are expected to harbor low genetic variation. To determine the amount of variation contained in the population I created inbred lines to separate the genetic variation within the population into iso-female lines. Chapter 2 I explored the production of increased and decreased thermal phenotypes of transgressive segregants by creating interpopulation crosses. Chapter 3, I measured gene expression with RNAseq in single copepods from thermal stress in positive transgressive segregants in reciprocal crosses of the Abalone Cove and Catalina Island populations from southern California. Chapter 4, using RNAseq data from Chapter 3, I genotyped individuals to determine the composition of parental chromosomes contained in positive thermal transgressive copepods from multiple recombinant inbred lines. In summary, the chapters of this dissertation provide insight on the variation for thermal tolerance in a range edge population of *Tigriopus californicus* and a greater understanding of the requirements for novel thermal phenotypes.

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Chapter 1

Exploring the genetic diversity for maximum thermal limit in a southern range edge population of tidepool copepod, *Tigriopus californicus*

Introduction

Global climate change is proceeding rapidly causing variable and extreme selection pressure on species and populations (IPCC 2018). Temperature is a key element of climate and determinant of species persistence, setting limits of where organisms can live and reproduce. Globally, temperatures are increasing, putting at risk organisms that are living close to their thermal maxima (Kingsolver, Diamond, and Buckley 2013; Williams et al. 2016; Neukom et al. 2019). In general, populations living closer to the equator (tropical and subtropical populations) exhibit higher thermal tolerances but lower thermal margins (Somero 2010; Kingsolver and Buckley 2017) consequently, the capacity of these populations to adapt to future rising temperatures is predicted to be limited (Somero 2010). However, even with a lower adaptive capacity, tropical and subtropical population may possess novel alleles that could contribute to the phenotypic responses of other populations in a species range (Provan and Maggs 2012; Nicotra et al. 2015; Macdonald et al. 2017).

The capacity to adapt to novel environments in a species or population is contingent on the ability to track changes to the environment. There are three main methods to track changes in the environment. Organisms can migrate to favorable habitat, acclimate to new novel conditions by phenotypic plasticity, and/or adapt by natural selection based on the genetic diversity of the population (Nicotra et al., 2015). Many populations are restricted in dispersal due to habitat fragmentation limiting movement to more favorable conditions or receiving new genotypes from other populations (Eckert, Samis, and Lougheed 2008; Mota et al. 2018). Phenotypic plasticity does maintain the population if individuals are able physiologically track environmental changes and then genetically adapt to the new environmental mean (Storz, Scott, and Cheviron 2010). Arguably the genetic diversity of a population is the most important

feature in attempting to determine vulnerability to extirpation (Razgour et al. 2019; Bennett et al. 2019; Ørsted et al. 2019). Genetic diversity is positively correlated with the expression of phenotypic diversity (Cheverud 1988; Forsman 2014; Sodini et al. 2018; Sun et al. 2019). Response to natural selection and demographic resilience may depend on the amount of variation (Hoffmann and Sgró 2011; Ørsted et al., 2019).

Adaptation can be limited by small population size, dearth of genetic variation, interactions with other species, high gene flow, and fitness trade-offs with other traits not directly under selection. Natural selection due to climate change will remove unfit genotypes from a population. Even if a population is receiving immigrants from other regions, diversity may not increase when selection pressures are strong (Bahn, O'Connor, and Krohn 2006). How do we determine if a population is resilient or vulnerable to increased temperature from a rapidly changing climate? Several studies have attempted to clarify this question using artificial selection on laboratory populations. This approach typically applies strong selection over several generations to measure capacity for adaptation. Studies like these are useful but may be hampered by the rapid removal of standing genetic variation as the population is pushed to a new trait mean. Furthermore, compounding the negative effects of rapidly removing genetic variation, most experiments use small numbers of individuals, effectively starting with limited genetic variation. Another approach to quantify adaptive genetic variation questions is to produce iso-female lines (David et al. 2005; Bridle, Gavaz, and Kennington 2009; Condon et al. 2015; Faria and Sucena 2017; Lockwood, Gupta, and Scavotto 2018; Rolandi et al. 2018). The distribution of phenotypes in iso-female lines can be quantitatively attributed to the genetic variation of the starting population without applying strong extrinsic stress (Nouhaud et al. 2016).

The high intertidal marine copepod *Tigriopus californicus* is an attractive system to study questions regarding variation in thermal limit within populations. This copepod has a geographical range from subtropical Baja California Sur, Mexico to the southern tip of Alaska, USA of North America spanning a latitudinal distance of ~3740 km (Edmands 2001; Peterson et al. 2013). *T. californicus* inhabit high intertidal splash pools in rocky outcrops and exhibit increasing temperature tolerance with decreasing latitude (Willett 2010; Kelly, Sanford, and Grosberg 2012; Schoville et al. 2012; Pereira, Sasaki, and Burton 2017; Healy, Bock, and Burton 2019). Rock outcrops are separated by unfavorable habitats of sandy beaches limiting immigration between locations and leading to high genetic divergence between rocky outcrops. In this species, very few polymorphisms are shared between populations indicating a high degree of local adaptation and low immigration (Pereira et al. 2016). Because of its broad physiological tolerances, *T. californicus* has proven to be an interesting model system for analysis of responses to environmental stresses such as salinity (Burton and Feldman 1982; Willett and Burton 2002; DeBiasse, Kawji, and Kelly 2018), temperature (Schoville et al. 2012; Wallace, Kim, and Neufeld 2014; Pereira, Sasaki, and Burton 2017) and hypoxia (Graham and Barreto 2019).

It has been argued the standing variation for thermal tolerance in natural populations of *Tigriopus californicus* has been depleted due to local adaptation (Kelly, Sanford, and Grosberg 2012; Kelly, Grosberg, and Sanford 2013). If populations are depleted of variation for thermal tolerance, then they will be more susceptible to extirpation from increased temperatures due to climate change. A study of *Tigriopus californicus* surmised populations were at risk for two reasons: (1) populations did not contain enough additive genetic variation for thermal response and (2) the populations may incur a high fitness cost of increased thermal tolerance (Kelly,

Grosberg, and Sanford 2013). The reasoning for this conclusion was due to one generation of copepods raised at higher temperatures to measure plasticity for thermal tolerance and five to ten generations of acute (LT50) thermal stress once per generation for several populations along the coast of North America. In both experiments the thermal tolerance was increased but it was not a large increase. On average, the LT (50) selection experiment removed 40-90% of the population each generation, decreasing the amount of variation dramatically over ten generations. Furthermore, it was concluded the degree of change for northern populations did not increase to levels of the southern populations and therefore this species would be at risk of extinction. Genetic variation within populations was determined using neutral makers and found to have low within population variation and high between population variation (Kelly, Sanford, and Grosberg 2012). An evolutionary response due to such extreme extrinsic selection pressures in small populations are useful but could be underestimating the adaptive potential of the population. Deploying methods to quantify population variation without extreme selection pressures could be a useful tool in our understanding of the genetic variation contained in a population and the adaptive potential. To address this, I selected a population from the southern part of the species range, where previous studies have found the greatest level of thermal tolerance. Similar to other species, it could be expected a low latitude population may possess unique adaptations to thermal stress but could also be expected to be depleted for adaptive variation (Macdonald et al. 2017). In this study I created inbred iso-female inbred lines from the San Roque, Baja California Sur, Mexico population. This subtropical population is near the southern end of the species range and is the most thermal tolerant population tested to date (Pereira, Sasaki, and Burton 2017; Healy, Bock, and Burton 2019). Our null hypothesis is that if genetic variation for thermal tolerance has been depleted, all inbred iso-female lines will

have similar thermal tolerance. To test thermal tolerance, I used a dynamic critical thermal limit test to measure the variation between independent iso-female lines after inbreeding allowing me to test the amount of genetic variation in the parental population.

Methods and Materials

Mature *Tigriopus californicus* males clasp immature virgin females with geniculate antennae. Upon female maturity the pair copulates and disengages. The female copepod will mate only once while the male can continue to perform several successful mating events (Egloff 1966; Burton 1985). Females will produce as many as 20 eggs sacs over a lifetime from one copulation event, the number of eggs produced in each egg sac can vary in quantity from as low as 1 egg to more than 100 (Egloff 1966; Vittor 1971). Females typically carry their egg sacs until they hatch. Development proceeds through six naupliar stages followed by 5 copepodite stages concluding development in the final adult stage and sexual maturity. This entire process can take place in as little as 2-3 weeks, leading to the possible rapid expansion of a population from very few mating pairs.

Population maintenance

For this study, I selected the San Roque population (27.17, -114.39) from Baja California Sur, Mexico, the southernmost portion of the *T. californicus* range (~38 km from end of range, Peterson et al. 2013). This population was selected due to its high thermal tolerance and sizeable individual variation in thermal tolerance (Pereira, Sasaki, and Burton 2017; Healy, Bock, and Burton 2019). The copepods were collected from tidepools in 2016 and maintained in 400 ml beakers with 200 ml of 0.45µ filtered seawater and fed ground Spirulina ad lib. The beakers were housed in incubators with a 12-hour light and 12-hour dark photoperiod at a constant temperature of 20 °C. Salinity was monitored and maintained at 35

psu. Monthly 1/3 volume seawater changes were conducted, at this time separate beakers were mixed to maintain similar genetic composition between each beaker.

Inbred line creation

Fifty-four inbred lines were created by placing a single clasped pair into a well of 6 well tissue culture dish (Corning Costar nontreated) with ~10 ml of seawater and ground Spirulina. Wells were monitored daily for gravid females; upon presence of a gravid female the male was removed. The female was removed once her first egg sac hatched. After the female was removed, larvae developed, and each well was checked daily for the appearance of clasped pairs. The first clasped pair that appeared for each well was moved to a new well, supplied dried spirulina and filtered seawater to create the next generation. Each inbred generation was produced from a single clasped pair. This process was repeated for three generations of inbreeding. The F3 generation of inbred copepods were moved to a 400 ml beaker with 200 ml of seawater and dried Spirulina and allowed expand to greater numbers. Generations were allowed to overlap as numbers increased to perform intrinsic stress and CTmax assays.

Fitness of inbred lines

Inbreeding increases homozygosity which can lead to the reduced fitness in an individual. To determine the effect of inbreeding on the fitness for each iso-female line, I measured several life history traits to quantify intrinsic stress. The number of nauplii per egg sac, survivorship fourteen days post hatch, and total of each life stage fourteen days post hatch was measured for each inbred line. If inbreeding reduced intrinsic fitness of an iso-female line, I would expect to see smaller numbers of hatching larvae (nauplii) in the first clutch, slower development, and lower survivorship.

For each iso-female line, twenty-four eggs sacs were assayed. To measure the fitness of only the first egg sac of each female, a clasped pair (male and virgin female) was placed into a single well of 24 well tissue culture dish. Once males disengaged, they were removed. The female remained to produce her first egg sac. Upon hatching, the female was removed, the number of nauplii were counted and the date of hatching was recorded. In the event the first egg sac did not mature to hatching in fourteen days, the female was removed and replaced with another male and virgin female pair.

The developing larvae were fed ground *Spirulina* ad libitum for fourteen days. At the end of fourteen days the number of remaining live nauplii, copepodites, and adults were counted. The number of eggs in the first egg sac and the total survivorship of each egg sac for each inbred line was tested for significant differences using a Kruskal-Wallis rank sum test. I conducted pairwise comparisons for differences between iso-female lines using Mann-Whitney U-test and adjusted for multiple comparisons with a Bonferroni corrected P-value. The number of copepods in each life stage was tested for homogeneity using the categorical variable life stage with a Pearson's Chi-square test of homogeneity. A post hoc analysis was conducted to determine which life stages deviate significantly from expected values of homogeneity (Beasley and Schumacher 1995; Agresti 2007). A Bonferroni correction was applied for multiple comparisons.

CTmax assay

To determine upper thermal limit of each iso-female line, I performed a dynamic thermal stress assay using equal numbers of male and female adult copepods. The upper thermal limit was measured as the point at which copepods stopped swimming and responding to external stimuli (Harada, Healy, and Burton 2019; Healy, Bock, and Burton 2019). The day

proceeding the experiment, copepods were moved from beakers into 6 well tissue culture dishes with filtered water and no food to clear their guts overnight. The next day the seawater was removed and replaced with fresh seawater. A single copepod was moved in a 100 ul aliquot of fresh seawater into individual 200 ul microcentrifuge tubes. The volume of 100 ul fills the microcentrifuge tube up to the portion of the tube where the angle changes providing a visibly marked location to check for evaporation. Tubes were left open to allow oxygen exchange and placed randomly in an Applied Biosystems SimpliAmp Thermal Cycler (Thermo Fisher Scientific, Waltham, MA). The temperature of the thermal cycler was held constant at 20°C for 5 minutes followed by an increase in temperature of +0.5 °C/min from 20.1°C to 32 °C, followed by +0.1 °C/min from 32.1°C to 45 °C. Individuals were monitored every 60 seconds for motion, upon cessation of motion, a micropipette was used to produce a jet of water to check responsiveness. In the event of no response, the temperature was recorded as the knockdown temperature;16 males and 16 females were assayed for each iso-female line. Volume of each tube was monitored for evaporation for the duration of the experiment, there was no appreciable level of evaporation witnessed.

The mean upper thermal limit of each line and the stock population culture were compared using a Kruskal-Wallis rank sum test. A pairwise post hoc comparison was conducted using a Dunn test in combination with a Bonferroni corrected P-value for multiple comparisons. A Pearson's correlation was used to detect relationships between extrinsic and intrinsic variables. All statistical analysis was performed in R version 3.6.3 (The R Foundation for Statistical Computing Platform). I expected the thermal phenotypes of most iso-female lines to fall within the range of the parental population.

Results

CTmax

The differences in average CT_{max} by sex in each line were not significant (Mann Whitney U Test; all $P > 0.07$) and therefore males and females within a line were combined as independent replicates. The average inbred line upper thermal limit ranged from 37.8 ℃ to 40.85 ℃, the parental San Roque population averaged 39.3 ℃ (Figure 1-1). There was a significant variation for CT_{max} among all inbred lines and the parental population (Figure 1-1; $X^2 = 205.85$; df = 25; P-value = 2.2e-16). Line 30, the most thermal tolerant line, was the only line significantly more thermal tolerant than the stock San Roque population (Dunn Test with Bonferroni correction; p-value= 0.0017).

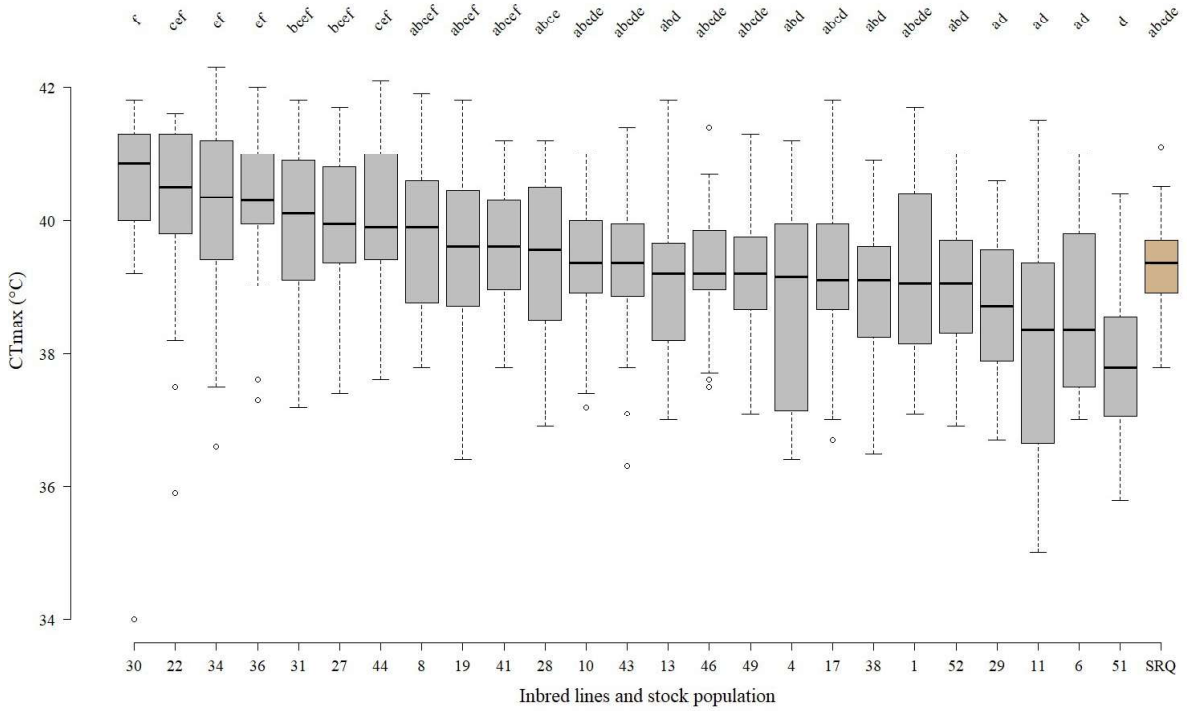

Figure 1-1. Combined male and female CTmax for San Roque inbred lines (gray) and parental population (gold). Data is displayed as standard box and whisker plots. The lowercase letters indicate the post hoc comparisons between groups (n=32 for each).

First clutch size

During three successive rounds of inbreeding, 25 of the initial 54 iso-female lines did not survive or did not maintain population sizes that were large enough to sufficiently assay. The number of nauplii produced in a females first clutch is positively correlated with fitness measures of the female (Barreto and Burton 2015; Powers et al., 2020). I did not observe significant differences in size of the first clutch of the first egg sac (df = 24, p-value = 0.2291). The inbred lines averaged 6.5 eggs to 11.5 eggs per sac (Figure 1-2). I did not observe egg sacs with partial hatching in any of my lines.

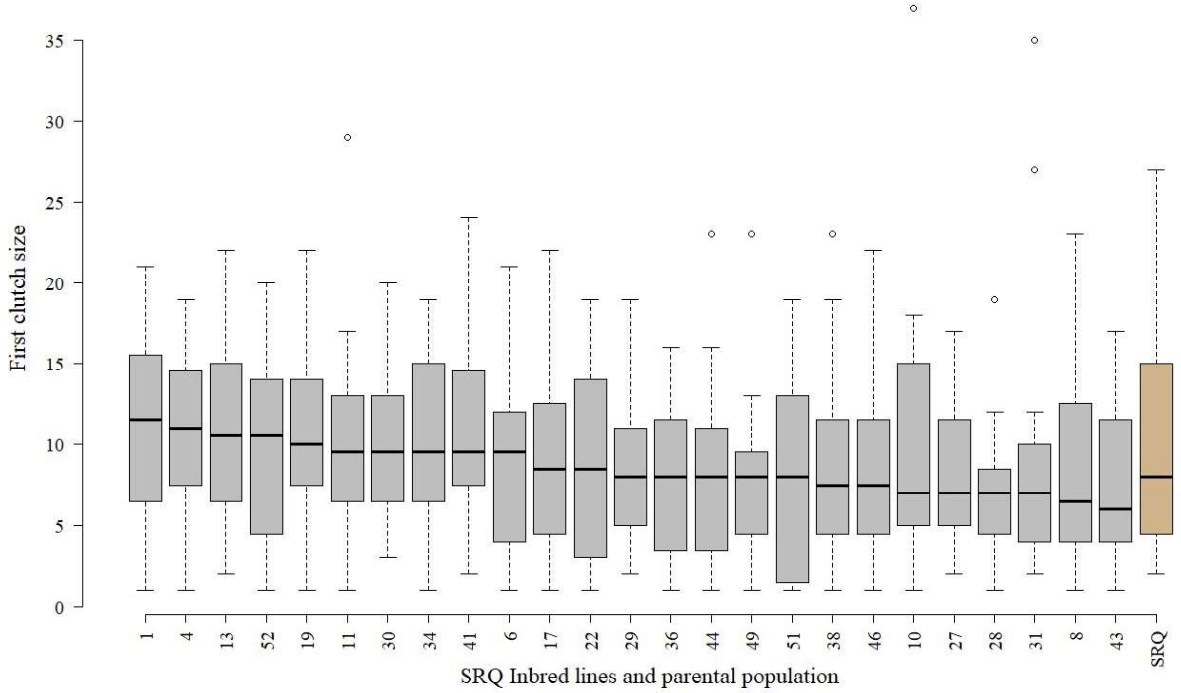

Figure 1-2. The average number of offspring produced in the first clutch of a female copepod. Data is displayed as standard box and whisker plot (n=24 egg sacs each line).

Percent survival and distribution of life stages 14 days post hatch

Inbreeding may reduce fitness and present as reduced survivorship and slow growth rates. The total percent of copepods alive at fourteen days post hatch did not differ significantly between lines (Figure 1-3; df = 24, p-value = 0.33). Grouping all life stages together, the average range of total survivorship at fourteen days post hatch was between 75% and 96%. The *T. californicus* egg to adult life cycle takes roughly 5-6 days in naupliar stages followed by 8-9 days in copepodite stages before reaching the final stage of adult. In a fourteen-day period it could be expected lines showing a reduced growth rate (more intrinsic stress) would present as fewer individuals reaching copepodite stage. The distribution of copepods by life stage in each line was significantly heterogeneous (Figure 1-4; df = 48, p-value < 2.2e-16). Several lines

were significantly different from each other for values in one or two life stages (4,11,22,27,29,30,34,36,38,46 and 52) and one line was different in all life stages (10) (Table 1-1, all P-value < 0.05). Line 10, 27, 36, 38, and 52 all had more nauplii than other lines indicating these lines developed slower. Line 11, 22 ,30, and 46 all showed reduced numbers of nauplii indicating faster development in these lines. Line 4 and 34 both showed faster development to the adult stage indicated by larger number of adults than other lines; line 34 also contained significantly less nauplii than expected (Figure 1-3, Table 1-1).

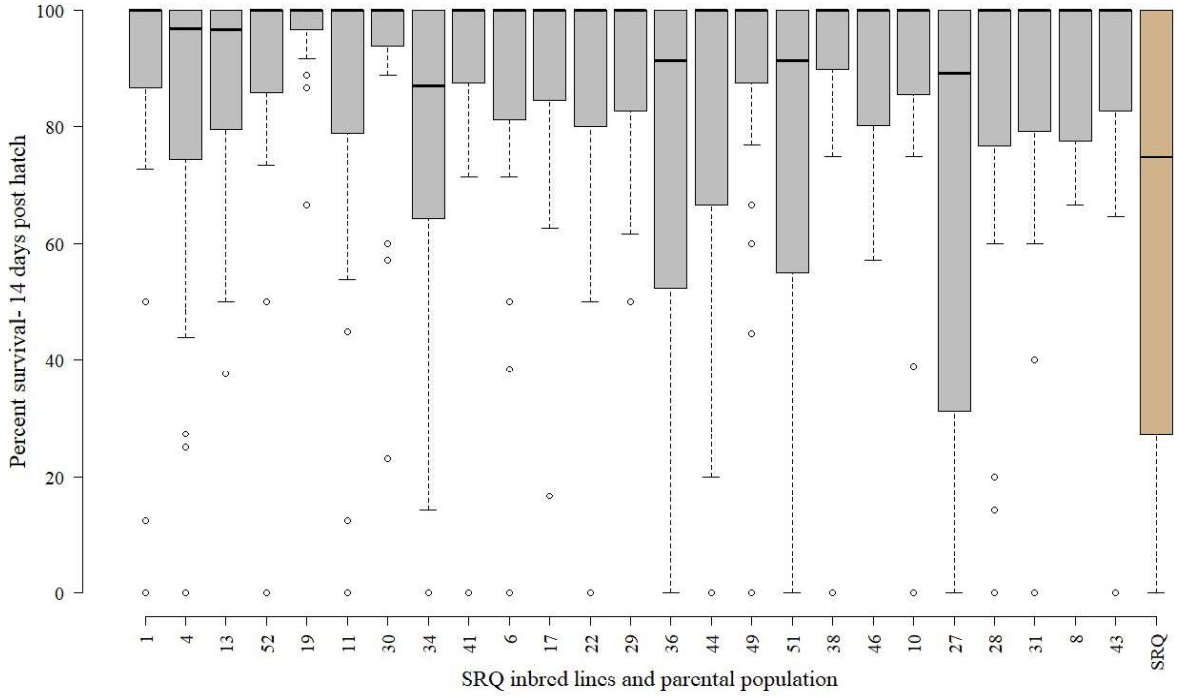

Figure 1-3. The percent survival of all inbred lines (grey) and parental population (gold) fourteen days after hatch, all life stages are combined. Data is displayed as standard box and whisker plots (n=24 egg sacs each line).

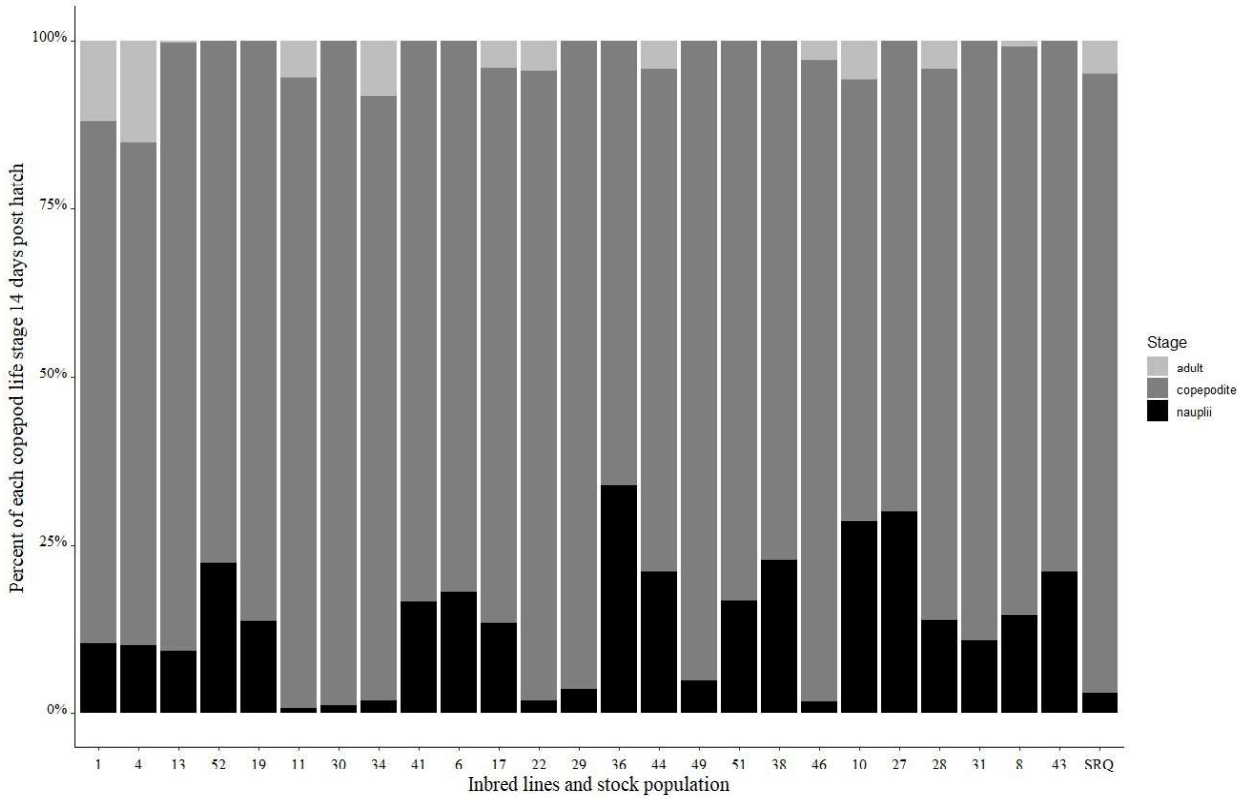

Figure 1-4. The percent of each life stage for inbred lines and parental population fourteen days post hatch. Data is displayed as standard stacked bar chart (n=24 egg sacs each line).

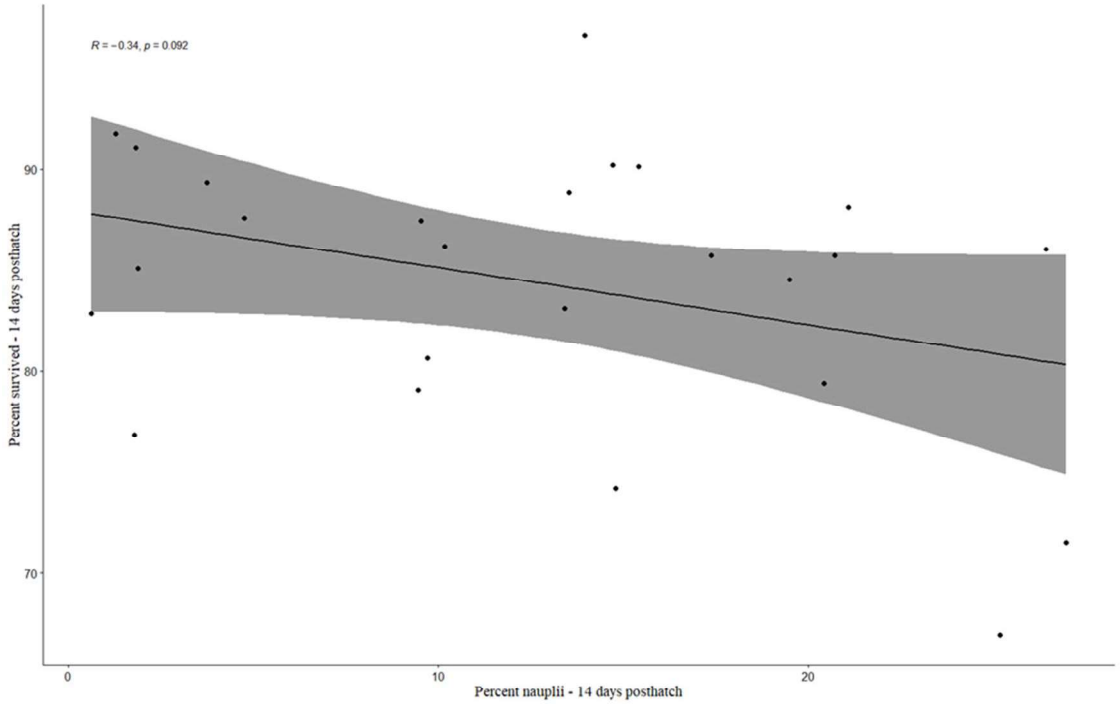

Figure 1-5. Scatterplot representing Pearson's correlation between the percent of nauplii of total copepods survived at fourteen days post hatch and percent of total survivorship of first egg sac. (n=24 egg sacs each line)

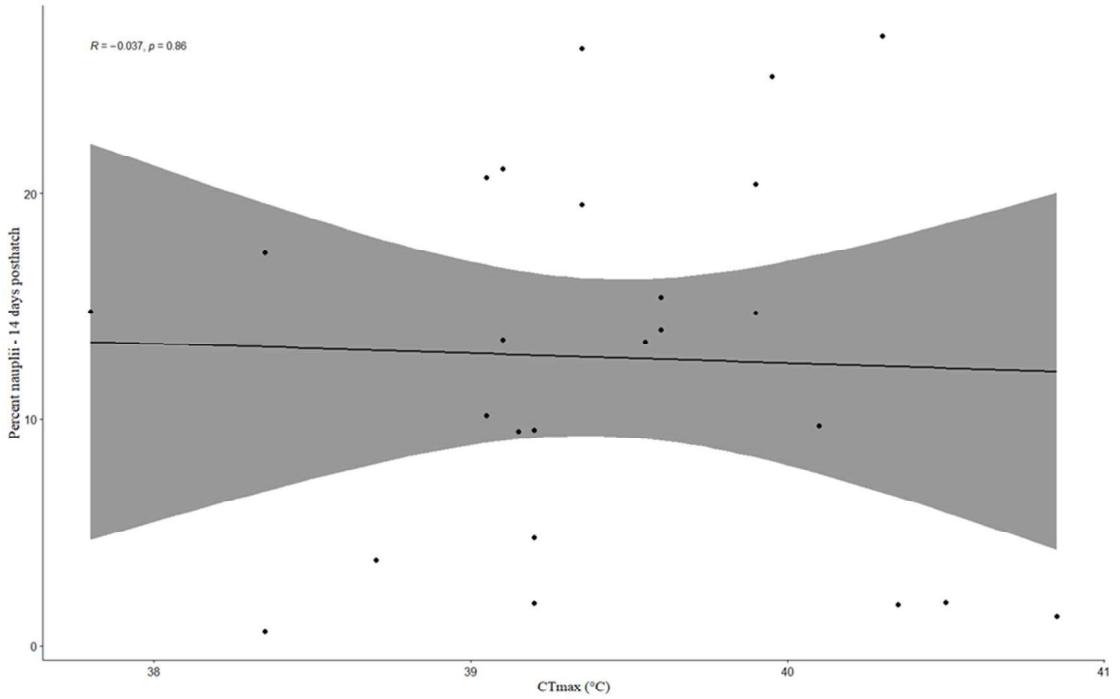

Figure 1-6. Scatterplot representing Pearson's correlation between the percent of nauplii of total copepods survived at fourteen days post hatch and CTmax (°C). (n=24 egg sacs each line).

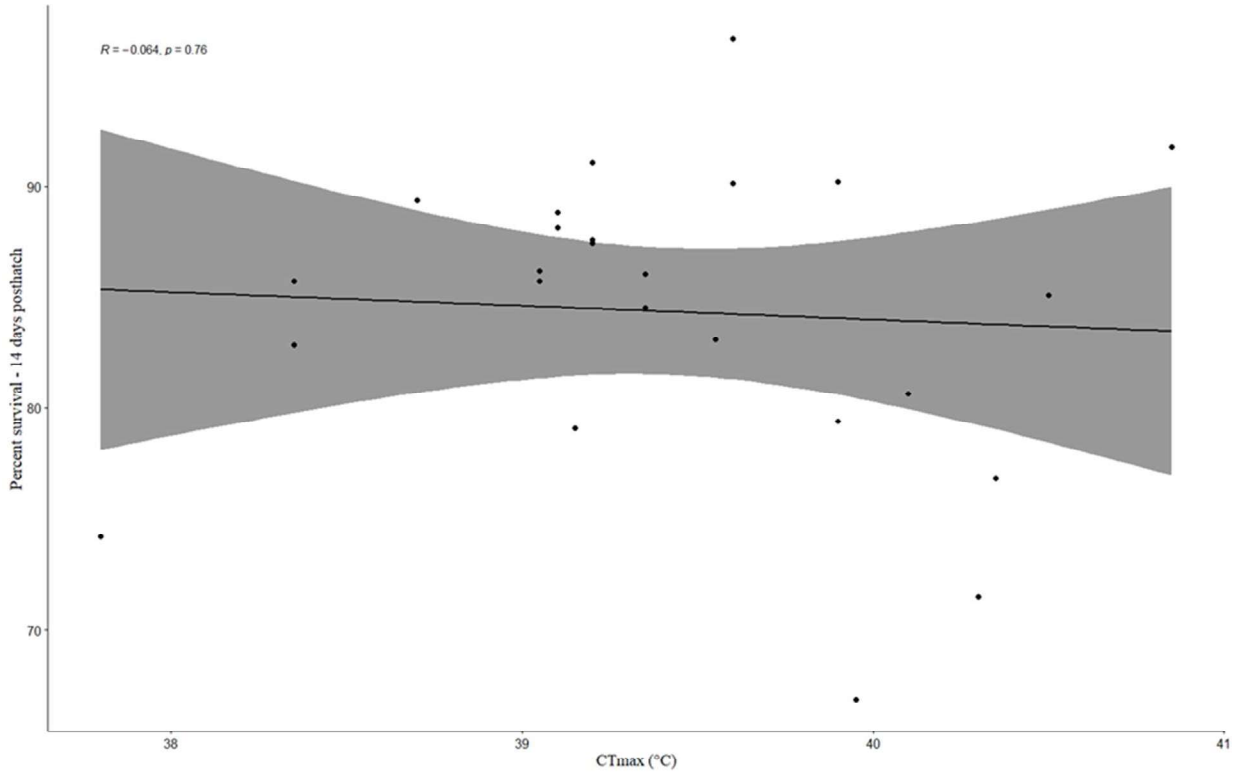

Figure 1-7. Scatterplot representing Pearson's correlation between the percent of copepods survived at fourteen days post hatch and CTmax (°C). (n=24 egg sacs each line).

Table 1-1. Post hoc multiple regression analysis of residuals from Pearson Chi-square test of homogeneity of life stages from inbred lines with Bonferroni correction for multiple tests. Significant heterogeneity in life stages from expected values in grey shading (α=0.05).

	Observed counts			Expected counts			Residuals			P-values		
Line number	nauplii	copepodite	adult	nauplii	copepodite	adult	nauplii	copepodite	adult	nauplii	copepodite	adult
1	24	193	9	33.91	188.22	3.87	-1.89	0.87	2.7	1	1	0.5256
4	15	155	21	28.66	159.07	3.27	-2.83	-0.81	10.1	0.3538	1	**<0.0001**
6	21	170	0	28.66	159.07	3.27	-1.58	2.16	-1.86	1	1	1
8	28	143	1	25.81	143.25	2.95	0.48	-0.05	-1.16	1	1	1
10	79	107	15	30.16	167.4	3.44	9.86	-11.67	6.42	**<0.0001**	**<0.0001**	**<0.0001**
11	2	192	3	29.56	164.07	3.37	-5.62	5.45	-0.21	**<0.0001**	**<0.0001**	1
13	24	215	1	36.01	199.88	4.11	-2.23	2.69	-1.59	1	0.5430	1
17	36	143	4	27.46	152.41	3.13	1.8	-1.9	0.5	1	1	1
19	41	200	0	36.16	200.71	4.13	0.9	-0.13	-2.1	1	1	1
22	5	182	1	28.21	156.57	3.22	-4.84	5.07	-1.27	**0.0001**	**<0.0001**	1
27	46	101	0	22.06	122.43	2.52	5.62	-4.81	-1.63	**<0.0001**	**0.0001**	1
28	18	123	1	21.31	118.26	2.43	-0.79	1.08	-0.94	1	1	1
29	9	162	0	25.66	142.41	2.93	-3.63	4.09	-1.76	**0.0209**	**0.0032**	1
30	4	217	0	33.16	184.06	3.78	-5.63	6.08	-2.01	**<0.0001**	**<0.0001**	1
31	27	149	0	26.41	146.58	3.01	0.13	0.5	-1.79	1	1	1
34	3	164	15	27.31	151.58	3.12	-5.15	2.52	6.93	**<0.0001**	0.8859	**<0.0001**
36	55	101	0	23.41	129.92	2.67	7.2	-6.31	-1.68	**<0.0001**	**<0.0001**	1
38	60	129	0	28.36	157.41	3.24	6.58	-5.65	-1.85	**<0.0001**	**<0.0001**	1
41	41	172	0	31.96	177.39	3.65	1.78	-1.01	-1.97	1	1	1
43	33	129	0	24.31	134.92	2.77	1.95	-1.27	-1.71	1	1	1
44	18	130	5	22.96	127.42	2.62	-1.14	0.57	1.51	1	1	1
46	5	185	4	29.11	161.57	3.32	-4.95	4.61	0.38	**0.0001**	**0.0003**	1
49	24	147	0	25.66	142.41	2.93	-0.36	0.96	-1.76	1	1	1
51	30	127	0	23.56	130.75	2.69	1.46	-0.82	-1.68	1	1	1
52	53	155	0	31.21	173.23	3.56	4.33	-3.47	-1.95	**0.0011**	**0.0397**	1

Discussion

Intrapopulation thermal variation

Standing genetic variation plays a key role in how populations and species will respond to climate change. The present study examined variation for thermal limit in a highly thermal tolerant population of the harpacticoid copepod *Tigriopus californicus*. Surprisingly the average CT_{max} of our inbred lines displayed a temperature range of ~ 3°C between the most and least thermal tolerant lines. The large range of average CT_{max} found in this study is comparable to the overall variation range of thermal limit across the latitudinal distribution of populations (Healy, Bock, and Burton 2019) and the range in thermal tolerance found in other studies of this species (Willett 2010; Kelly, Sanford, and Grosberg 2012; Pereira, Barreto, and Burton 2014; Pereira, Sasaki, and Burton 2017; Foley et al. 2019). The range of thermal limit of our lines fell largely within the range of the thermal limit for the parental population with one line exceeding the parental phenotype (Figure 1-5). This result supports the usefulness of our procedure to segregate the genetic variation for thermal limit in this population.

A useful tool to study the allelic variation in a population for a polygenic trait is the creation of iso-female lines (David et al. 2005; Rolandi et al. 2018). This process uses inbreeding to force homozygosity at genic loci leading to random fixation of the population's alleles into individual lines. This process is not without its limitations, homozygosity across the genome increases the chance for fixation of deleterious alleles leading to poor fitness. Measures of intrinsic stress are important to tease apart reduced overall fitness (measured by life history traits) from reduced environmental stress phenotypes.

Effects of Intrinsic selection stress from inbreeding

Inbreeding increases homozygosity across the genome and frequently reduces fitness in offspring, often witnessed by reduced growth rates, survival, and fecundity (Charlesworth and Charlesworth 1987; Charlesworth and Willis 2009). As recessive alleles are more frequently expressed due to rising homozygosity they may be removed from the population (Hedrick 1994; Glémin 2003; Xue et al. 2015). Removal or purging of deleterious alleles may allow a population to return to an optimal fitness level (Larsen et al. 2011). The size of the population influences the speed of inbreeding, small populations have a faster rate of inbreeding than large population (Reed and Frankham 2003). Small populations are greatly affected by random genetic drift which also increases the rate of negative fitness consequences due to inbreeding (Pekkala et al. 2012). In our study, approximately half of the lines did not survive three generations of inbreeding (25 of 54 survived). The high number of lines lost in this study is most likely due to the method used to create the inbred iso-female lines. Using only a single clasped pair to begin each generation represents a very small starting population size in which I would expect the speed of inbreeding to be very rapid. Every inbred line and successive generation were kept under the same temperature, light, and food regime; I can infer the lines lost were due to increased homozygosity resulting in reduced fitness and termination of those lines. In the lines that did survive, some of the negative effects of small population size were most likely offset by selecting the first clasp pair in each generation. Selecting the first clasped pair to create the next generation biased for faster developing individuals from each egg sac. The most fit siblings in each generation would be expected to mature faster which points to a lower possibility of carrying highly deleterious alleles.

The twenty-five surviving lines were similar in percent survivorship fourteen days after hatching (Figure 1-1) but some differed significantly in development rates of naupliar life stage (Figure 1-3, Table 1-1). These two measures of intrinsic stress are weakly negatively correlated but not significantly ($r = -0.34$, $p = 0.092$). The diminished rate of development in some lines could be due to alleles that are mildly deleterious but are not affecting overall survival. The long-term repercussions of these genetic combinations may lead to lower population growth rates in the slower developing lines since it would take longer for them to become reproductively mature. Interestingly there was no correlation between development time or survival with thermal limit ($r = -0.064$, $p = 0.76$; $r = -0.037$, $p = 0.86$). The fitness cost of increased thermal limit does not appear to have tradeoffs with development or survival. This result bodes well for the adaptability of this population. If increased thermal tolerance comes with a high fitness cost that would further compound the stressors on individuals in the population and likely lead to reduced ability to evolve and adapt to environmental change (Bridle et al. 2010; Hoffmann and Sgró 2011; Hackett and Bonsall 2016).

Variation for CTmax

The capacity for populations to display plasticity in environmentally relevant traits is an important key in our understanding of their ability to respond to rapid environmental shifts and give hints at the genetic variation within a population. Phenotypic plasticity can enhance survival during seasonally predictable unfavorable conditions but this may come at a cost by limiting a populations ability to adaptively evolve when conditions are unpredictable due to climate change (Hendry 2016; Oostra et al., 2018; Scheiner et al., 2019). The San Roque population was shown to demonstrate a modest degree of adaptive plasticity for increased thermal tolerance (+1.34°C) and a positively sloped thermal reaction norm when raised at 25°C

vs 20°C (Pereira, Sasaki, and Burton 2017). Regional populations, those within 10 km of San Roque, also display moderate amounts of plasticity (0.5-1.0°C) for increased thermal tolerance (Kelly, Sanford, and Grosberg 2012). The increased temperature tolerance due to a plastic response found by Pereira et al. (2017) is greater than that found in regionally close populations by Kelly et al. (2012) but it is much smaller than the interpopulation difference of 3.83 °C due to genetic variation (Pereira, Sasaki, and Burton 2017). The positive slope of the San Roque thermal reaction norm and no discernable reduction in fitness indicate little to no cost for increased thermal tolerance from plasticity. Intertidal animals experience a thermally heterogenous habitat that may promote thermal plasticity selection. Even subtropical and tropical intertidal animals, where thermal regimes are considered stable for terrestrial animals, experience thermal selection that is similar terrestrial temperate zones (Tomanek and Helmuth 2002; Somero 2010; Gareth et al., 2011;. Brahim et al., 2018). Furthermore, genetic variation is a minimum requirement to maintain phenotypic plasticity by natural selection (Gotthard and Nylin 1995; Dewitt et al. 1998; Gomez-Mestre et al., 2013).

Rocky intertidal habitats are a mosaic of interconnected tide pools that vary in size and shape creating many microhabitats in a relatively small geographic area. Temperature in tidepools can increase quickly during warm months but not all pools at a site will heat at the same rate or to the same temperature. The depth, solar orientation, prevailing winds, presence of ledges, deep cracks, and width are some variables that vary within an outcrop of tidepools creating unique habitat in each pool. When living in such heterogenous habitats, populations have been shown to persist with high levels of genetic variation because selection pressures are heterogeneous (De Jong and Gavrilets 2000; Agashe 2009). Results of our study demonstrate the sizeable amount variation for CTmax between lines is most likely explained by genetic

variation for thermal tolerance in this subtropical population. The polygenic nature of environmental thermal response systems allows a population to produce a wide variation of response with moderate genetic variation. Polygenic traits can be composed complex genetic architectures that add up to different values for the trait depending on the polymorphisms in the population (Zan et al., 2017).

Recombination during sexual reproduction creates genetic variation with every generation as it breaks up linkage on chromosomes. Experiments involving strong selection pressures such as LT50 may remove a large portion of genetic variation from a study. These studies are useful but can be limited in interpretation of population response. The study population may not have sufficient time to produce useful genetic combinations for environmentally relevant traits from the starting variation because these traits are composed of many loci of small effect. If known, one must consider the rate of recombination in the study system. The rate of recombination will affect the speed at which variation can be generated (Battagin et al., 2016). Slower recombination rates produce genetic variation slower than faster recombination. *Tigriopus californicus* is a species where an understanding of recombination is important in the design of experiments. In this species, meiotic recombination only occurs in the male sex effectively reducing recombination events by 50% (Ar-rushdi 1962; Burton, Feldman, and Swisher 1981).

Based on their results from selection experiments, Kelly et al. (2012) concluded that *T. californicus* populations show only limited response to future climate change. However, the use of extremely strong selective pressure (50% mortality per generation) and small laboratory populations may underestimate evolutionary potential. These type of tests are informative because extreme events are predicted to increase in the future with climate change (Dahl et al.,

2019). Extreme heat events, which are most likely to happen during the summer months, can lead to reduced population numbers. The remainder of the year is less likely to have such extreme heat events and during this time the population will undergo multiple generations of recombination (as discussed above) and mutation to replenish variation lost during extreme events. Although recombination of standing genetic variation is likely a more important contributor to genetic and phenotypic variance, novel mutations should not be totally discounted given the natural populations of *T. californicus* are very large, frequently exceeding 10^5 -10^6 in most local populations and the polygenic nature of traits like thermal tolerance provide many targets for potentially adaptive mutations (Weber and Diggins 1990; Weber 1990). Both mutation and recombination were severely restricted in the Kelly et al. (2012) selection experiment.

Historical trends also show the average daily temperature is increasing causing a different regime of temperature selection on populations (IPCC 2018). Studies demonstrate *T. californicus* can increase thermal tolerance in a single generation due to plasticity alone (Kelly, Sanford, and Grosberg 2012; Pereira, Sasaki, and Burton 2017; Healy, Bock, and Burton 2019). Temperature means fluctuate from year to year, in times of higher daily averages phenotypic plasticity may provide a buffer to the population for them to recover. In this study I demonstrate the San Roque population, one of the most thermal tolerant in the *Tigriopus californicus* range, contains a sizeable amount of genetic variation when measured using inbreeding to segregate genetic variation for thermal limit. Contrary to the interpretations from extreme selection pressure over multiple generations for a duration of less than a year, the results of this study coupled with the plastic thermal response from Pereira et al. (2017) suggest there is sizeable genetic variation for thermal adaptation in this subtropical population.

Conclusion

This study demonstrates the utility of creating iso-female lines to experimentally quantify population variation for environmentally important adaptive traits. *Tigriopus californicus* populations span a large latitudinal gradient of temperature and are locally adapted to the environmental conditions in which each population is found. Each location varies in the composition of tidepools and environmental variables on a macroscale (latitudinal distribution) and microscale (single location). To predict future response to climate change for this intertidal species it is important to understand intrapopulation adaptive genetic diversity to better forecast future population response. I show this subtropical population of *Tigriopus californicus* does contains significant additive genetic variation for thermal limit. The line with the higher thermal limit (30) developed faster than many of the other lines showing that increased thermal tolerance does not appear to come at increased intrinsic stress. It still needs to be determined if the patterns I found in this population are similar in other populations in the latitudinal range.

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Chapter 1, in full is currently being prepared for submission for publication of the material. Blackwell, Reginald C.; Burton, Ronald. The dissertation author was the primary investigator and author of this material.

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Chapter 2

Positive and negative transgressive segregants for maximum thermal limit in interpopulation crosses of *Tigriopus californicus*

Introduction

Hybridization plays a major evolutionary role in the creation of genetic and phenotypic diversity in plants and animals (Barton and Hewitt 1985; Barton 2001; Mallet 2007; Abbott et al., 2013). Through sexual reproduction, genomes of two divergent species or populations are combined in one organism producing new genetic combinations. Evolution can occur quickly through hybridization by increasing variation from new genetic combinations faster than the increase in variation from mutations alone (Grant and Grant 2019). New combinations in the first-generation hybrids (F1) normally display phenotypes intermediate between the parentals; since F1 hybrids contain a full haploid complement of both parental genomes, this often allows coadapted genomic complexes to function properly. In some cases, the fitness of F1 hybrids may differ from the parents. Increased fitness or hybrid vigor can be the result of masking of deleterious recessive alleles from the parental populations, overdominance from heterozygote advantage, or positive epistatic interactions. The vigor witnessed in the F1 generation is normally transient and lost in successive generations when new genetic combinations are formed during recombination. A reduction in fitness due to genetic mismatches (intrinsic fitness) or phenotypic mismatch with the environment (extrinsic fitness) is also possible but is more frequently witnessed in the F2 and later generations also due to recombination.

Recombination may form genetic novelty in F2 and later generation hybrids, also known as transgressive segregants (Rieseberg, Archer, and Wayne 1999; Soltis 2013; Nieto Feliner et al., 2017). Transgressive segregants produce phenotypes that are more extreme than either of the parents. The novel phenotypes of transgressive segregants have not been tested by selection in the environment of either parent. The hybrids may outcompete the parents for resources or be able to occupy a space that is unusable to the parents (Stelkens et al., 2014). The movement into

regions inaccessible to the parents solidifies the hybrid genotype by removing instances of backcrossing with the parents and can lead to speciation of the transgressive segregants (Seehausen 2004; Keller and Seehausen 2012; Dittrich-Reed and Fitzpatrick 2013). If hybrids outcompete one or both parental species it may lead to the extinction of one or both parents (Rhymer and Simberloff 1996; Todesco et al. 2016). Transgressive hybrids from population crossing has been proposed by some researchers to increase environmental tolerance of some species that might be threatened by rapid climate change (Nicotra et al., 2015). Hybridization events may become more common as the environmental conditions rapidly change, increasing the likelihood of divergent populations coming into contact.

Unlike heterosis, which is frequently restricted to the F1 hybrid generation, the extreme phenotypes of transgressive segregants are heritably stable (Lewontin and Birch 1966; Rieseberg et al., 1999). Transgressive segregation is witnessed in the F2 and later generations due to recombination and segregation of standing variation from the parental populations in hybrids lineages. This genetic phenomenon has been more widely studied in plants but is starting to be found more frequently in animals (Rieseberg et al., 1999; Stelkens and Seehausen 2009; Dittrich-Reed and Fitzpatrick 2013). The type of traits in which transgression segregants are found in hybrids are generally any quantitative trait such as stress response, behavior, growth rate, and morphology. Quantitative genetic research of transgressive segregates has shown the predominant mechanisms underlying the extreme phenotypes are due to epistasis, complementary gene action, or a combination of the two (Rieseberg et al. 1996; Rieseberg et al., 1999; Stelkens et al., 2009; Dunn et al., 2013; Reyes 2019). In one instance, unmasking of recessive alleles was shown to produce a transgressive phenotype (Rick and Smith 1953). Mutation has also been shown to contribute to transgressive phenotypes by activating a

transposable element in a *Drosophila* study (Engels 1983). The last two mechanisms do produce transgressive segregants but are generally rare in the literature; complementary gene action and epistasis are believed to be the main mechanisms underlying transgressive segregation (Rieseberg et al. 1999).

Epistasis is a phenomenon that occurs when a genes effect on phenotype is dependent on the presence or absence of one or more genes at different locations in the genome (Phillips 2008). In general epistasis is not considered to contribute as often as complementary gene action to the production transgressive segregants but this may have to do with the difficulty of finding evidence of epistasis (Ehrenreich 2017). However, several recent studies have been able to attribute epistasis as one of the mechanisms leading to transgressive segregants in plant hybrids (Rowe et al., 2008; Mao et al., 2011; Koide et al., 2019). Complementary gene action is frequently found to be the mechanism of extreme phenotypes in hybrids due to antagonizing quantitative trait loci found in the parents (Rieseberg et al., 1999; Rieseberg et al., 2003). A simple model for transgressive segregant is a phenotype produced from five loci, one parent carries three negative (---) and two positive loci (++), the other carries three positive (+++) and two negatives (--) for the trait. The hybrids, by chance, may contain only positive loci (+++++) or negative loci (-----) for the trait, producing an extreme value.

Many genes respond to the environment to maintain function in an organism. Random mutations can lead to changes in these genes that may increase/decrease function or have no perceivable effect at that current time during the conditions that are experienced. When populations are not exchanging alleles frequently, they can diverge due to random mutations and the effects of genetic drift over time. Genetic mechanisms that have evolved in one population may not be the same that have evolved in another population even if they are undergoing similar

selection. In other words, even if both populations express similar phenotypes, they may evolve different underlying mechanisms (Manceau et al. 2010).

Most environmentally relevant traits are polygenic, composed of many loci of small effect. It is reasonable to assume hybrids between geographically close populations that are not frequently exchanging alleles would be expected to produce transgressive segregants for thermal tolerance. Populations with large differences in thermal phenotypes are most likely diverging under different selection pressures and the combination of these thermal phenotypes in hybrids is expected to be intermediate of the parental populations (Rieseberg et al., 1999). Hybrids may also display reduced fitness from breaking up coadapted gene complexes creating outbreeding depression in interpopulation crosses and this could lead lower thermal tolerance in hybrids (Edmands 2007; Pekkala et al., 2012).

The intertidal copepod *Tigriopus californicus* is an ideal system to study the effects of hybridization on extrinsic responsive phenotypes (i.e., maximum thermal limit). This species lives in splash pools from Baja California, Mexico to the southern tip of Alaska resulting in varying degrees of ecological and genetic divergence across its range (Edmands 2001; Willett and Ladner 2009; Peterson et al. 2013; Barreto et al. 2018). Populations are locally adapted to the environmental conditions in which the population is found and do not diapause to escape unfavorable conditions (Dethier 1980; Dybdahl 1994; Burton 1997). Due to the size of the *T. californicus* distribution, populations span a large latitudinal thermal range and biogeographical regions with several populations in the same region (Blanchette et al. 2008; Willett 2010; Peterson et al. 2013; Healy, Bock, and Burton 2019). Each region is delimited by the similar oceanographic conditions and the geographical variation of the communities of species (Blanchette et al. 2008).

In previous work, Pereira et al. (2014) found that hybrids of *Tigriopus californicus* from either the same region or from distant regions produced F1 hybrids that displayed parental level or somewhat increased thermal tolerance indicating heterosis. However, while the hybrids between geographically close and ecologically similar populations maintained a level of thermal tolerance greater than the parents in isofemale lines at the F9 stage, the geographically distant and ecologically dissimilar cross did not show such transgressive phenotypes. These results suggest that although phenotypically similar, the ecologically similar populations apparently evolved different mechanisms for mitigating thermal stress; when combined they produced transgressive segregants, most likely from complementary gene action. Notably, this study only carried out a single cross of each type - ecologically similar populations and ecologically dissimilar populations.

In this study, I expand the work of Pereira et al. (2014) and carry out several crosses between *Tigriopus californicus* populations within the same biogeographic region and between distant biogeographic regions. The crosses were performed reciprocally to track mitochondrial heritage. I tested (1) whether F7+ recombinant inbred lines displayed thermal limits (extrinsic fitness) outside of the average range of the parental population (negative or positive transgression), (2) whether fitness of recombinant inbred lines was higher or lower within a cross. I found that hybridization within a biogeographic range produced transgressive hybrid lines, both negative and positive transgressive segregants. Hybridization between distant biogeographic zones did not produce transgressive segregants and most hybrid crosses were unable to be maintained.

Methods

i-Button tidepool temperature monitoring

Maxim Integrated™ i-Button models DS1922L and DS1921G were deployed in tidepools containing *Tigriopus californicus* at three California locations of San Diego, Bird Rock, and Santa Cruz. The temperature loggers were coated in PLASTI DIP rubber sealant to waterproof and attached to the rock surface with Splash Zone epoxy. The San Diego and Bird Rock tidepool locations were monitored with two of each of DS1921G and DS1922L i-Buttons. The Santa Cruz tidepool temperatures were measured with 4 DS1922L i-Buttons. The i-Buttons were programmed to record temperature every 17 minutes with a resolution of 0.5 °C, upon completion of designated recording period each logger was removed and replaced with a new logger. The DS1922L were collected and replaced ~ 96 days and the DS1921G were collected and replaced ~ 24 days.

Tigriopus californicus collection and maintenance

Copepods were collected from high intertidal rock pools at 6 locations stretching ~820 km of latitudinal distance from Pescadero, California to La Bufadora, Baja California Sur, Mexico. The locations consisted of 6 from California (Pescadero, Santa Cruz, Catalina Island, Abalone Cove, Bird Rock, and San Diego) and 1 from Baja California, Mexico (La Bufadora). Populations were maintained in 400 ml beakers with ~250 ml 0.2um filter seawater at 34 ppt and fed dried *Spirulina* powder ab libitum. The beakers were housed in incubators with a 12-hour light and 12-hour dark photoperiod at a constant temperature of 20 ℃. Monthly 1/3 volume seawater changes were conducted, at this time single population beakers were mixed to maintain similar genetic composition among a population distributed across multiple beakers. All populations were housed in incubators for thirty days (approx. one full generation) to remove environmental acclimation before conducting any experiments.

Male *Tigriopus californicus* are identified by their distinct geniculate antennae which they use to clasp immature virgin females. Upon female maturity the pair copulates, the male disengages to have repeat mating events with other virgin females. Females of this species mate only once allowing easy construction of crosses of between genetically distinct populations in which mitochondrial heritage can be tracked (Egloff 1966, Burton 1985). Females can produce as many as 20 eggs sacs over her reproductive lifetime from one copulation event. The females typically carry their egg sacs until they hatch, and each egg sack can vary in quantity from as low as 1 egg to more than 100 (Egloff 1967, Vittor 1971). After hatching, development to an adult copepod takes approximately 3-4 weeks at 20^{o}C. The entire life cycle consists of six naupliar stages followed by 5 copepodite stages concluding in the final adult stage and sexual maturity (Huizinga 1971; Powlik 2000). Male and female copepods are visually indistinguishable until adult stage when males develop geniculate antennae and female display dark greenish to black striping indicating gonadal maturation (Vittor 1971; Burton 1987).

Fitness of parental populations

CTmax assay (extrinsic stress)

To determine upper thermal limit of each parental population, I performed a dynamic thermal stress assay using equal numbers of male and female adult copepods. The upper thermal limit was measured as the point at which copepods stopped swimming and responding to external stimuli, also known as the knockdown temperature (Harada, Healy, and Burton 2019; Healy, Bock, and Burton 2019). The day preceding the experiment, copepods were moved from beakers into 6 well tissue culture dishes with filtered water and no food to clear their guts overnight. The next day the seawater was removed and replaced with fresh seawater. A single copepod was moved in a 100 ul aliquot of fresh seawater into individual 200 ul microcentrifuge

tubes. Tubes were left open to allow oxygen exchange and placed randomly in an Applied Biosystems SimpliAmp Thermal Cycler (Thermo Fisher Scientific, Waltham, MA). The temperature of the thermal cycler was held constant at 20℃ for 5 minutes followed by an increase in temperature of +0.5 ℃/min from 20.1℃ to 32 ℃, followed by +0.1 ℃/min from 32.1℃ to 45 ℃. Individuals were monitored every 60 seconds for motion, upon cessation of motion, a micropipette was used to produce a jet of water to check responsiveness. In the event of no response, the temperature was recorded as the knockdown temperature;16 males and 16 females were assayed for each population.

Clutch size and development (intrinsic stress)

I measured several components of fitness of the parental populations to understand the health of the populations before hybridization. The measures examined were the number of nauplii per egg sac, survivorship fourteen days post hatch, and percent of each life stage of the survivors fourteen days post hatch. The number of eggs produced in the first egg sac and survivorship of the first clutch fourteen days are good predictors of health of later life stages (Powers et al., 2020).

For each parental population, twenty-four eggs sacs were assayed. To measure the fitness of only the first egg sac of each female, a clasped pair (male and virgin female) was placed into a single well of 24 well tissue culture dish. Once males disengaged, they were removed. The female remained to produce her first egg sac. Upon hatching, the female was removed, the number of nauplii were counted and the date of hatching was recorded. In the event the first egg sac did not mature to hatching in fourteen days, the female was removed and replaced with another male and virgin female pair. The developing larvae were fed ground *Spirulina* ad libitum for fourteen days. At the end of fourteen days the number of remaining live nauplii, copepodites,

and adults were counted. The rate of development is a plastic trait and can be sped up or slowed down depending on the temperature at which copepods are maintained (Pereira et al. 2016; Healy, Bock, and Burton 2019). Because of this all cultures were maintained in the same incubator to maintain common conditions between them.

Recombinant inbred line creation

Population crossing scheme was designed after measuring the CT_{max} of each of the six parental populations. The differences in tolerances between populations were used to place crosses into three tolerance category bins: low = 0.0 to 0.5, medium = 0.51 to 0.99, high = > 1.0 °C. Latitudinal distance between populations was measured with Google Earth. To produce each reciprocal cross, clasped pairs were removed from stock 400 ml beakers and separated into petri dishes containing only males or only virgin females for each population. Populations were crossed by placing 20 males from one population and 20 females from the other population into new petri dishes with ~30 ml of filtered seawater and dried *Spirulina*, this process was replicated for a second petri dish for a total of 40 males and 40 females for each cross. Copepods were allowed to pair, and dishes were checked daily; upon the appearance of gravid females the males were removed, the females were maintained to produce several egg sacs and were removed once F_1 offspring were visible (copepodite stage). When F_1 hybrids produced breeding pairs, the pairs were teased apart and placed into a new dish with the opposite sex from the second F_1 petri dish to prevent inbreeding. Hybrids were maintained in Petri-dish mass cultures with discrete non-overlapping generations until the F_3 generation. When F_3 females became gravid, they were isolated in a 6 well tissue culture dish (Corning Costar nontreated) with ~10 ml of seawater and ground *Spirulina* to begin an iso-female recombinant inbred line (RIL). Fifty recombinant inbred lines were created for each reciprocal cross, each from a single F_3 gravid female. In the event an

egg sac did not hatch or produced less than 5 nauplii, the female was removed and replaced with another gravid F_3 female from that cross. Wells were monitored daily for hatching and development; upon the presence of nauplii the female was removed, and the well was continually monitored for the appearance of clasped pairs. The first clasped pair that appeared in a well was moved to a new well, supplied dried spirulina and filtered seawater to create the next generation from a single clasped pair. Although not a goal of the study, this process does selects for the fastest developers and is likely to select for the healthiest individuals from each clutch. I repeated this process for three generations of inbreeding. The F_7 generation of copepods were moved to a 400 ml beaker with 200 ml of seawater and dried Spirulina to allow expansion of a line to greater numbers. Generations were allowed to overlap as numbers increased in order to perform intrinsic stress and extrinsic stress assays. The intrinsic and extrinsic measurements of RILs were conducted in the same manner as the parental populations (description above).

Statistical analysis

The parental populations phenotypic range were defined as the highest and lowest value of CTmax plus the standard error of the two parental populations used to create hybrid crosses. The RILs were considered to display a transgressive phenotype if the mean CTmax and standard error did not overlap the range of the parental populations. Statistical analysis was conducted on the closest parental population value to the RIL value. A RIL that displayed a transgressive phenotype higher than both parents was compared to the highest parent value, a RIL that showed a transgressive phenotype lower than the parental range was compared to the lowest parent from the parental range. The CTmax of males and females in each population and RIL were tested in pairwise comparisons for differences in thermal limit due to sex using a Mann Whitney U-test. All comparisons of RIL and parental upper thermal limit were made using a Kruskal-Wallis rank

sum test. A pairwise *post hoc* comparison was conducted using a Dunn test in combination with a Benjamini-Hochberg procedure for false discover rate (FDR) corrected P-value for multiple tests.

Similar to the CTmax of the parental lines, the mean and standard error of the number of eggs in the first egg sac of each female from both populations were used to determine the parental range. The average percent survival fourteen days after hatching and the standard error of the parental populations was used to define the parental range of survivorship. Significance for the number of eggs in the first egg sac in each RIL and percent survivorship fourteen days post hatch were compared to the highest or lowest parental value depending on the direction of transgression of the RIL using a Kruskal-Wallis rank sum test. I performed a Dunn test for post hoc pairwise comparisons adjusted for multiple comparisons using an FDR corrected P-value. The number of individuals of each life stages in a RIL was tested for differences between RILs within a cross with a Chi-square test of homogeneity. In the event of significant deviation in homogeneity within a cross was found, a post hoc analysis was conducted by multiple regression of the Chi-square residuals with a Bonferroni corrections was applied to determine which line and life stage deviated significantly (Beasley and Schumacher 2007; Agresti 2007).

A Spearman correlation was conducted using extrinsic fitness (CTmax) measures and intrinsic fitness measures (percent survival/percent nauplii) to determine if there was correlation between these different measures of fitness of RILs. All statistical analyses were performed in R version 3.6.1 (The R Foundation for Statistical Computing Platform).

Results

i-Button data

A seasonal warming trend is present during the summer months in San Diego (SD), Bird Rock (BR), and Santa Cruz (SCN). The Santa Cruz tidepools displayed no days of minimum daily temperatures above 20°C. Temperature recordings indicate the San Diego tide pools are warmer than the Bird Rock location even though they are only 8 km apart. In comparison to the San Diego and Bird Rock locations, the Santa Cruz location rarely warmed past 36 °C during this time period. The temperatures experienced during the year may explain the difference in CTmax for SCN in comparison to BR and SD (Figure 2-1 and Figure 2-2).

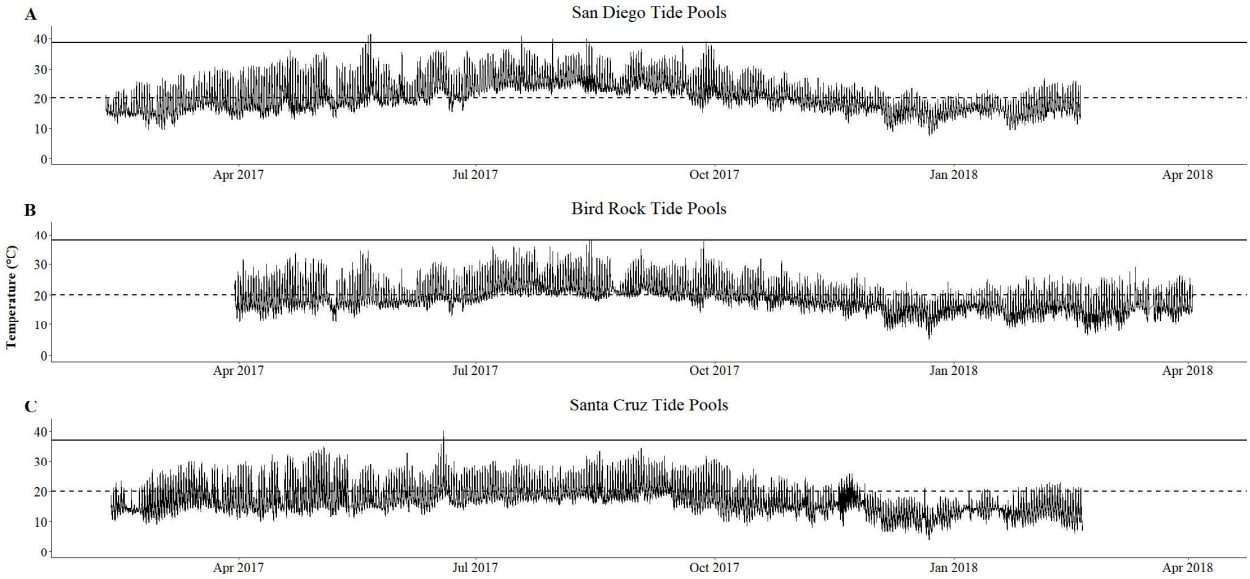

Figure 2-1. Temperature recordings at three *Tigriopus californicus* tidepool rock outcrop locations in California. The solid line denotes the average CTmax for the population and the dashed line indicates the incubator temperature where copepods are housed.

CTmax

Parental populations

Similar to other thermal tolerance studies of populations across several degrees of latitude in this species (Willett 2010; Pereira et al. 2016; Healy, Bock, and Burton 2019), the populations displayed a temperature phenotype of increasing tolerance with decreasing latitude (Figure 2-2). There were substantial differences in variation between populations (P-value < $2.2x10^{-16}$; Figure 2-2), although there was no significant difference in CT_{max} variation between the sexes within a population (all P-value > 0.08).

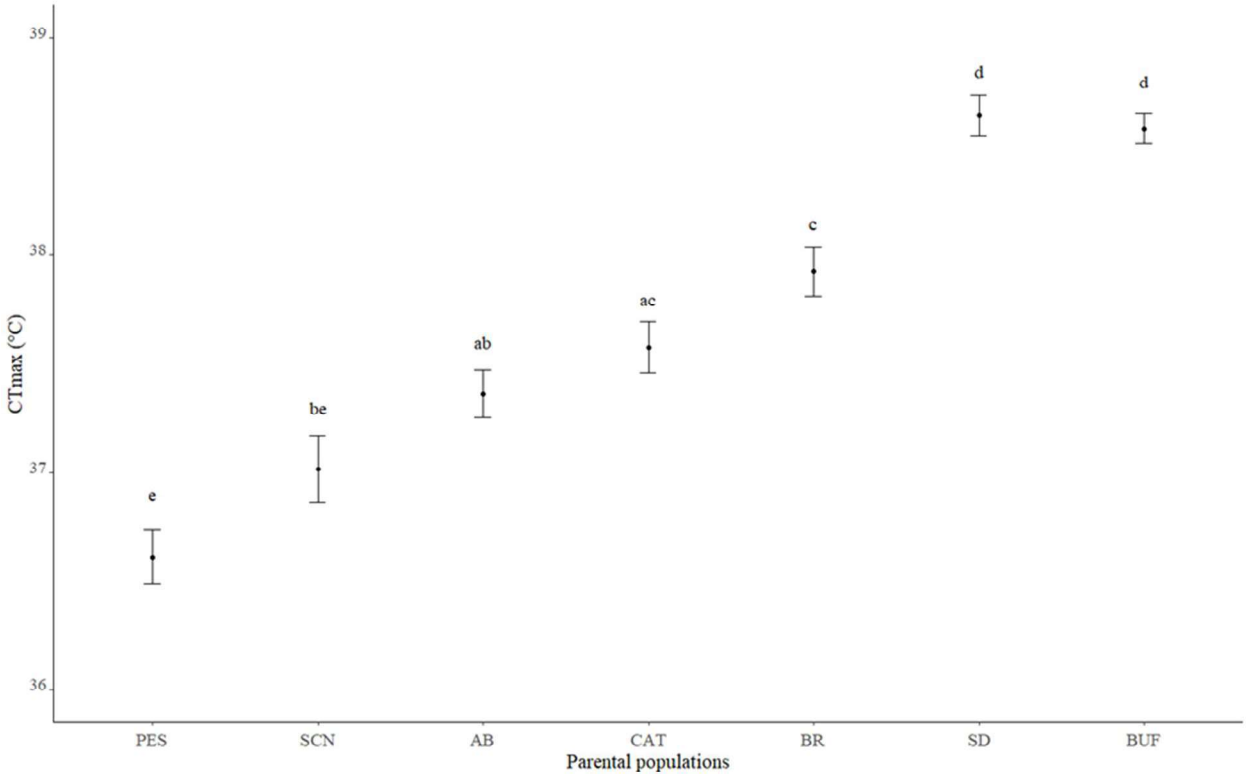

Figure 2-2. The CT_{max} with standard error bars of the parental populations from northern to southern locations from the left to right. The distribution of CT_{max} among populations follows an inverse relationship of decreasing thermal tolerance with increasing latitude. Lowercase letters above error bars indicate post hoc results from population comparisons (n=32 for all populations).

Table 2-1. The CTmax of each population separated by sex from northern to southern locations, top to bottom.

Population	Sex	N	CTmax	sd	se	ci
PES	female	16	36.68	0.62	0.15	0.33
PES	male	16	36.54	0.79	0.2	0.42
SCN	female	16	37.03	0.88	0.22	0.47
SCN	male	16	37.01	0.87	0.22	0.46
AB	female	16	37.62	0.51	0.13	0.27
AB	male	16	37.11	0.64	0.16	0.34
CAT	female	16	37.77	0.72	0.18	0.38
CAT	male	16	37.39	0.56	0.14	0.3
BR	female	16	38.07	0.56	0.14	0.3
BR	male	16	37.79	0.73	0.18	0.39
SD	female	16	38.69	0.44	0.11	0.23
SD	male	16	38.6	0.6	0.15	0.32
BUF	female	16	38.59	0.33	0.08	0.18
BUF	male	16	38.58	0.47	0.12	0.25

Table 2-2. The difference in CTmax between sexes for each population used to create recombinant inbred line and distance between the populations. Tolerance differences are based on combined male and female CTmax each population (n = 16 for each sex). Tolerance difference categories are structured as: low = 0.0 to 0.5, medium = 0.51 to 0.99, high = > 1.0 ℃. Transgression for CTmax in any line within a cross is indicated by a “Yes” or “No”.

Populations hybridized (♀x♂)	Δ Tolerance (℃)	Tolerance category	Geographic distance (km)	Transgressive lines
SDxBR	0.91	medium	7.9	Yes
BRxSD	0.53	medium	7.9	Yes
CATxAB	0.66	medium	33.85	Yes
ABxCAT	0.23	low	33.85	Yes
SDxBUF	0.11	low	123.99	Yes
BUFxSD	0.01	low	123.99	Yes
ABxBR	0.17	low	144.88	Yes
BRxAB	0.96	medium	144.88	Yes
SCNxSD	1.58	high	639.92	No
SDxSCN	1.69	high	639.92	No
PESxAB	0.43	low	666.48	No
ABxPES	1.08	high	666.48	No

Recombinant inbred lines

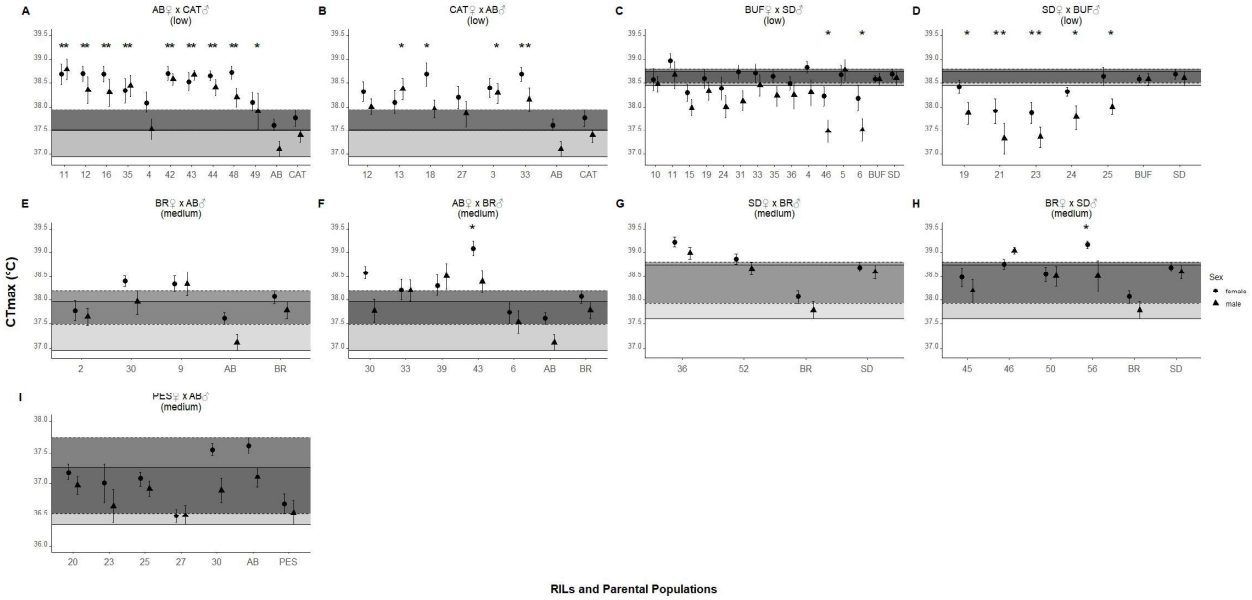

Figure 2-3. The CTmax and standard error of the RILs and parental populations separated by sex, females are represented with black circles and males are represented with black triangles. Thermal tolerance categories indicated below name of each cross. The RILs are listed as numbers and parental populations are listed as two or three letter codes. The light grey horizontal band outlined with solid lines indicate male parental range (+/- SE), medium grey band outlined by dashed line indicates female parental range (+/- SE). Values that are above or below the grey band indicated positive or negative transgression respectively (n=16 for each sex). RILs with "*" indicate statistically significant CTmax compared to the parental population.

In each of the RILs created I witnessed variable survivorship, not every line of the starting 50 survived to able to be tested. Some reciprocal crosses did not survive at all (SD x SCN and AB x PES) while others (SCN x SD) only a single line survived (Table A2-3). The single SCN x SD line was removed from the analysis due to not enough lines to compare within RILs for this cross. Positive or negative transgressive segregants for maximum thermal limit were found in all hybrid crosses between populations separated by less than 150 km (Figure 2-3, Table 2-1). There was no correlation between increased maximum thermal limit (positive transgressive segregants) and percent nauplii or survivorship 14 days post hatch (Figures A2-

1:8). I did find a positive correlation between decreased maximum thermal limit (negative transgressive segregants) and percent of surviving copepods in naupliar stage in the SD x BUF RILs ($R = 0.97$, $P = 0.0048$, Figure A2-8B). There were differences between sexes in the CTmax within a RIL (p-value < 0.05) therefore male and female were analyzed separately.

ABxCAT and CATxAB

The Abalone Cove and Catalina Island hybrids produced the most RILs exhibiting positive transgressive segregants (Figure 2-3A:B). In the ABxCAT RILs there were 9 lines displaying positive transgression, not all lines showed transgression in both males and females. There were 9 lines in which males were transgressive (all p-value < 0.05) and 8 lines where females were transgressive (all p-value <0.05) (Figure 2-3A). There were 6 CATxAB RILs where the males (3 were significant p-value < 0.05) were transgressive for thermal tolerance and 5 CATxAB RILs where females were transgressive (2 were significant p-value < 0.05) (Figure 2-3B).

BUFxSD and SDxBUF

The San Diego and La Bufadora hybrids produced the second most transgressive lines, unlike the Catalina Island and Abalone Cove hybrids, most of the San Diego and La Bufadora lines displayed negative transgression for thermal tolerance (below parental range). The BUFxSD RIL males were negative transgressive in 5 lines (3 are significant p-value <0.05) and the females were negative transgressive in 3 lines and positive transgressive in 1 line (Figure 2-3C). The reciprocal SDxBUF RILs displayed only negative transgressive lines, in 5 lines males (all p-value > 0.05) were negative transgressive and in 3 lines females (2 significantly p-value > 0.05) were also negative transgressive (Figure 2-3D).

BRxAB and ABxBR

The BR x AB RILs produced 1 line in which females were positive transgressive and 1 line in which males were positive transgressive but in different lines (Figure 2-3E). The AB x BR RILs produced 3 lines where the males were positive transgressive and 2 lines where the females (1 significantly, p-value > 0.05) were positive transgressive for thermal tolerance (Figure 2-3F).

SD x BR and BR x SD

The SD x BR RILs produced 1 positive transgressive line, both males and females, and 1 parental level line (Figure 2-3G). The reciprocal side of the cross, the BR x SD RILs there was 1 line that males were positive transgressive and 1 line the females (p-value > 0.05) were positive transgression for thermal tolerance but from different RILs (Figure 2-3H).

PES x AB

The CTmax for both sexes in the PES x AB were all intermediate knockdown temperature phenotypes similar to parental populations indicating no transgressive thermal phenotypes in these RILS (Figure 2-3I).

First clutch size and Percent survival and Percent of life stages 14 days post hatch

Parental lines

The average size of a female's first egg sac differed significantly among the parental populations, ranging from a low of 8 (CAT) to a high of 20 (PES) (Figure 2-3A, P-value = 6.207e-05). The remainder of the populations were similar in size of first clutch, averaging between 11 and 15 individuals in a clutch (Figure 2-4A). The SCN, CAT, BR, and SD populations all maintained > 75 % survival after 14 days post hatch while the PES, AB, and BUF each suffered more than 50% mortality (Figure 2-4B).

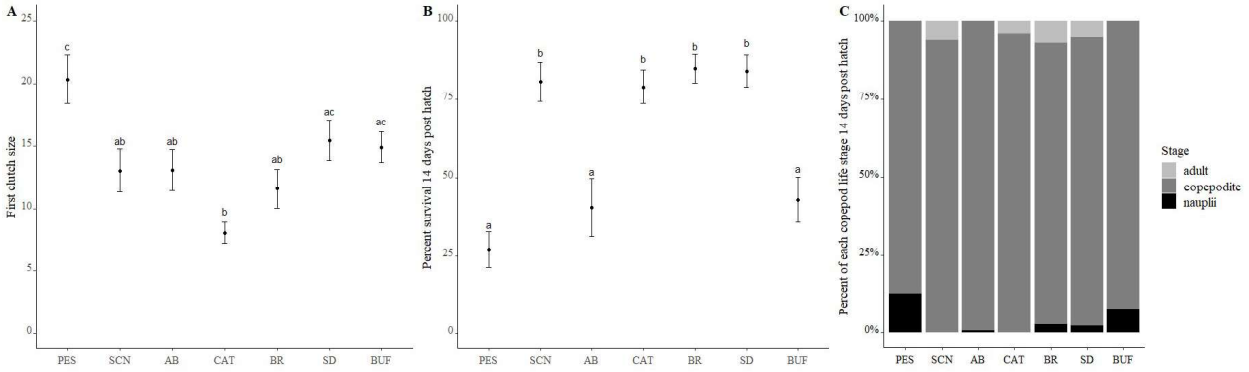

Figure 2-4. Parental populations plotted from north to south (left to right): Pescadero, CA (PES), Santa Cruz, CA (SCN), Abalone Cove, CA (AB), Catalina Island, CA (CAT), Bird Rock, CA (BR), San Diego, CA (SD), La Bufadora, MX (BUF). Lowercase letters displayed are results of post hoc comparisons among populations (n=24 for all populations). (A)The average clutch size (+ SE), of the first egg sac in the parental populations. (B) The percent of copepods surviving fourteen days after hatching (+ SE), all life stages combined. (C) The percent of each copepod life stage alive fourteen days post hatch.

Recombinant inbred lines

Size of first clutch

To determine the effect of hybridization and inbreeding on the fitness for each recombinant inbred line, I counted the number of nauplii per egg sac, survivorship fourteen days post hatch, and number of each life stage fourteen days post hatch. If fitness is decreased in a RIL I would expect lower survivorship and smaller first egg sacs (less eggs).

BUF x SD and SD x BUF

The BUF x SD RILs first clutch size for most lines was in the parental range. Line 31 and 5 both showed a trend toward positive transgression for clutch size but neither case was significant (Figure 2-5E, all P-value > 0.05). The percent survival for the BUF x SD RILs was also intermediate except for line 10 and line 33 which were negative transgressive for survival

(Figure 2-6E). There were significant deviations from homogeneity of life stages in the BUF x SD RILs (Figure 2-7E; p-value < 2.2e-16). Line 31 developed faster, this is evident by less nauplii and more copepodites than expected (Table A2-4, P-value <0.05). Line 4 developed slower and had more nauplii and fewer copepodites than expected (Table A2-4, P-value <0.001). Line 24 developed faster resulting in more adults than expected (Table A2-4, P-value < 0.001). The reciprocal of this cross, the SD x BUF RILs, only 5 lines survived. Two lines (RIL 21 and 23) displayed a trend toward negative transgression in clutch size but neither significantly different from the lowest parental populations (Figure 2-5D, P-value > 0.05). No RILs in the SD x BUF cross were transgressive in survivorship compared to the parental populations (Figure 2-6D). There were significant deviations in the distribution of life stages in the SD x BUF RILs (Figure 2-7D; p-value = 8.273e-09). Line 21 developed faster producing less nauplii and more copepodites than expected (Table A2-5, p-value < 0.001). Line 25 developed slower than other RILS and produced more nauplii and less copepodites than expected (Table A2-5, p-value < 0.001).

PES x AB

In the PES x AB RILS, line 20 and 25 had smaller clutches than the parental range indicating negative transgression, the remaining 3 lines were in the low end of the parental range (Figure 2-5I). Although there were some lines with negative transgression for clutch size in this cross, none of these values were significant (all P-value > 0.05). There were 4 lines (20,23,25, & 30) that showed positive transgression for survival (Figure 2-6I; all P-value < 0.05). RIL 27 has slightly higher survival but still within the parental range. There was a significant difference in life stage distributions of the PES x AB RILs than expected (Figure 2-7I; p-value = 0.02219). Line 30 developed faster than the rest of the PES x AB RILs and had lower amount of nauplii

and higher amount of copepodites that expected (Table A2-7; p-value = 0.02 and 0.01). The reciprocal, AB x PES, did not survive till the F_7 generation to perform tests of fitness.

BR x AB and AB x BR

The BR x AB lines were not different in clutch size from the parental BR or AB populations, although line 9 was negative transgressive (Figure 2-5A; p-value > 0.05). All RILs displayed survivorship within the parental range (Figure 2-6A). There was no deviation in distribution of life stages between the RILs of this cross (Figure 2-7A; p-value 0.1636, Table A2-7). The reciprocal AB x BR RILs all showed a parental range for clutch size and survivorship (Figure 2-5B and 2-6B). The AB x BR RILS also did not deviate from the expected distribution of life stage among lines (Figure 2-7B, Table A2-8, p-value = 0.2328).

CAT x AB and AB x CAT

The CAT x AB RILs all were within the parental range for clutch size and survivorship (Figure 2-5G, 2-6G). There were significant deviations in the distributions of life stages within the lines (Figure 2-7G; p-value < 2.2e-16). Line 3,12,18 and 33 all developed faster than the rest of the lines. Line 3 and 12 had less nauplii and more copepodites, line 18 had more copepodites, and line 33 had more adults than expected (Table A2-9, all p-value < 0.001). Line 13 developed slower than other lines, there were more nauplii and less copepodites than expected (Table A2-9, both p-value < 0.001). In the reciprocal AB x CAT RILs, all 9 lines displayed a similar non transgressive or intermediate phenotype for clutch size and survivorship (Figure 2-5G, 2-6G). There were significant deviations in the distribution of life stages (Figure 2-7G; p-value < 2.2e-16). Line 11 developed faster and contained less nauplii than expected at the end of fourteen days (Table A2-10, p-value = 0.02). Line 16 and 49 developed slower than expected. Line 16 had more nauplii and less copepodites, line 49 had more nauplii (Table A2-10, all p-value < 0.001).

SD x BR and BR x SD

In the SD x BR RILs only had 2 lines survived, one displayed non-significant negative transgression for first clutch size and survivorship (RIL 36, both p-value > 0.05) and the other was on the low end of intermediate phenotype (RIL 52) (Figure 2-5F, 2-6F). There were no significant deviations in the distribution of life stages in the SD x BR RILs (Figure 2-7F; p-value = 0.1887; Table A2-11, p-value > 0.05). The reciprocal BR x SD RILs was similar, 3 of the 4 lines were within the low end of the range of the parentals and one line (RIL 56) was below the range indicating negative transgression that was non-significant (Figure 2-5C; p-value > 0.05). The BR x SD RILs had two lines with lower survivorship than the parents (45 and 50) and the other two were in the bottom edge of the parental range (46 and 56) (Figure 2-6C). Line 45 was significantly lower (P-value < 0.05) than the SD population. There was a significant deviation in the distribution of life stages, but I did not have enough power to detect which life stage and which line (Figure 2-7C, p-value = 0.02593; Table A2-12, all p-value > 0.05).

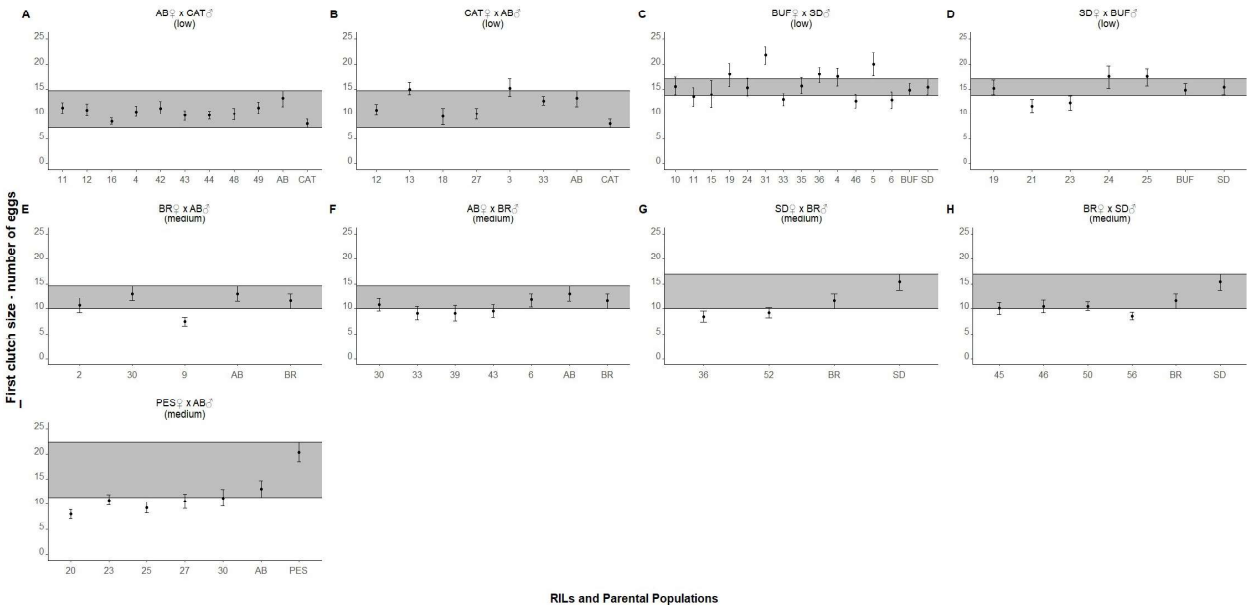

Figure 2-5. The size of the first clutch of eggs for the parental populations and RILs. The RILs are listed as numbers and parental populations are listed as two or three letter codes. Thermal tolerance categories indicated below name of each cross. Grey horizontal band indicates parental range (+/- SE).

Percent survival 14 days post hatch

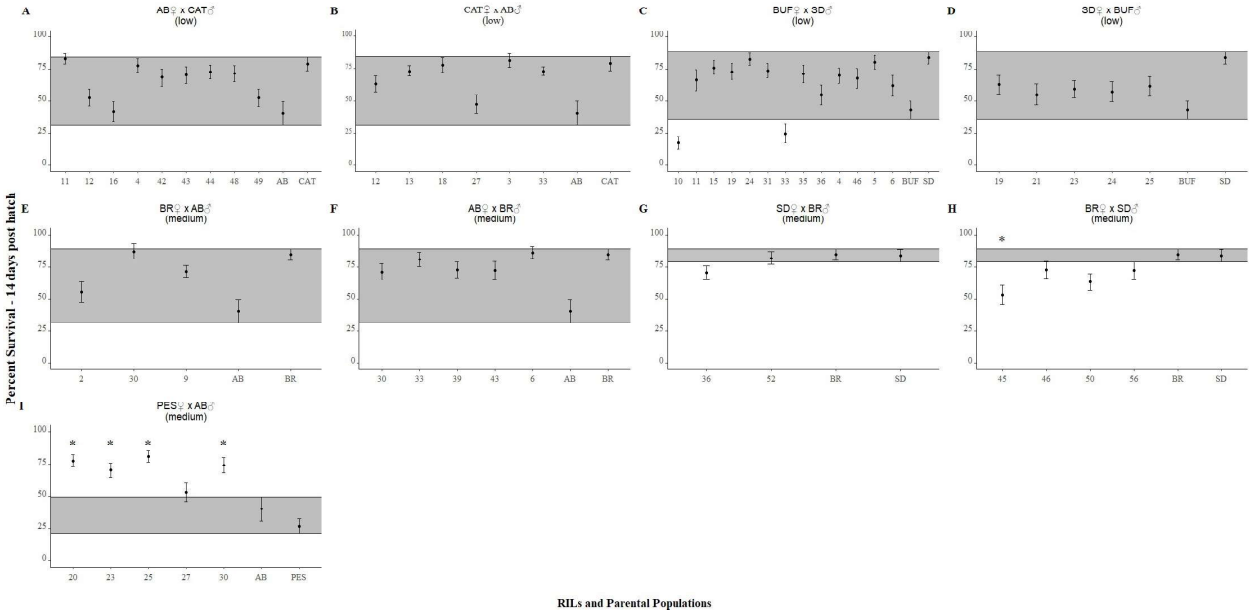

Figure 2-6. The percent of total copepods alive 14 days post hatch for parental populations and RILs. The RILs are listed as numbers and parental populations listed as two or three letter code. Thermal tolerance categories indicated below name of each cross. Grey horizontal band indicates parental range (+/- SE), an '*' above a RIL indicates significantly lower or higher survivorship compared to nearest parental mean (α=0.05).

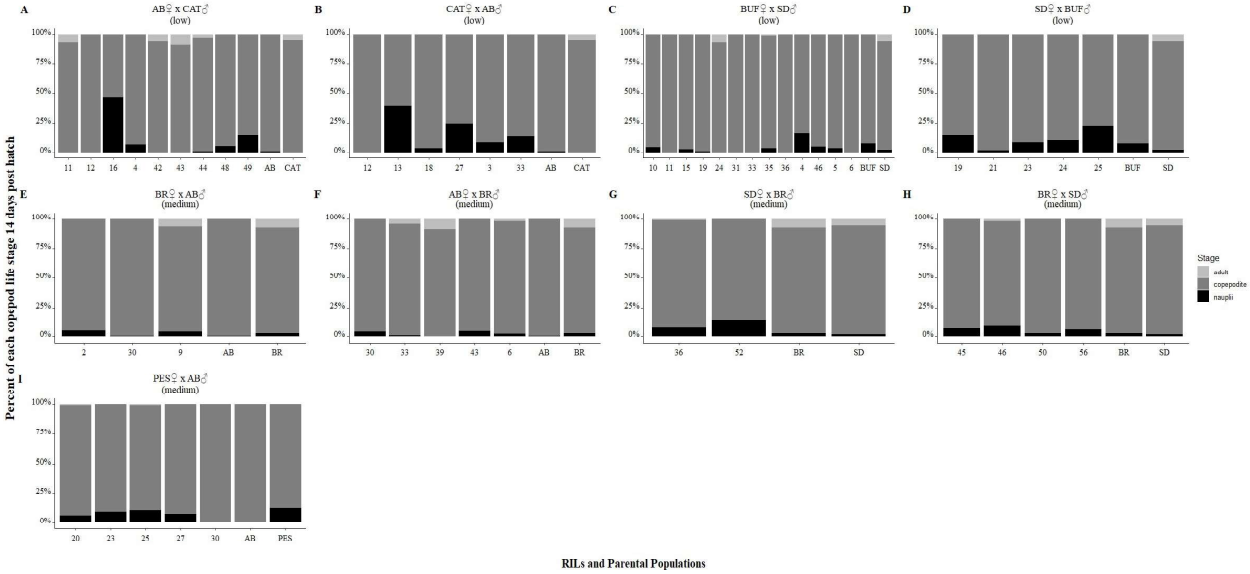

Figure 2-7. Data displayed as stacked bar chart of the percent of each copepod life stage alive 14 days post hatch for parental populations and RILs. The RILs are listed as numbers and parental populations hybridized are listed as two or three letter codes. Thermal tolerance categories indicated below name of each cross.

Discussion

In agreement with previous work (Pereira et al. 2014) our study demonstrates transgressive segregants for thermal tolerance in hybrids between geographically close populations and no transgressive segregants in hybrids of geographically distant populations (Figure 2-2). This study shows evidence for positive and negative transgression in thermal tolerance in hybrids between populations of low (0 – 0.50 ℃) and medium (0.51-0.99 ℃) thermal limit phenotype differences. Hybrids between populations with large differences in thermal limit phenotype differences (>1.0 ℃) displayed no transgressive segregants. In most RILs in this study there was no correlation between CTmax increase/decrease and the size of the first egg sacs of females or the survivability of her offspring when measured fourteen days after

hatching. I did find a positive correlation between CTmax and the number of surviving copepods at 14 days post hatch of the naupliar stage in the SDxBUF RILs ($R = 0.97$, $p = 0.0048$; Figure A2-8B). This result suggests increasing CTmax in this cross slowed development but did not correlate with survivorship in the time frame I measured.

The Pereira et al. (2014) study found hybrids between populations of different thermal phenotypes and in different biogeographic regions (Bird Rock (BR) and Santa Cruz (SCN)) did not produce thermal transgressive segregants. The population crosses with similar thermal phenotypes and in the same biogeographic region did produce transgressive segregants (San Diego (SD) and Bird Rock (BR)). The BR and SCN populations are separated by > 600 km of coastline while the BR and SD populations are regionally close, only ~ 8 km. To investigate the pattern of thermal transgressive segregants found by Pereira et al. (2014) further, I created hybrids between the Pescadero (PES) and Abalone Cove (AB) populations and the San Diego (SD) and Santa Cruz (SCN) populations. Both crosses are between populations separated by > 600 km of coastline but the thermal divergence between the two crosses is different (Table 2-1). The thermal limit of the PESxAB was the only cross with a low difference in maximum thermal limit between populations ($\Delta = 0.43$ °C). The three other crosses (ABxPES, SDxSCN, SCNxSD) were high thermal divergence ($\Delta > 1.0$ °C; Table A2-3) indicating differences in habitat temperature selecting for different levels of CTmax. The low level of thermal phenotype difference of the PESxAB cross also did not produce transgressive segregants further indicating populations in different biogeographic zones are divergent in thermal response thus producing intermediate parental thermal phenotype. Tidepool temperatures from the SD and SCN locations indicate higher average temperature and more frequent extreme temperatures in the SD location (Figure 2-1). *T. californicus* populations are locally adapted, different mechanism of thermal

tolerances are evident in gene expression presenting as different levels of upregulation in thermal tolerant and thermal sensitive populations (Schoville et al. 2012) and differential use of some chaperone proteins between thermal tolerant populations (Lima and Willett 2017).

The remainder of the crosses I developed were between low and medium thermal phenotypes and less than 150 km distance and within the same biogeographic region. The SD and BR RILs produced positive transgressive segregants similar to Pereira et al. (2014) but in our study only six lines survived (including reciprocals). The difference in CTmax for this cross was in the medium range for both reciprocals but SDxBR cross was near the high end of the range (Figure 2-1, SDxBR $\Delta = 0.91°C$, BRxSD $\Delta = 0.53°C$). Interestingly, four of our low thermal phenotype difference crosses produced opposite transgressive segregant thermal phenotypes. The ABxCAT ($\Delta = 0.23°C$) and ABxBR ($\Delta = 0.17$) RILs were positive transgressive segregants and most of the SDxBUF ($\Delta = 0.11°C$) and BUFxSD ($\Delta = 0.01°C$) were found to display negative transgressive thermal limit phenotypes (Figure 2-2). Based on similar thermal phenotypes and both populations within the same biogeographic region I expected to see positive transgressive phenotypes in the SD and BUF hybrids. The southern location (closer to the equator) of the SD and BUF populations in the *T. californicus* range may indicate the upper limits for thermal tolerance in this species and there is less thermal margin available to reach the maximum temperature tolerance that can be gained by hybridizing.

Few studies show transgressive segregation for physiological traits, most are morphological (Rieseberg et al. 2003). A possible reason for this is the difference in the evolution of morphological genes compared to physiological genes. In an analysis of genes that only affect morphological or physiological processes in mice and humans, Liao et al. (2010) found genes involved in morphology were more essential and pleiotropic (i.e., transcription

regulator, structural), evolve faster expression patterns but the protein sequence evolved slower. Genes involved in physiological process (i.e., catalytic activity, receptor activity, ion transport, and channel/pore class transporter) have more paralogues, evolve faster in cis regulatory sequence, have lower purifying selection and stronger positive selection (Liao, Weng, and Zhang 2010). Selection drives populations to allele frequencies that theoretically will result in optimal fitness. Populations will accumulate genetic differences over time, a needed step for transgressive segregation to occur in hybrids. Genetic pathways that respond to environmental physiological stresses (i.e., heat stress response) are composed of many genes of small effect. If physiological genes have lower purifying selection, there is the opportunity for slightly deleterious alleles to remain in a population instead of being purged. Because heat stress response is composed of many genes of small effect, mildly deleterious genes may not dramatically affect the thermal phenotype. Hybridizing can remove some of the deleterious alleles but the small effect each has in the response to stress will make it harder to detect the underlying mechanism to transgressive phenotypes.

Overall, I did experience a large amount of die off in our independent lines and no cross maintained greater than thirteen of the original fifty lines that were started before the three consecutive rounds of inbreeding. Populations may accumulate differences that are incompatible and detrimental when interacting in the same cellular environment in hybrids (Dittrich-Reed and Fitzpatrick 2013). The genetic conflicts from the original hybridization between divergent genomes or the stress of inbreeding may have contributed to such high rate of loss. In the lines that did survive, selecting the first breeding pair to start each generation increased the probability of selecting the fittest individuals in each egg sac. This design could be expected to increase the occurrence of positive transgression in thermal limit, survivability, and rate of development.

Because I selected the fastest developing pair for each generation, genotypes are not expected to be a completely random selection of parental combinations. The genes that underlie thermal limit (extrinsic fitness) are not expected to be the same that underlie cytonuclear incompatibilities (intrinsic fitness). I would expect genotypic combinations that reduce the speed of development, increase prevalence sterility, or were highly inviable (lethal) to not be selected for in our design. An example of the effects of this is the San Diego (SD) and La Bufadora (BUF) hybrids, most displayed a reduced thermal limit indicating genetic mismatches for extrinsic fitness but not for the measurements of intrinsic fitness. This is not to say I did not see patterns of intrinsic stress in our study. I did see complicated patterns of intrinsic stress phenotypes in all our hybrids. For instance, in both of our crosses of populations separated by > 600 km of distance, the mitochondrial background (ABxPES & SDxSCN) of the cross of the more thermal tolerant populations did not survive. The reciprocal crosses, PESxAB and SCNxSD hybrids, both had lines survive, but only a single line survived in the SCNxSD cross (Table A2-3B). The PESxAB hybrids were ~75 % survival for most lines but both parental populations had < 50% survival (Figure 2-5I). The PES and AB populations appear to be less healthy by this measure of fitness than other populations (Figure 2-3B).

The BUF population also survived at ~50 % fourteen days post hatch but when paired with the SD population most of their hybrids, regardless of mitochondrial background, survived and produced clutch sizes that were intermediate of the parental populations (Figure 2-3B, 2-5D, E). The difference in fitness of the BUF and SD hybrids compared to the SCNxSD and PESxAB hybrids may be due to the geographic distance between them. The BUF and SD populations are ~124 km apart instead of > 600 km. Considering biogeography, the BUF and SD populations are

both found in the Ensenadian region while the SCNxSD and PESxAB crosses are between the Ensenadian and Montereyan region of coastal California (Blanchette et al. 2008).

Conclusion

Understanding the mechanisms that underlie hybrid phenotypes outside the range of the parents (transgressive segregants) and more suited to current and future conditions is important in predictions of tolerance limits and persistence of species. Given the regularity of transgressive segregation for thermal tolerance between populations within the same biogeographic range, it is evident in *T. californicus* populations are most likely diverging in response to thermal stress. Combining the response of populations with similar thermal phenotypes produced transgressive segregants most likely by complementary gene action instead of epistasis, mutation, or overdominance (Rieseberg et al. 1999). Hybridization can disrupt the effects of genetic drift, inbreeding depression, and break up coadapted genomic complexes creating new genetic combinations that are affected by both intrinsic and extrinsic selection (Abbott et al., 2013; 2016). In some genetic combinations, hybrid phenotypes will be more suited for predicted climate change (Hoffmann and Sgró 2011; Provan and Maggs 2011).

Acknowledgements

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Chapter 2, in full is currently being prepared for submission for publication of the material. Blackwell, Reginald C.; Burton, Ronald. The dissertation author was the primary investigator and author of this material.

Appendix

Table A2-1. The difference in CTmax between sexes from each population used to create recombinant inbred line and distance between the populations. Tolerance differences are based on combined male or female CTmax from each population used in the cross (n=16 for each sex in a RIL). Tolerance difference categories are structured as: low = 0.0 to 0.5, medium = 0.51 to 0.99, high = > 1.0 ℃. Transgression for CTmax in each line by sex is indicated by the number of lines that displayed transgression and the direction of transgression.

Cross (♀x♂)	Δ Tolerance (℃)	Geographic distance (km)	Tolerance category	Lines remaining (50)	Lines with Positive Transgressive ♂	Lines with Positive Transgressive ♀	Lines with Negative Transgressive ♂	Lines with Negative Transgressive ♀
SDxBR	0.91	7.9	medium	2	1	1	0	0
BRxSD	0.53	7.9	medium	4	1	1	0	0
CATxAB	0.66	33.85	medium	6	5	6	0	0
ABxCAT	0.23	33.85	low	10	9	8	0	0
SDxBUF	0.11	123.99	low	5	0	0	3	5
BUFxSD	0.01	123.99	low	13	0	1	3	5
ABxBR	0.17	144.88	low	5	3	2	0	0
BRxAB	0.96	144.88	medium	3	1	1	0	0
SCNxSD	1.58	639.92	high	1	0	0	0	0
SDxSCN	1.69	639.92	high	0	0	0	0	0
PESxAB	0.43	666.48	low	5	0	0	0	0
ABxPES	1.08	666.48	high	0	0	0	0	0

Table A2-2. BUFxSD RILs Chi-square test of homogeneity observed and expected counts for each life stage in each line. Residuals from Chi-square used in post hoc multiple regression analysis. Grey shaded P-values indicate significant deviations from expected values of homogeneity in a life stage (α=0.05).

	Observed counts			Expected counts			Residuals			P-values		
Line number	nauplii	copepodite	adult	nauplii	copepodite	adult	nauplii	copepodite	adult	nauplii	copepodite	adult
10	1	80	0	1.73	79	0.27	-0.57	0.72	-0.53	1	1	1
11	0	203	0	4.33	197.99	0.68	-2.17	2.34	-0.85	1	0.7513	1
15	4	218	0	4.74	216.52	0.74	-0.35	0.66	-0.89	1	1	1
19	3	309	0	6.66	304.3	1.05	-1.51	1.8	-1.08	1	1	1
24	0	287	9	6.32	288.69	0.99	-2.66	-0.66	8.44	0.3019	1	**<0.001**
31	0	416	0	8.88	405.73	1.39	-3.22	3.47	-1.27	**0.0495**	**0.0201**	1
33	0	90	0	1.92	87.78	0.3	-1.42	1.53	-0.56	1	1	1
35	3	279	2	6.06	276.99	0.95	-1.31	0.8	1.13	1	1	1
36	0	248	0	5.29	241.88	0.83	-2.42	2.61	-0.95	0.608	0.3574	1
4	45	247	0	6.23	284.79	0.98	16.45	-14.93	-1.04	**<0.001**	**<0.001**	1
46	4	225	0	4.89	223.35	0.77	-0.42	0.73	-0.91	1	1	1
5	10	394	0	8.62	394.03	1.35	0.51	-0.01	-1.24	1	1	1
6	0	204	0	4.35	198.96	0.68	-2.18	2.35	-0.86	1	0.7391	1

Table A2-3. SD x BUF RILs Chi-square test of homogeneity observed and expected counts for each life stage in each line. Residuals from Chi-square used in post hoc multiple regression analysis. Grey shaded P-values indicate significant deviations from expected values of homogeneity in a life stage (α=0.05).

	Observed counts			Expected counts			Residuals			P-values		
Line number	nauplii	copepodite	adult	nauplii	copepodite	adult	nauplii	copepodite	adult	nauplii	copepodite	adult
19	17	227	1	27.61	216.3	1.09	-2.42	2.4	-0.09	0.23	0.24	1
21	3	148	1	17.13	134.2	0.67	-3.9	3.74	0.43	**<0.001**	**<0.001**	1
23	10	176	1	21.07	165.1	0.83	-2.8	2.71	0.21	0.08	0.1	1
24	41	236	1	31.33	245.44	1.23	2.11	-2.03	-0.24	0.52	0.64	1
25	56	208	1	29.86	233.96	1.18	5.81	-5.67	-0.19	**<0.001**	**<0.001**	1

Table A2-4. PES x AB RILs Chi-square test of homogeneity observed and expected counts for each life stage in each line. Residuals from Chi-square used in post hoc multiple regression analysis. Grey shaded P-values indicate significant deviations from expected values of homogeneity in a life stage (α=0.05).

	Observed counts			Expected counts			Residuals			P-values		
Line number	nauplii	copepodite	adult	nauplii	copepodite	adult	nauplii	copepodite	adult	nauplii	copepodite	adult
20	4	129	1	5.46	128.21	0.33	-0.7	0.37	1.27	1	1	1
23	12	153	0	6.72	157.87	0.41	2.33	-2.09	-0.72	0.3	0.55	1
25	10	158	1	6.89	161.7	0.42	1.36	-1.57	1.02	1	1	1
27	7	144	0	6.15	144.48	0.37	0.39	-0.21	-0.68	1	1	1
30	0	191	0	7.78	182.75	0.47	-3.26	3.36	-0.79	**0.02**	**0.01**	1

Table A2-5. BR x AB RILs Chi-square test of homogeneity observed and expected counts for each life stage in each line. Residuals from Chi-square used in post hoc multiple regression analysis (α=0.05).

	Observed counts			Expected counts			Residuals			P-values		
Line number	nauplii	copepodite	adult	nauplii	copepodite	adult	nauplii	copepodite	adult	nauplii	copepodite	adult
2	1	120	0	1.12	119.43	0.45	-0.13	0.52	-0.76	1	1	1
9	1	125	2	1.19	126.34	0.47	-0.2	-1.2	2.54	1	1	0.1
30	3	287	0	2.69	286.23	1.08	0.28	0.58	-1.53	1	1	1

Table A2-6. AB x BR RILs Chi-square test of homogeneity observed and expected counts for each life stage in each line. Residuals from Chi-square used in post hoc multiple regression analysis. (α=0.05).

	Observed counts			Expected counts			Residuals			P-values		
Line number	nauplii	copepodite	adult	nauplii	copepodite	adult	nauplii	copepodite	adult	nauplii	copepodite	adult
6	7	225	1	3.54	228.09	1.36	2.17	-1.66	-0.37	0.45	1	1
30	1	178	0	2.72	175.23	1.05	-1.18	1.62	-1.15	1	1	1
33	2	145	2	2.27	145.86	0.87	-0.2	-0.54	1.33	1	1	1
39	1	136	2	2.11	136.07	0.81	-0.84	-0.05	1.44	1	1	1
43	2	153	0	2.36	151.74	0.91	-0.26	0.78	-1.06	1	1	1

Table A2-7. CATxAB RILs Chi-square test of homogeneity observed and expected counts for each life stage in each line. Residuals from Chi-square used in post hoc multiple regression analysis. Grey shaded P-values indicate significant deviations from expected values of homogeneity in a life stage (α=0.05).

	Observed counts			Expected counts			Residuals			P-values		
Line number	nauplii	copepodite	adult	nauplii	copepodite	adult	nauplii	copepodite	adult	nauplii	copepodite	adult
3	9	294	1	39.12	261.02	3.86	-5.92	6.23	-1.68	**<0.001**	**<0.001**	1
12	1	167	1	21.75	145.11	2.15	-5.12	5.19	-0.85	**<0.001**	**<0.001**	1
13	92	173	1	34.23	228.39	3.38	11.91	-10.98	-1.47	**<0.001**	**<0.001**	1
18	12	173	1	23.93	159.7	2.36	-2.83	3.03	-0.97	0.08	**0.04**	1
27	22	84	1	13.77	91.87	1.36	2.48	-2.28	-0.32	0.23	0.4	1
33	26	190	11	29.21	194.91	2.88	-0.7	-1.03	5.31	1	1	**<0.001**

Table A2-8. ABxCAT RILs Chi-square test of homogeneity observed and expected counts for each life stage in each line. Residuals from Chi-square used in post hoc multiple regression analysis. Grey shaded P-values indicate significant deviations from expected values of homogeneity in a life stage (α=0.05).

	Observed counts			Expected counts			Residuals			P-values		
Line number	nauplii	copepodite	adult	nauplii	copepodite	adult	nauplii	copepodite	adult	nauplii	copepodite	adult
4	6	192	0	7.84	188.67	1.49	-0.72	1.2	-1.32	1	1	1
11	0	226	3	9.07	218.21	1.72	-3.35	2.65	1.07	**0.02**	0.22	1
12	0	129	0	5.11	122.92	0.97	-2.42	2.65	-1.03	0.42	0.22	1
16	30	45	0	2.97	71.47	0.56	16.43	-14.8	-0.77	**<0.001**	**<0.001**	1
42	0	171	2	6.85	164.85	1.3	-2.84	2.35	0.66	0.12	0.51	1
43	0	163	4	6.62	159.13	1.25	-2.79	1.5	2.61	0.14	1	0.24
44	1	169	2	6.81	163.89	1.29	-2.42	1.96	0.67	0.42	1	1
48	7	171	0	7.05	169.61	1.34	-0.02	0.52	-1.24	1	1	1
49	14	129	0	5.67	136.26	1.07	3.76	-3.02	-1.1	<0.001	0.07	1

Table A2-9. SD x BR RILs Chi-square test of homogeneity observed and expected counts for each life stage in each line. Residuals from Chi-square used in post hoc multiple regression analysis (α=0.05).

	Observed counts			Expected counts			Residuals			P-values		
Line number	nauplii	copepodite	adult	nauplii	copepodite	adult	nauplii	copepodite	adult	nauplii	copepodite	adult
36	6	124	1	9.17	121.39	0.44	-1.45	1.16	1.14	0.89	1	1
52	15	154	0	11.83	156.61	0.56	1.45	-1.16	-1.14	0.89	1	1

Table A2-10. BR x SD RILs Chi-square test of homogeneity observed and expected counts for each life stage in each line. Residuals from Chi-square used in post hoc multiple regression analysis (α=0.05).

	Observed counts			Expected counts			Residuals			P-values		
Line number	nauplii	copepodite	adult	nauplii	copepodite	adult	nauplii	copepodite	adult	nauplii	copepodite	adult
45	7	119	0	4.54	120.84	0.62	1.32	-0.93	-0.89	1	1	1
46	10	165	3	6.42	170.7	0.88	1.71	-2.56	2.7	1	0.12	0.08
50	3	162	0	5.95	158.24	0.81	-1.44	1.73	-1.06	1	1	1
56	2	139	0	5.09	135.22	0.69	-1.59	1.83	-0.95	1	0.81	1

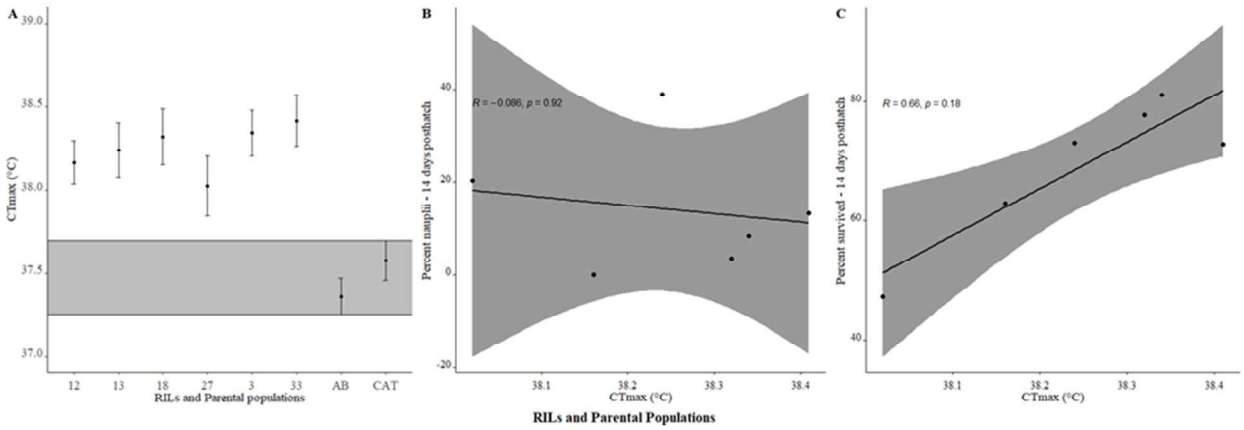

Figure A2-1. The combined CTmax of male and female CATxAB RILs (numbers) and parental populations, 16 male and 16 females for each RIL and parental population (A). The results of Spearman correlation between the CTmax and percent of total survivors at fourteen days that were nauplii stage, 24 egg sacs for each RIL (B). A Spearman correlation between CTmax and the percent of total copepods alive fourteen days post hatch in the first egg sac a female, 24 egg sacs for each RIL (C).

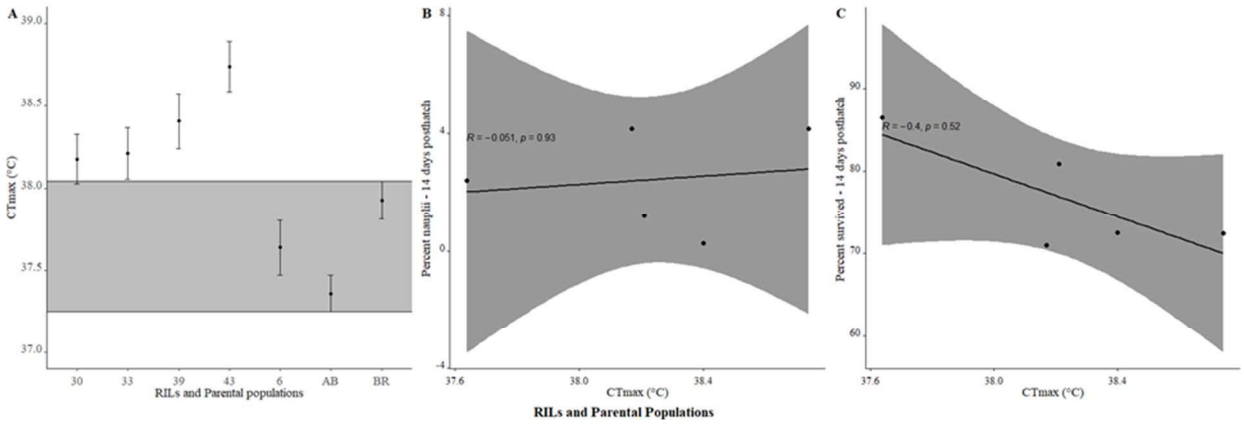

Figure A2-2. The combined CTmax of male and female ABxBR RILs (numbers) and parental populations, 16 male and 16 females for each RIL and parental population (A). The results of Spearman correlation between the CTmax and percent of total survivors at fourteen days that were nauplii stage, 24 egg sacs for each RIL (B). A Spearman correlation between CTmax and the percent of total copepods alive fourteen days post hatch in the first egg sac a female, 24 egg sacs for each RIL (C).

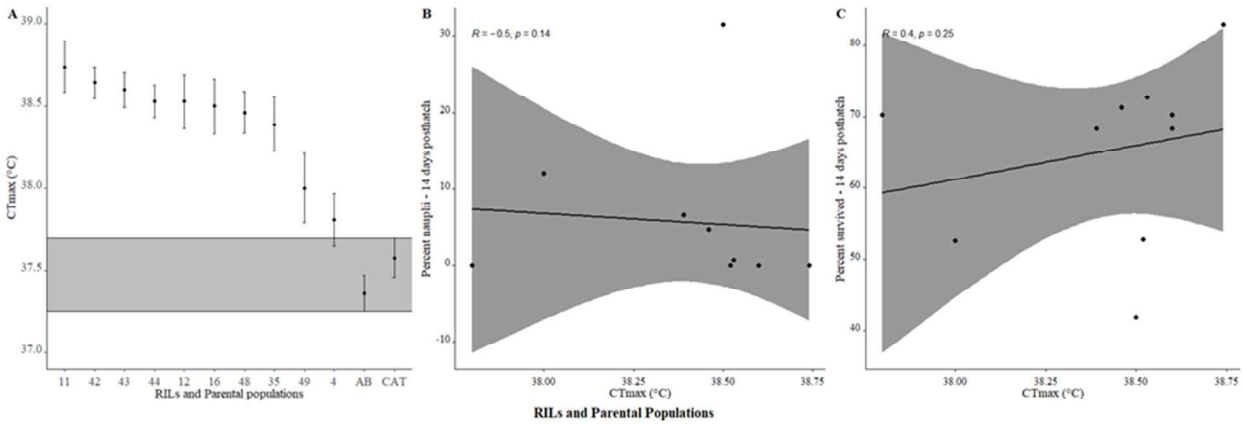

Figure A2-3. The combined CTmax of male and female ABxCAT RILs (numbers) and parental populations, 16 male and 16 females for each RIL and parental population (A). The results of Spearman correlation between the CTmax and percent of total survivors at fourteen days that were nauplii stage, 24 egg sacs for each RIL (B). A Spearman correlation between CTmax and the percent of total copepods alive fourteen days post hatch in the first egg sac a female, 24 egg sacs for each RIL (C).

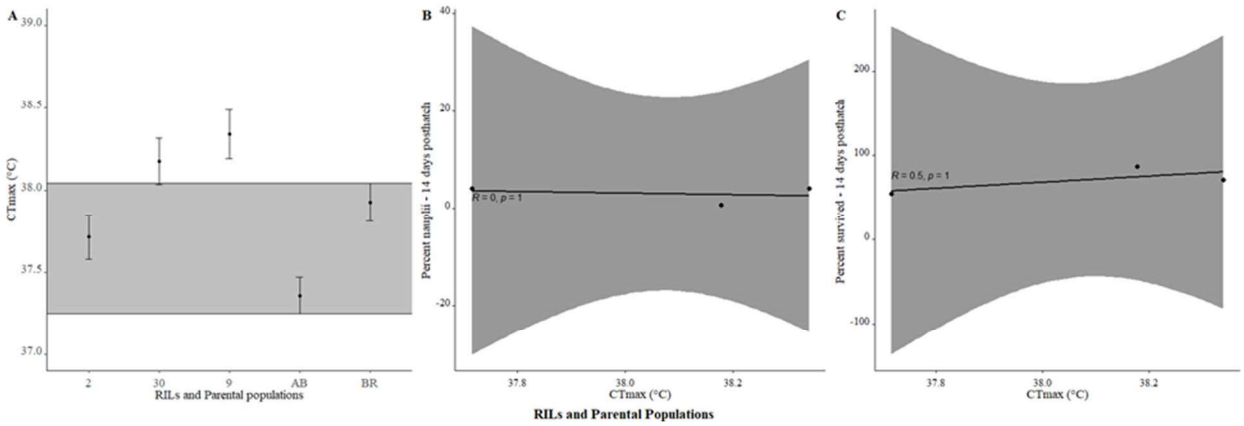

Figure A2-4. The combined CTmax of male and female BRxAB RILs (numbers) and parental populations, 16 male and 16 females for each RIL and parental population (A). The results of Spearman correlation between the CTmax and percent of total survivors at fourteen days that were nauplii stage, 24 egg sacs for each RIL (B). A Spearman correlation between CTmax and the percent of total copepods alive fourteen days post hatch in the first egg sac a female, 24 egg sacs for each RIL (C).

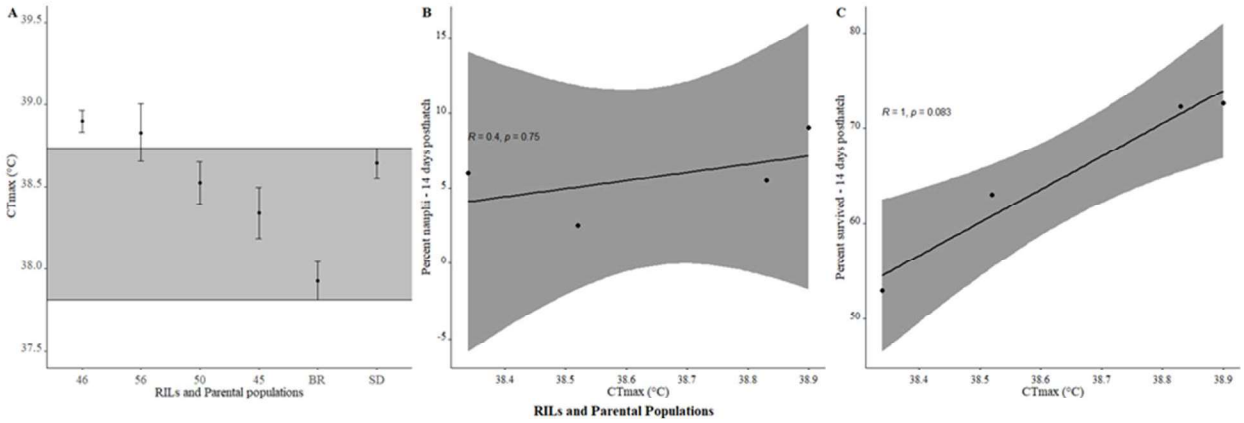

Figure A2-5. The combined CTmax of male and female BRxSD RILs (numbers) and parental populations, 16 male and 16 females for each RIL and parental population (A). The results of Spearman correlation between the CTmax and percent of total survivors at fourteen days that were nauplii stage, 24 egg sacs for each RIL (B). A Spearman correlation between CTmax and the percent of total copepods alive fourteen days post hatch in the first egg sac a female, 24 egg sacs for each RIL (C).

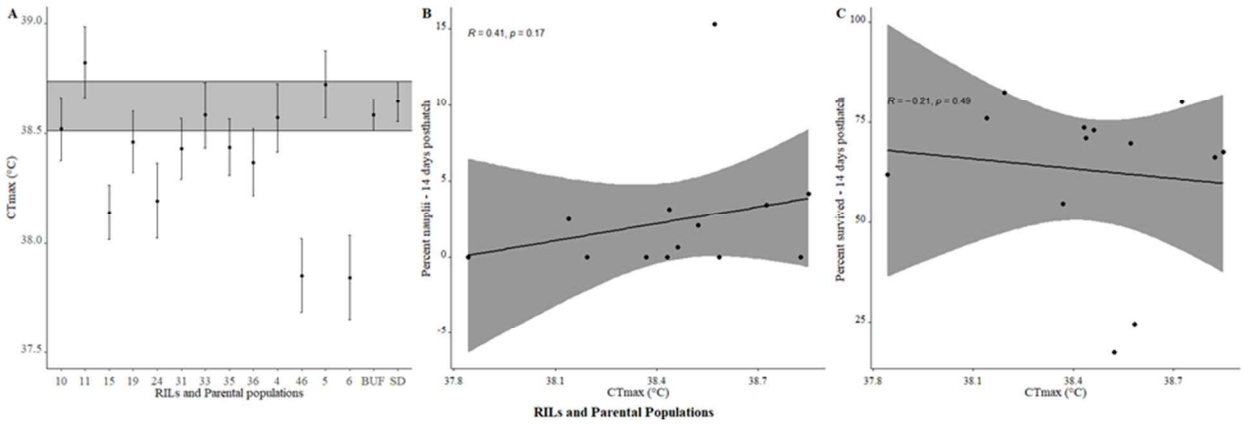

Figure A2-6. The combined CTmax of male and female BUFxSD RILs (numbers) and parental populations, 16 male and 16 females for each RIL and parental population (A). The results of Spearman correlation between the CTmax and percent of total survivors at fourteen days that were nauplii stage, 24 egg sacs for each RIL (B). A Spearman correlation between CTmax and the percent of total copepods alive fourteen days post hatch in the first egg sac a female, 24 egg sacs for each RIL (C).

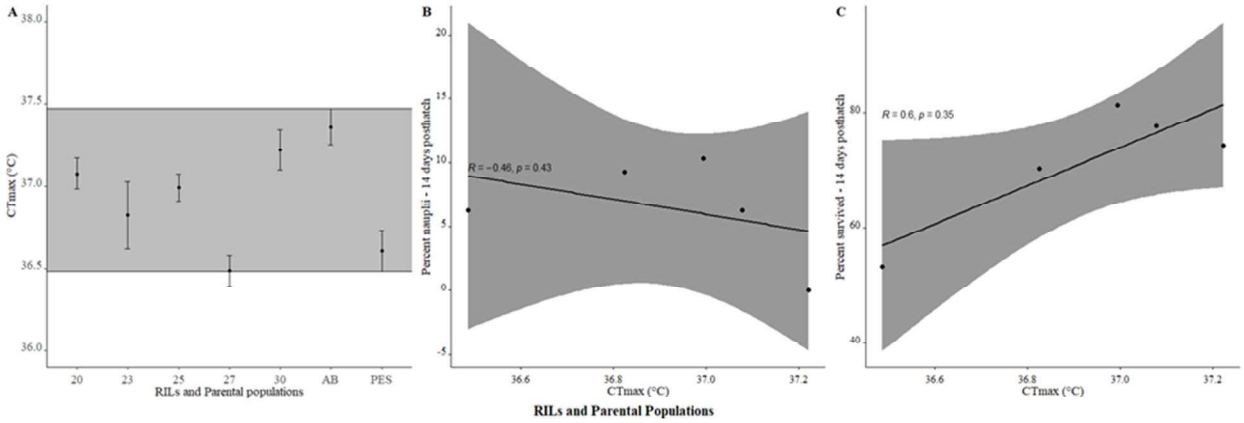

Figure A2-7. The combined CTmax of male and female PESxAB RILs (numbers) and parental populations, 16 male and 16 females for each RIL and parental population (A). The results of Spearman correlation between the CTmax and percent of total survivors at fourteen days in the nauplii stage, 24 egg sacs for each RIL (B). A Spearman correlation between CTmax and the percent of total copepods alive fourteen days post hatch in the first egg sac a female, 24 egg sacs for each RIL (C).

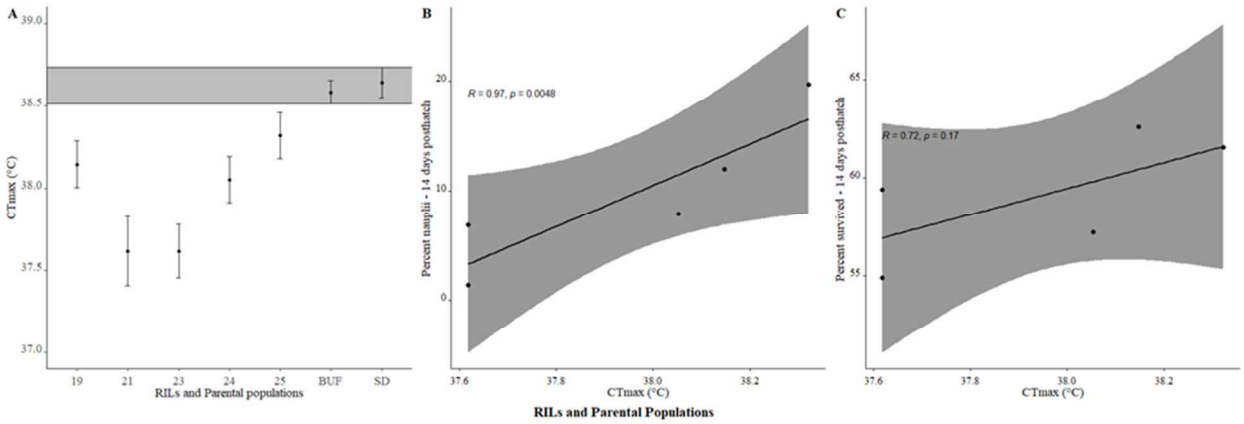

Figure A2-8. The combined CTmax of male and female SDxBUF RILs (numbers) and parental populations, 16 male and 16 females for each RIL and parental population (A). The results of Spearman correlation between the CTmax and percent of total survivors at fourteen days that were nauplii stage, 24 egg sacs for each RIL (B). A Spearman correlation between CTmax and the percent of total copepods alive fourteen days post hatch in the first egg sac a female, 24 egg sacs for each RIL (C).

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Chapter 3

Transcriptome response of increased thermal limit in transgressive segregants of the copepod *Tigriopus californicus*

Introduction

Hybridization combines genomes of divergent taxa creating new genetic variation with ecological and evolutionary consequences. Hybrid genomes are a mosaic of the parental genomes and this new variation has not been tested by selection in either of the parent's environments. Most genotypic combinations are expected to produce hybrids with intermediate fitness and a competitive disadvantage. Hybridization can disrupt the effects of genetic drift and mutation load releasing hybrids from lower fitness of the parentals (Abbott, Barton, and Good 2016). Hybridization may also lead to novel and extreme phenotypes that are outside of the parental range and in some cases the development of new species (Rieseberg et al. 1999; Renaut et al. 2014; Dittrich-Reed and Fitzpatrick 2012; Pereira, Barreto, and Burton 2014; Feliner, Casacuberta, and Wendel 2020). The movement of organisms around the globe from escaping the effects of climate change to the anthropogenic assisted travel is increasing contact between taxa (IPCC 2018). Understanding the adaptive potential of hybrid genotypes for future climate change is an important question in evolutionary biology.

Predicting the outcome of a hybrid genome response for environmentally relevant traits is complicated. Species and populations are evolving independently, accumulating differences, and diverging in fixed alleles based on mutation and selection pressure experiences. Geographically close parents can be experience similar selection events but evolve different response mechanisms (Lima and Willett 2017). Broadly speaking, alleles that are selectively favorable to the environmental conditions of a region are dispersed between the parental populations. Alleles that are mildly deleterious can be expected to reside in both populations. Hybridization events can create progeny with an increased number of alleles that can increase or decrease a trait value outside the range of either parent.

The accumulation of genetic changes at loci from mutation, selection, and the effects of genetic drift are random and scattered across the genomes of divergent populations. In each population, changes in one location of the genome are compensated for by the rest of the genome to maintain proper function and fitness. Intrinsic stress can be created by hybridizing the divergent population and placing alleles that have never interacted together before in the same genetic background (Orr 1996). Hybrid genotypes can also be mismatched with the environment, reducing fitness from an extrinsic stress in comparisons to the parental populations. The evolutionary potential and persistence of the hybrids is contingent on their interactions with the parents or the availability of underutilized resources (Barton and Hewitt 1985; Mallet 2007).

The first-generation hybrids (F1) will most often resemble one of the parents or display traits intermediate of both. The F1 hybrids contain a full haploid complement of both parental genomes. Vigor in F1 hybrids is normally the result of heterosis or heterozygote advantage (Bar-Zvi et al. 2017). Although, normally occurring in the second generation, hybrids displaying reduced fitness in the first generation can occur, generally a consequence of genomic mismatches or Dobzhansky Muller Incompatibilities (DMIs) at multiple locations in the genome (Fierst and Hansen 2010). Recombination occurring in the F2 and later generations may form genetic novelty that is evident in phenotypes that are more extreme than either parent (Slatkin and Lande 1994; Rieseberg et al. 1999; Soltis 2013). Parental gene interactions can also be interrupted leading to beneficial phenotypes and increased fitness (Dagilis, Kirkpatrick, and Bolnick 2019).

Gene expression varies greatly across species and the extent to which it is driven by adaptive changes or neutral evolution is controversial (Khaitovich, Pääbo, and Weiss 2005; Hoekstra and Coyne 2007; Carroll 2008; Nourmohammad et al. 2017; Yang et al. 2017). Genotypic changes in coding and noncoding regions will not always change the phenotype

presented suggesting that these changes are neutral and not adaptive. On the other hand, a change in a single gene can change the entire gene network and influence several adaptive traits (Knight et al. 2006; Stearns 2010). Gene misexpression can act as a speciation boundary, resulting in lower fitness than parental populations and unlikely ability of hybrids to outcompete the parents for resources (Michalak and Noor 2003; Renaut et al. 2009; Barreto et al. 2015). Mixing parental alleles can affect protein-protein interactions (Rawson and Burton 2002) possibly altering the binding ability of cis factors and trans elements resulting in gene expression in hybrid diverging from parental levels (Wittkopp et al. 2008; Meiklejohn et al. 2014; Zhang et al. 2019). Measuring the expression of hybrid genomes may indicate the mechanisms underlying the extreme phenotypes (Landry et al. 2007).

Transgressive segregation is primarily the result of multiple genes that additively produce an extreme phenotype also known as a complementary gene model (Rieseberg et al. 1999). If following the complementary gene model, hybrids would be expected to combine expression patterns of the parents to produce a more extreme phenotype (Tzin et al. 2015). To explore this further, I made recombinant inbred lines (RIL) by crossing allopatric populations of *Tigriopus californicus* with similar and differentiated maximum thermal limit in order to produce transgressive hybrids. The high intertidal copepod, *T. californicus* is a unique system to evaluate transgressive phenotypes. Populations span a large latitudinal thermal gradient from Baja California to the southern tip of Alaska (Edmands 2001; Peterson et al. 2013). Populations experience low migration between rock outcrops creating high genetic diversity between populations of even short distances (Pereira et al. 2016). Gene expression profiles of early stage hybrids (F3) of thermal sensitive and thermal tolerant populations produced hybrids of reduced fitness with transgressive gene expression (Barreto et al. 2015). In another study of thermal

sensitive and thermal tolerant hybrids, the late stage (F9) hybrids did not produce transgressive segregants for thermal phenotypes although intrinsic survivorship was lower indicating hybrid breakdown (negative transgression) (Pereira et al. 2014). In the same study, hybrids of similar thermal phenotypes produced positive transgressive segregants (Pereira et al. 2014). Unlike the two previous studies that applied no selection pressure, selection for increased thermal tolerance in F4 hybrids of thermal sensitive and thermal tolerant populations did increase the LT_{50} (Kelly et al. 2017).

A study of local adaptation to thermal stress in *Tigriopus californicus* measured the transcriptional response to an acute heat stress of a high thermal tolerant Southern California population (San Diego (SD)) and low thermal tolerant population in Central California (Santa Cruz (SCN)) (Schoville et al. 2012). Both populations significantly regulated several gene categories such as heat shock genes, proteolysis genes, cuticle genes, and mitochondrial genes. A significant result of this study showed both populations differentially regulated heat shock proteins (HSPs), but the intensity of the response was very different. For example, the SD population upregulated an HSP 70 gene to an extreme fold change of 224 whereas the SCN upregulated an orthologue to a fold change of 7. This same pattern was evident in most genes known to be a part of the heat stress system. This example of differential gene regulation in ecologically dissimilar *Tigriopus* populations may underlie the differences in thermal tolerance.

Gene expression studies are an important way to link differences in genotypes to different phenotypes. Recently, Lima and Willett (2017), showed gene expression profiles in population sets of *T. californicus* with similar thermal phenotypes are very different. These results indicate some *Tigriopus* populations have evolved different mechanisms to survive heat stress while still presenting similar thermal phenotypes. If two populations evolve different mechanisms to

tolerate the same stress, then hybrids of the two populations may acquire both mechanisms increasing the chance for transgression to occur. In this study I found gene expression patterns of transgressive segregants to incorporate expression patterns of both parental populations with other differentially regulated genes that were line specific.

Methods

Tigriopus californicus collection

Copepods were collected from high intertidal rock pools at two locations in southern California separated by ~ 34 km of ocean. The populations are from Abalone Cove (33.736857, -118.373817) from the California coast and Catalina Island (33.446687, -118.484823) from the Channel Islands. Populations were maintained in 400 ml beakers with ~250 ml 0.2um filter seawater at 34 ppt and fed dried *Spirulina* powder ab libitum. Both populations completed at least two generations in the (approx. 60 days) to remove environmental acclimation before performing any experiments. Water changes were conducted monthly by removing 1/3 volume of seawater and detritus. During water changes, beakers were mixed to maintain similar genetic composition among beakers. The beakers were maintained in constant 20 °C incubators with 12-hour light and 12-hour dark photoperiod.

Tigriopus californicus copepods are sexually dimorphic in the adult morphology and grow to a size of ~1 mm. The female has thin antennae and dark greenish to black striping indicating gonadal maturation, the male is identified by their geniculate antennae which they use to clasp immature virgin females (Vittor 1971; Burton 1987). Copulation occurs after the female matures and the pair of copepods release after wards. The female will mate only once allowing the development of crosses between genetically distinct populations in which mitochondrial heritage can be tracked (Egloff 1966, Burton 1985).

Recombinant inbred line creation

Hybrid copepod lines were created reciprocally, and mitochondrial heritage was tracked for all generations of hybrids. To produce each reciprocal cross, 20 males from one population and 20 virgin females from the other were placed into a petri dish with ~ 30 ml of filtered seawater and dried Spirulina. This step was repeated for a second petri dish for a total of 40 males and 40 females for each cross. Petri dishes were visually inspected daily for pairing, once gravid females appeared non paired males were removed. The females were maintained in the petri dish until the presence of F1 offspring were visible (copepodite stage). The F1 copepods were allowed to mature to adult and when breeding pairs were formed, the pairs were teased apart and placed into new dishes with the opposite sex from the second F1 dish. The hybrids were maintained in Petri dish mass culture with discrete non-overlapping generations until F3 generation. When the F3 females became gravid the gravid female was isolated in a 6 well tissue culture dish (Corning Costar non treated) with ~ 10 ml of seawater and ground Spirulina. This process was repeated for both reciprocal crosses to produce 50 iso-female lines for each cross. In wells that the female produced less than 5 nauplii or the egg sac did not hatch, the female was replaced with another gravid female from that cross. Wells were inspected daily, once nauplii hatched the female was removed and the well was monitored for the appearance of breeding pairs. The first breeding pair that appeared in a well was moved to a new well to create the next generation by full sib mating. Selecting the first pair selects for the fastest developers and likely the healthiest individuals from each clutch. Controlled inbreeding was conducted until the F7 generation, at this stage copepods were moved to 400 ml beakers with 200 ml of seawater and dried Spirulina to allow the recombinant inbred line to expand in numbers with overlapping generations.

CTmax

The thermal limit of each parental population and recombinant inbred line was measured with sixteen male copepods. The upper thermal limit was the point at which copepods stopped swimming and responding to external stimuli (Harada et al. 2019; Healy et al. 2019). Copepods were moved into 6 well tissue culture dishes with fresh filtered seawater and no food the day before the thermal limit test to clear their guts. The day of the experiment, the seawater was removed and replaced with fresh sweater. Copepods were moved in 100 ul aliquots of fresh seawater into a 200 ul microcentrifuge tube. Each tube contained a single copepod. Tubes were placed into a SimpliAmp Thermal Cycler (Thermo Fisher Scientific, Waltham, MA) with the lids open to allow oxygen exchange. The temperature of the thermal cycler was held constant at 20℃ for 5 minutes followed by an increase in temperature of +0.5 ℃/min from 20.1℃ to 32 ℃, followed by +0.1 ℃/min from 32.1℃ to 45 ℃. Individuals were monitored every 60 seconds from movement, when no movement was detected, a jet of water from a micropipette was used to check responsiveness. In the event the copepod did not respond, the temperature was recorded as the thermal limit (Figure A3-1).

Gene expression

Lines for gene expression measurements from each reciprocal cross were selected that displayed positive transgressive segregants for positive thermal tolerance from the previous measurement of CTmax. Before measuring gene expression, RILS and parents were retested to select the copepods with highest thermal limits in each group. Sixteen male copepods for each group were tested in the same CTmax assay design as above. The thermal limit of each individual was assessed, and they were moved from the 200 ul microcentrifuge tube into a well of a 24 well tissue culture plate (Corning, nontreated) with fresh seawater and dried *Spirulina*.

Copepods were returned to a 20℃ incubator to rest and monitored for three days. The second day after CTmax measurement, water and food was removed and replaced with fresh seawater and no food to clear their guts overnight. The next day, the seawater was removed and replaced with fresh seawater. Six copepods from each RIL and from each parental population were moved in 100 ul aliquots of seawater into 200 ul microcentrifuge tubes. Three microcentrifuge tubes were placed into a SimpliAmp Thermal Cycler (Thermo Fisher Scientific, Waltham, MA) with the lids open to allow oxygen exchange and three were placed in a 20℃ incubator. The temperature of the thermal cycler was held constant at 20℃ for 5 minutes followed by an increase in temperature of +0.5 ℃/min from 20.1℃ to 32 ℃, followed by +0.1 ℃/min from 32.1℃ till 34℃ for one hour. The one-hour hold temperature was experimentally determined as the temperature at which copepods were responsive and no copepods died during the test or up to three days after.

After the one-hour temperature hold, heat treated copepods were move to 1.5 ml microcentrifuge tubes and the seawater was removed. Immediately 250 ul of QIAzol was added and ~60 ul of 100 um silica and zirconium beads and the sample was homogenized and stored at -80 ℃. The control copepods were removed from the 20 ℃ incubator and processed the same as the heat-treated samples. Total RNA was extracted following manufactures standard the protocol using a miRNeasy Micro Kit (Qiagen Inc.). Genomic DNA was removed from samples with Turbo DNA-free kit (Thermo Fisher Scientific) using standard protocol. Total RNA was quantified fluorometrically with a Qubit 2.0 (Thermo Fisher Scientific).

Whole transcriptome library preparation

The QIAseq UPX 3’ Transcriptome libraries (Qiagen Inc.) were prepared with 5 ng of total copepod RNA from each individual copepod according to manufacturer’s handbook. There

were three biological replicate control samples and three biological treatment samples for every RIL and both parental populations. Briefly, each sample was tagged with a unique identifier and all RNA molecules were tagged with a unique molecular index (UMI). The samples were reverse transcribed to cDNA and individually tagged samples were pooled into six libraries. The final libraries were quantified using the QIAseq Library Quant kit (Qiagen Inc.) and quality control was performed by capillary electrophoresis on a TapeStation System (Agilent Technologies) using a High Sensitivity D5000 Screen Tape. The 6 libraries were sequenced with a custom asymmetric run (read 1 = 100 bp and read 2 = 27 bp, following the QIAseq protocol) on 2 lanes of a NovaSeq 6000 (Illumina) conducted at the IGM Genomics Center, University of California, San Diego, La Jolla, CA.

Read mapping

Libraries were demultiplexed and trimmed with standard Qiagen UPX 3' workflow in CLC Genomics Workbench 20.0.3 (www.qiagenbioinformatics.com/) modified to replace human genome with the *Tigriopus californicus* reference genome. A high-quality reference genome for *Tigriopus californicus* is available for transcriptome mapping and includes ~ 15600 annotated protein coding genes (Barreto et al. 2018). Because there is a high degree of divergence between *Tigriopus* populations, population specific references were created for read mapping (Burton 1997; Edmands 2001; Peterson et al. 2013; Pereira et al. 2016; Barreto et al. 2018). Population reference genomes were equalized with a custom python script to control for any differences in assemblies to reduce mapping bias (Lima and Willett 2017; Healy and Burton 2020). The parental population trimmed reads were aligned to their respective genomes using STAR (Dobin et al. 2013) and only uniquely mapped exon reads were kept. The hybrid (RILs) copepod reads were aligned to a custom reference genome built from merging the equalized genomes of

Catalina Island and Abalone Cove and the corresponding maternal mitochondrial genome. Read files from hybrids were mapped to each mitochondrial genome independently to verify correct mitochondrial background. The uniquely mapped reads and multi-mapped reads to exons were kept for the hybrids. Multi-mapped reads were only kept if they mapped to a maximum of two locations, each read counted as 0.5 of a read and added to the count of uniquely mapped reads. This procedure controlled for regions of the parental genomes that contained very similar sequence. The BAM files of mapped exon reads counts were exported to a text file with featureCounts (Liao et al. 2014). The population indicator tags and any reads mapping to mitochondrial ribosomal proteins were removed from the read counts and counts were consolidated by gene name using Excel. Mitochondrial ribosomal mRNA is a dominant signal from the mitochondrial genome, removal of these transcripts allows assessment of total read counts to the mitochondria. Samples with high percent of read counts to the mitochondrial genome (>50%) may indicate poor sample quality due to library prep or death during thermal stress.

Differential expression

Samples were filtered before analysis to remove low quality samples (UMI < 50000), seven of sixty samples were removed. Normalization and differential expression were conducted in R statistical environment (v.3.6.1) with Bioconductor package edgeR package (Chen et al. 2020). A grouping factor of treatment and line was constructed for the design matrix (i.e. AB.control, AB.treatment, AC11.control). The count matrix was filtered using the design matrix as a grouping factor to remove low expression genes with the edgeR function “filterByExp” and reads were normalized with trimmed mean of M- values (Robinson and Oshlack 2010). A total

of 10201 of the 15665 genes remained for the 53 samples, each RIL or parental group contained a minimum of two control and two treatment samples.

Samples were tested for differential expression within a line for response to thermal treatment and between treatments with a linear model using a quasi-likelihood F-test. The p-values were corrected and significance for comparisons was assessed at FDR adjusted p-value < 0.05 (Benjamini and Hochberg 1995). Lists of differentially expressed genes were compared for overlap between parental populations and RILs using Venny ver 2.1 (Oliveros 2007-2015). A heatmap was constructed with Pheatmap (Kolde 2015) comparing the log2 fold change of genes that overlap all samples from pairwise comparisons within each sample.

Results

There were sixty transcriptomes from single copepods (3 replicates *(1 treatment + 1 control) * 10 lines (8 RILs + 2 parents)) created from only 5 ng of total RNA per copepod. The samples were composed of 4 AB♀ x CAT♂ (AC) RILs, 4 CAT♀ x AB♂ (CA) RILs, Catalina Island (CAT), and Abalone Cove (AB) parental populations. The distribution of thermal limit of the six males selected from each RIL for gene expression was 0.48 ℃ to 1.78 ℃ above the parental range (Figure 3-1). Trimming and removal of low-quality reads yielded samples with 235 single reads up to 8.2 million reads with an average length of ~ 90 bp. All clean reads were mapped to the hybrid reference genome or parental equalized genomes producing alignment rates from 29.8% to 84.8% (Table A3-1). Samples yielding < 50,000 mapped reads to coding regions were removed leaving 53 samples for differential expression analysis. The *Tigriopus californicus* genome is not fully functionally annotated resulting in a few transcripts with unknown function. The protein sequence for differentially regulated unknown transcripts were analyzed for functional domains with PANNZER (Koskinen and Holm 2012). There were no

functional domains found in any differentially regulated unknown transcripts indicating these transcripts could be copepod specific stress response genes.

The Abalone Cove population was found to significantly regulate 23 genes in response to heat stress, one downregulated and remainder upregulated. The parental population downregulated the small nucleolar RNA-associated protein *UTP15-U3* gene (Table 3-1 -5.4 log2 fold change). The genes upregulated include 6 large chaperone proteins (*Hsc70-4, Hsp83, Hspa5(78kDa),*3-*Hsp70*s), 7 small chaperone proteins (*SIP1, Hsp17.8, CRYAB, Samui BAG, Hsp23, DnaJ-1, Unc-45B*), *esterase FE4, Sqstm 1, Snx 2, Gls, and NA(+)/H(-) exchanger*. The *Sqstm 1* (sequestosome) protein is involved in autophagy, the *Snx 2* (sorting nexin) works in intracellular transport from the Golgi network, endosomes, and protein recycling. The *Gls* (glutaminase kidney isoform) works in maintaining acid-base homeostasis. *NA(+)/H(-) exchanger* moves NA and H+ to prevent intracellular acidification. The *Hspa5 (Hsp70*) is a glucose regulated chaperone protein of the endoplasmic reticulum.

The Catalina Island population was found to upregulate 14 genes in response to thermal stress. The genes upregulated include 5 large chaperone proteins (*Hsc70-4, Hsp83,* 2-*Hsp70s, Hsp67Bb*), 5 small chaperone proteins (*SIP1, Hsp17.8, CRYAB, Samui BAG, Hsp23*), *MstA, esterase FE4*, a solute transporter *Slc16a1*. The *Slc16A1* transports pyruvate and lactate across cell membranes. The *MstA* gene produces a protein that is found in the cellular membrane and is responsible for transporting sugars across cellular membranes. The gene response of both Catalina Island and Abalone Cove are common genes that respond during thermal stress, differences in populations may indicate regulation of different mechanisms to buffer heat stress (Girardot et al. 2004; Fulda et al. 2009; Perdrizet et al. 2009; Vabulas et al. 2010; Pakos-Zebrucka et al. 2016).

The AB♀xCAT♂ RIL 11 significantly regulated 26 genes in response to thermal stress. The response was found to involve eight large chaperones (*Hsc70-4, Hsp83, 4-Hsp70s, Hsp67Bb, Hspa5 (78kDa)*), nine small chaperones (2-*SIP1*, 2-*CRYAB*, *Hsp17.8*, *Hsp23, DnaJ-1, MstA, Samui BAG*), three transporter proteins (*Slac5a6, Slc16a1, Snx2*), the transcription factor *Xbp1*, and the detoxification enzyme *esterase FE4*, and degradation pathway genes *Sqstm 1* and *HERC4* (Table 3-1).

The AB♀xCAT♂ RIL 35 upregulated 9 genes in during thermal stress. The genes upregulated include four different large chaperone proteins (3- *Hsp 70s*, 1- *Hsp 83*), five small chaperone proteins (*Hsp 17.8, CRYAB, Hsp 23, SIP 1, Samui Bag*).

The AB♀xCAT♂ RIL 43 produced the second largest response of the AB♀xCAT♂ RILs with 22 genes significantly regulated (Figure 3-2). The stress response included six large chaperones (*Hsp70 cognate-4(Hsc70-4), Hsp83, Hspa5(78kDa)*, 3-*Hsp70*s), eight small chaperones (*SIP1, Hsp17.8, CRYAB, Samui BAG, Hsp 23, DnaJ-1, Hsp16.48, DnaJA1*), an immune system gene *IFRD1* interferon, two transporter proteins (*Slc16a1 & Snx2*), and the helicase SEN1 that is important in mitochondrial function and redox homeostasis (Sariki et al. 2016). There were no differentially regulated genes detected in the AB♀xCAT♂ RIL 42, possibly due to the amount of variability between individuals in this line. Genotyping individuals from this line indicate lower similarity within group than witnessed with all other RILs (Chapter 4 Figure 4-3C).

The CAT♀xAB♂ RIL 13 produced the largest gene response to our thermal stress with 28 genes significantly regulated (Table 3-1). The response in CAT♀xAB♂ RIL 13 included 8 large chaperones (*Hsc70-4, Hsp83, Hspa5(78kDa)*, *Hsp67Bb,* 4-*Hsp70s*), 9 small chaperones (2-

SIP1, Hsp17.8, CRYAB, Hsp23, Samui BAG, Unc45, Hsp16.48, DnaJ1), 2 transporters (*Slc16a1* & *Snx2*), *esterase FE4, Sqstm 1*, and a stress activated protein kinase *MAPKAP1* (Table 3-1)

The CAT♀xAB♂ RIL 3 thermal response showed significant regulation in 27 genes. The CAT♀xAB♂ RIL 3 response involved 6 large chaperones (*Hsc70-4, Hsp83, Hspa5(78kDa),* 3-*Hsp70s*), 11 small chaperones (2-*SIPs, Hsp17.8, 2-CRYAB, Samui BAG, Hsp23, DnaJ-1, Aha1*(Hsp90 activator), *Hsp16.48, DnaJA1*), the transcriptional regulator *WDR13*, *esterase FE4, Sqstm 1*, 2 transporters (*Snx2* & *Slc16a1*), *Xbp1*, and *Gls* (Table 3-1).

The CAT♀xAB♂ RIL 33 upregulated 21 genes during heat stress response. The response was composed of 6 large chaperones (Hsc70-4, Hsp83, Hspa5(78kDa), 3-Hsp70s), 8 small chaperone genes (2-*SIP1, MstA, Hsp17.8, CRYAB, Samui BAG, Hsp23, DnaJ-1*), *esterase FE4, Sqstm 1*, 2 transporters (*Slc16a1* & *Snx2*), *Gls*, and *Xbp1* (Table 3-1).

The CAT♀xAB♂ RIL 27 significantly expressed 18 genes from our thermal stress experiment. The significant genes in CAT♀xAB♂ RIL 27 consisted of 6 large chaperones (*Hsc70-4, Hsp83, Hspa5(78kDa*), 3-*Hsp70s*), 7 small chaperone genes (*SIP1, Unc-45, Hsp16.48, 2-CRYAB, MstA, Hsp17.8*), *esterase FE4, Sqstm 1, and Xbp1* (Table 3-1).

I did not detect differences in most of the pairwise treatment comparisons investigating increased gene expression as a contributor to our increased thermal phenotypes. The only significant regulation of genes between thermal treatments was found in the CAT♀ x AB♂ 13 RIL (CA 13) vs AB pairwise comparison, the CAT♀ x AB♂ RIL 13 vs CAT pairwise comparison, and the CAT vs AB comparison (Table 3-2). There was one gene involved in calcium homeostasis (*regucalcin*) downregulated in the CA 13 RIL vs AB and in the CAT vs AB comparisons. The CA 13 RIL vs CAT comparison showed one upregulated gene involved in acid-base homeostasis (*Gls*).

There was an overlap of eight differentially expressed genes in response to the thermal treatment from the parental population and RILs (Table 3-1). These genes include four different large chaperone proteins (3- *Hsp 70s*, 1- *Hsp 83*), three small chaperone proteins (*Hsp 17.8*, *CRYAB*, and *SIP 1*). The log2 fold change of these genes was ranged from 2.9 to 6.9 (Table 3-1, Figure 3-2). The Catalina Island and Abalone Cove parental populations have similar thermal limits, both populations overlap by eleven genes in their response to thermal stress (Figure 3-3A). The Abalone Cove population was found to regulate twelve additional genes not found to be significant in the Catalina Island thermal response. The gene regulation in Abalone Cove, outside of the chaperone proteins, points to acid-base regulation and protein degradation as other pathways significantly regulated during thermal stress. The Catalina Island population was found to upregulate three genes not found in the thermal response in the Abalone Cove population. The non-chaperone genes upregulated in the Catalina Island population point to transport of sugars across the cell membrane.

The overlap in gene regulation of both parents and each RIL ranged from 9 to 11 genes (Figure 3-3B:H). The CA13, CA3, and AC11 RILs overlapped by the same 11 genes the CAT and AB populations overlapped. The CA27, AC43, and AC35 overlapped by 9 genes with the CAT and AB populations, these RILs did upregulate TCALIF_04876 (*esterase FE4*) or TCALIF_12628 (unknown protein). The CA33 response to thermal stress overlapped the CAT and AB populations by 10 genes, the TCALIF_12628 (unknown protein) was not found to be differentially expressed (Table 3-2). The overlap of differential expression was 13 genes when comparing all CAT♀xAB♂ RILs (Figure A3-2) and 9 genes when comparing all the AB♀ x CAT♂ RILs (Figure A3-3).

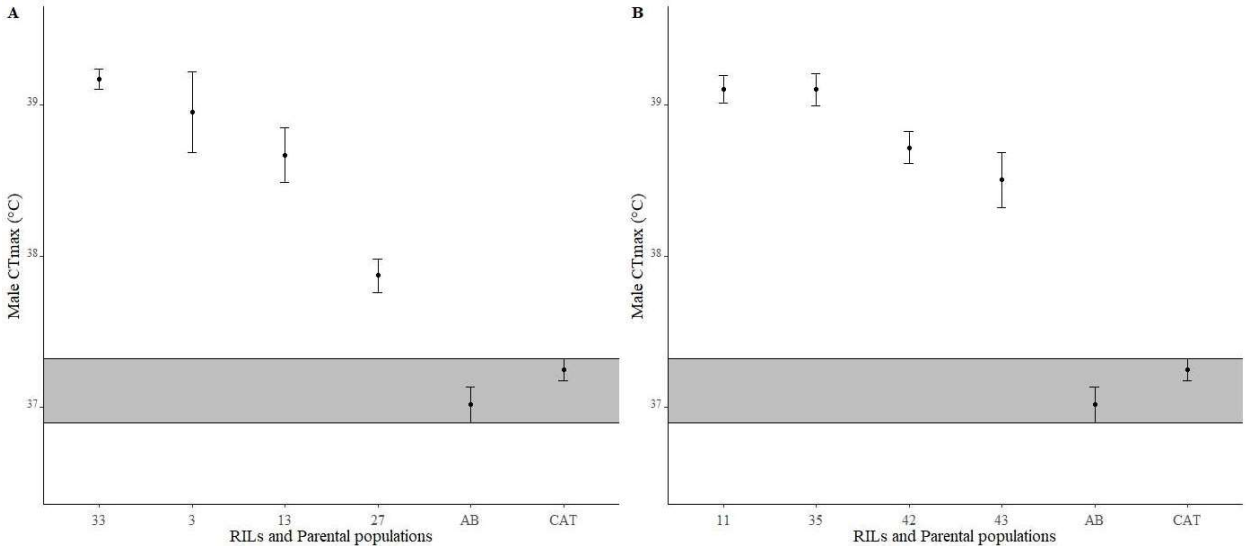

Figure 3-1. The CTmax and standard error for males from the CAT♀xAB♂ (A) and AB♀xCAT♀ (B) RILs compared to the CTmax of the parental populations AB (Abalone Cove) and CAT (Catalina Island). The numbers on the x-axis indicate the individual RILs and letters represent the parental population crossed to create the RILs. The grey band indicates the parental CTmax range, values that are above or below the grey band indicated positive or negative transgression respectively (n=6 for each).

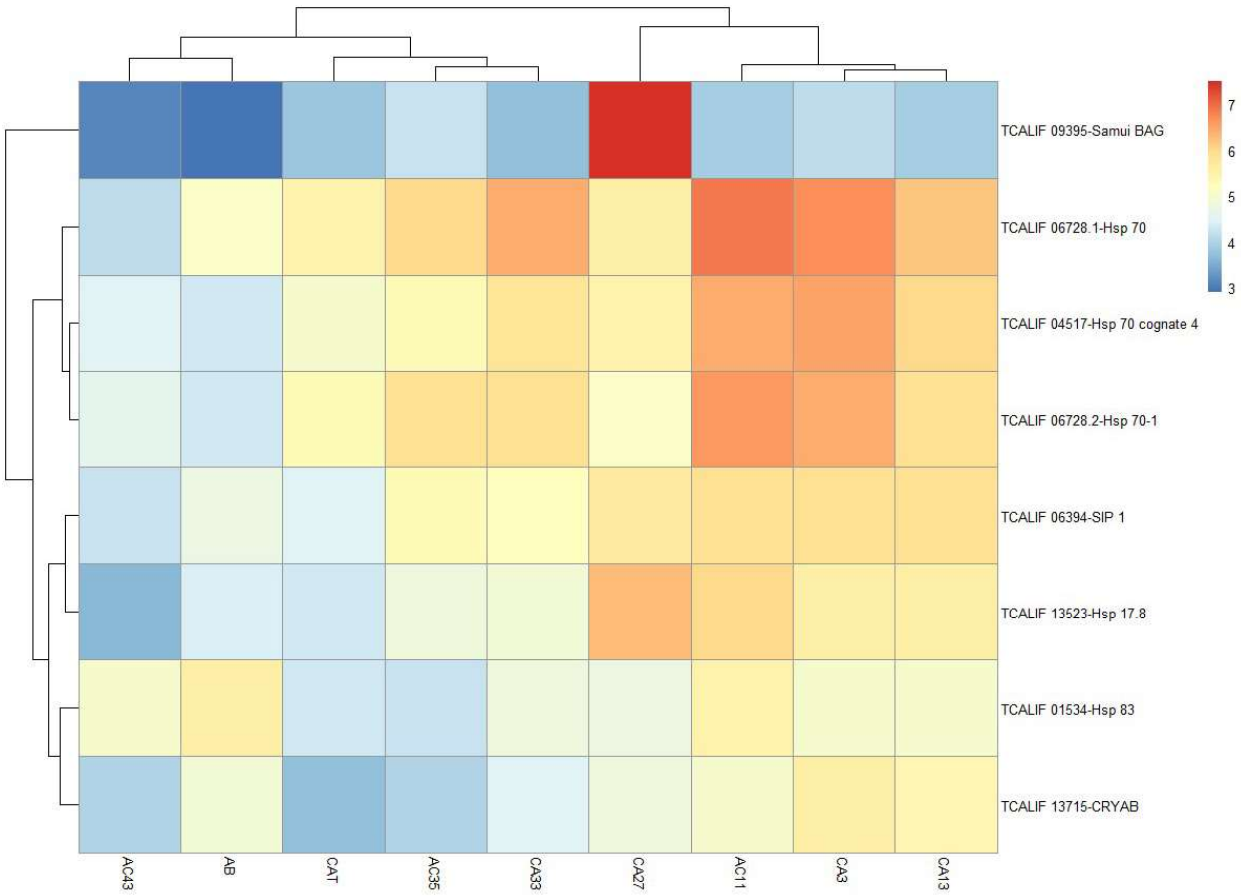

Figure 3-2. Heatmap of log2 fold change of overlapping differentially expressed genes in parental population and RILs.

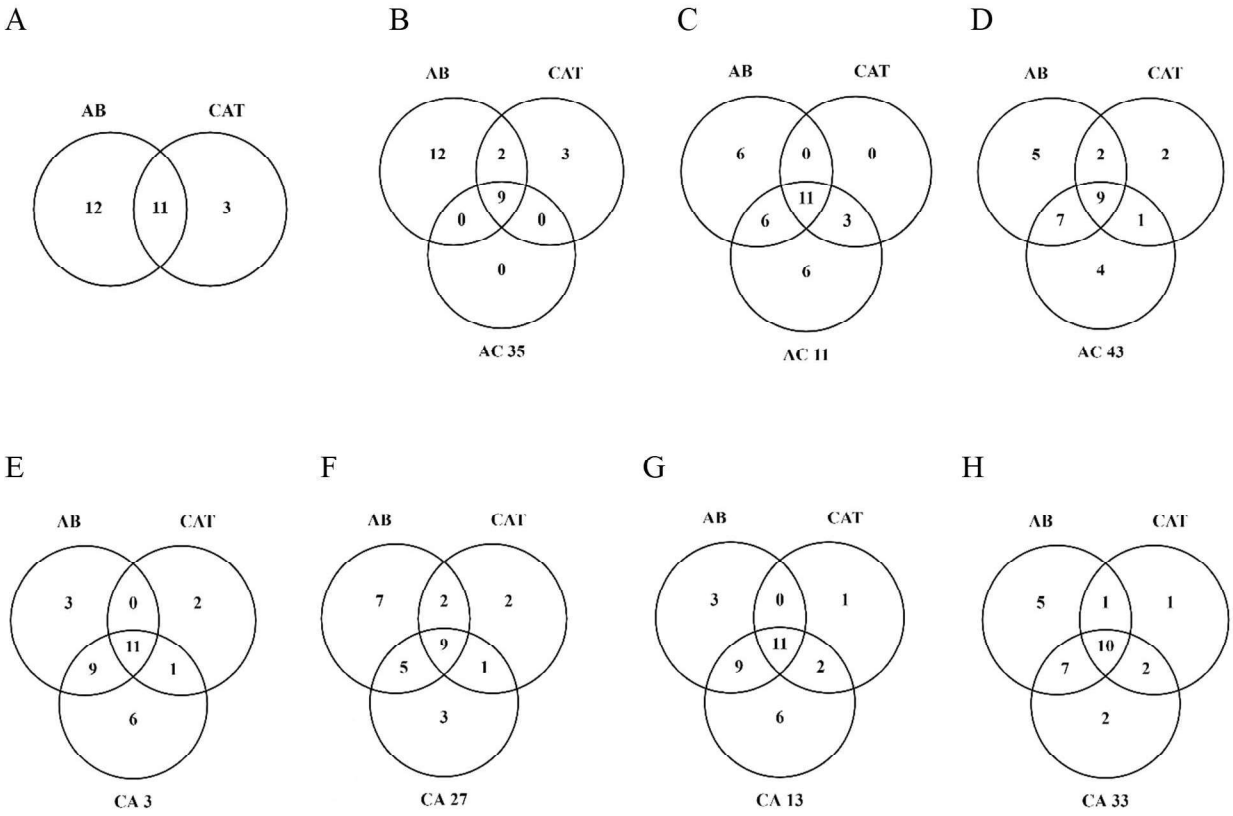

Figure 3-3. Venn diagrams of the shared number of significantly regulated genes in control vs treatment for each population (α=0.05). (A) Catalina Island (CAT) vs Abalone Cove (AB) (B) AB♀xCAT♂ RIL 35 vs Catalina Island (CAT) vs Abalone Cove (AB). (C) AB♀xCAT♂ RIL 11 vs Catalina Island (CAT) vs Abalone Cove (AB). (D) AB♀xCAT♂ RIL 43 vs Catalina Island (CAT) vs Abalone Cove (AB). (E) CAT♀xAB♂ RIL 3 vs Catalina Island (CAT) vs Abalone Cove (AB). (F) CAT♀xAB♂ RIL 27 vs Catalina Island (CAT) vs Abalone Cove (AB). (G) CAT♀xAB♂ RIL 13 vs Catalina Island (CAT) vs Abalone Cove (AB). (H) CAT♀xAB♂ RIL 3 vs Catalina Island (CAT) vs Abalone Cove (AB).

Table 3-1. Significantly regulated genes in heat stress treatment for each line. Values indicate log2 fold change and direction, an "-" indicates no significant value for the gene. The populations are: AB = Abalone Cove, CAT = Catalina Island, AC = AB♀xCAT♂, CA = CAT♀xAB♂ (grey = parental populations).

Gene ID	Gene name	AC 11	AC 35	AC 43	AB	CAT	CA 3	CA 13	CA 33	CA 27
TCALIF 04517	Hsp 70 cognate 4	6.4	5.3	4.5	4.3	5.0	6.5	6.0	5.8	5.5
TCALIF 06394	SIP 1	5.9	5.3	4.2	4.7	4.5	5.9	5.9	5.2	5.7
TCALIF 13523	Hsp 17.8	6.0	4.8	3.6	4.4	4.3	5.6	5.6	4.9	6.3
TCALIF 01534	Hsp 83	5.5	4.2	5.0	5.6	4.3	5.0	5.0	4.8	4.7
TCALIF 13715	CRYAB	5.0	4.0	4.0	4.9	3.7	5.6	5.4	4.5	4.8
TCALIF 06728.1	Hsp 70	6.9	6.0	4.1	5.1	5.5	6.7	6.2	6.4	5.6
TCALIF 06728.2	Hsp 70-1	6.6	5.9	4.6	4.3	5.3	6.4	5.9	5.9	5.1
TCALIF 09395	Samui BAG	3.9	4.2	3.1	2.9	3.8	4.1	3.9	3.7	7.5
TCALIF 11866	Hsp 23	7.3	5.4	3.8	3.6	5.7	6.6	6.1	6.2	-
TCALIF 04976	Esterase FE4	4.4	-	-	4.4	4.9	3.5	4.7	3.2	5.2
TCALIF 00753	Hspa5 (78 kDa)	3.9	-	3.6	3.3	-	5.7	3.2	4.4	5.3
TCALIF 03946	Hsp 70	3.3	-	3.5	3.1	-	4.1	4.1	3.1	4.6
TCALIF 05480	Sqstm 1	3.9	-	3.5	3.1	-	4.7	3.5	3.3	4.1
TCALIF 14754	Unknown	7.6	-	4.5	4.7	-	5.6	4.8	3.5	11.5
TCALIF 05614	DnaJ-1	5.3	-	6.8	4.5	-	7.3	4.1	4.3	-
TCALIF 11295	Snx 2	5.5	-	4.0	4.1	-	4.7	4.1	3.7	-
TCALIF 11469	SLC16A1	4.0	-	6.2	-	8.5	6.0	4.4	4.0	-
TCALIF 12628	Unknown	4.8	-	-	4.7	5.3	4.7	5.3	-	-
TCALIF 00957	Msta	4.2	-	-	-	4.5	-	-	3.6	4.1
TCALIF 09115	Xbp1	4.4	-	-	-	-	4.5	-	4.5	6.3
TCALIF 10081	SIP 1	7.6	-	-	-	-	5.5	6.2	5.5	-
TCALIF 04316	Gls	-	-	-	7.3	-	5.5	-	6.2	-
TCALIF 12367	Unc-45 homolog B	-	-	-	4.3	-	-	4.2	-	6.1
TCALIF 06906	Unknown	-	-	-	6.5	-	5.1	5.1	-	-
TCALIF 00461	Unknown	-	-	6.8	6.6	-	-	5.4	-	-
TCALIF 03439	Hsp67Bb	4.8	-	-	-	7.5	-	6.0	-	-
TCALIF 04918	Hsp 16.48	-	-	3.8	-	-	-	3.3	-	7.0
TCALIF 13714	CRYAB	3.5	-	-	-	-	3.3	-	-	5.1
TCALIF 12107	Na(+)/H(+) exchanger B		-	-	6.8	-	9.1	-	-	-
TCALIF 10670	Hspa4L (70 kDa)	3.0	-	-	-	-	-	3.2	-	-
TCALIF 04424	DNAJA1	-	-	3.5	-	-	3.6	-	-	-
TCALIF 12086	Utp15-U3 homolog 15	-	-	-	-5.4	-	-	-	-	-
TCALIF 08964	Unknown	-	-	-	-	-	-	3.6	-	-
TCALIF 11523	Unknown	-	-	-	-	-	-	4.7	-	-
TCALIF 08135	MAPKAP1	-	-	-	-	-	-	3.4	-	-
TCALIF 04182	Hsp90 activator ATPase	-	-	-	-	-	5.2	-	-	-
TCALIF 02250	WDR13	-	-	-	-	-	6.0	-	-	-
TCALIF 13035	SEN1 Helicase	-	-	4.8	-	-	-	-	-	-
TCALIF 12655	IFRD1 interferon	-	-	4.4	-	-	-	-	-	-
TCALIF 04992	Herc4	6.1	-	-	-	-	-	-	-	-
TCALIF 09415	Slc5a6 transporter	3.6	-	-	-	-	-	-	-	-

Table 3-2. Differentially regulated genes, comparison of thermal treatments between populations (adjusted FDR p-value < 0.05). The populations are denoted by CA = CAT♀xAB♂ RIL 13, CAT = Catalina Island, AB = Abalone Cove.

Gene ID	Gene name	Population comparsion	log2 Fold change
TCALIF 01383	Regucalcin	CA13 vs AB	-6.0
TCALIF 04316	Gls Glutaminas kidney isoform mitochondrial	CA13 vs CAT	7.2
TCALIF01383	Regucalcin	CAT vs AB	-6.0

Discussion

This study demonstrates the ability to sequence the transcriptomes of small (~1mm) individual copepods using low input of mRNA of only 5ng. Recent gene expression analysis of single copepods used ≥ 10 ng of mRNA and were able to fully analyze the transcriptome response per individual with a minimum of 2 million reads (Li, Arief, and Edmands 2019; Li et al. 2020). The samples in our study did not reach the 2 million mapped reads mark found in the above *Tigriopus* studies. In our study, the use of UMI's allows lower mapped reads counts for gene expression analysis because of the elimination of PCR duplication bias found in standard transcriptome library preparation (Parekh et al. 2016; Sena et al. 2018). Every RNA molecule is uniquely tagged during library preparation to easily facilitate removal of duplicated reads and an accurate representation of the gene expression at the time point sampled. This technique is also not hampered by gene size, where larger genes transcripts can overinflate the number transcripts for those genes in comparison to smaller genes.

I measured the transcriptomic response to heat-stress in parental populations and recombinant inbred transgressive segregants displaying increased thermal limit. Similar to other studies of acute heat shock (Schoville et al. 2012; Leong, Sun, and Edmands 2018) and moderate heat stress (Lima and Willett 2017), some *T. californicus* populations have a similar phenotypic response but populations also are divergent in their genetic response (Figure 3-2A). The transgressive segregants displayed maximum thermal limit phenotype outside of the parental range (+/- SE). To gain a better understanding of the genetic mechanism producing positive transgressive phenotypes I selected RILs from the Abalone Cove and Catalina Island reciprocal crosses. These crosses produced the most surviving lines with reciprocal mitochondrial backgrounds.

The main transcriptomic response of the transgressive segregants in this study was the regulation different combinations of small and large chaperone proteins in response to thermal stress. Heat shock proteins are highly conserved class of proteins that can be constitutively expressed or induced in response to cellular signals (Vabulas et al. 2010; Thibault and Ng 2013). To date, all *T. californicus* sequenced genomes have been shown to contain homologs of these proteins but regulate them to different levels (Schoville et al. 2012; Lima and Willett 2017; Barreto et al. 2018), even when the populations are from similar thermal environments and geographically close (Lima and Willett 2017; Graham and Barreto 2019). Immigration between populations is very low (Burton and Swisher 1984; Burton 1997), contributing to few shared polymorphisms between populations (Pereira et al. 2016). Combining divergent genomes that respond differently to similar stresses does produce positive thermal transgressive segregants in other populations of *T. californicus* (Pereira et al. 2014). Each recombinant inbred line is a unique combination of the parental genomes indicating there are several possible genetic architectures for increased thermal tolerance in hybrids. This study found several different overlaps in the thermal response of the transgressive segregants and the parental response.

The Abalone Cove and Catalina Island populations overlapped by 11 genes in their response to the thermal stress. The Abalone Cove response included 12 more genes not found in the Catalina Island response, while there were 3 genes found in the Catalina Island response not found in the Abalone Cove response. Of the 11 genes similarly regulated between Abalone Cove and Catalina Island, 9 genes were also differentially expressed in all the RILs. When comparing individual lines to the parental populations I found the RILs expression patterns overlapped the parental expression patterns by 9 to 11 genes, depending on the comparison.

The Abalone Cove population thermal gene response included Hspa5, Hsp70, DnaJ-1 chaperone proteins that were not found to be significantly regulated in the Catalina Island population. All these genes were found in the response of every RIL except for AB♀xCAT♂ RIL 35. The Catalina population upregulated Slc16a1 and the Abalone Cove population did not, this gene was found to be significant in all RILs except for AC35 and CA27. The Catalina population also upregulated the Hsp67Bb chaperone protein, similarly this gene was upregulated in the CA13 and AC11 but not the Abalone Cove population. The transgressive segregants appear to be using a combination of the thermal stress response from both parental populations. If true, this indicates the complementary gene model as the mostly likely genetic mechanism for the extreme positive thermal phenotypes in our F7+ RILs (Rieseberg et al. 1999; Tzin et al. 2015).

I cannot rule out the possible regulatory role of miRNAs in our hybrids or new genetic diversity due to intragenic crossovers during meiosis as the reason for the transgressive thermal phenotypes. The 3' sequencing of ~ 100bp is limited in the amount of each transcript sequence that can be identified. Intragenic crossover events can create novel genetic combinations and transgressive gene expression patterns (Liu et al. 2018). Populations of *Tigriopus californicus* have been shown to utilize different miRNAs in regulation of gene expression in response to temperature stress (Graham and Barreto 2019). Based on the type of miRNA inherited, hybrids could upregulate the expression of genes normally not contributing to the thermal phenotype in one the parent populations. In our study, the small chaperone protein, Stress Inducible Protein 1 (SIP1, TCALIF_10081 Table 3-1) was found to expressed in the ABxCAT RIL 11 and CATxAB RILs 3,13, and 33 but not in either parents or the other RILs. Barreto et al. (2015) knocked down expression of SIP1 in the San Diego population using RNAi greatly reducing the survivorship

from acute heat stress, indicating the importance of its upregulation during thermal stress response in this population. The use of SIP1 in some of our RILs showing the highest thermal tolerance may indicate a gain of function by upregulation of this gene in response to heat stress not incorporated in the thermal response of either parental population.

Conclusion

Transgressive segregation has been a useful tool in plant breeding to increase desirable traits in plants (Mackay et al. 2020). In animals, transgressive segregation has been proposed as a possible conservation tool to mitigate the loss of threatened species (Provan and Maggs 2012; Nicotra et al. 2015; Macdonald et al. 2017; Gaitán-Espitia and Hobday 2021). Positive transgressive segregants for maximum thermal limit were identified in our crosses between populations with similar thermal phenotypes and within the same biogeographic zone. Results from our study provide an understanding of gene regulation contributing to increased thermal tolerance as a result of hybridization.

Acknowledgements

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Chapter 3, in part is currently being prepared for submission for publication of the material. Blackwell, Reginald C.; Healy, Timothy; Burton, Ronald. The dissertation author was the primary investigator and author of this material.

Appendix

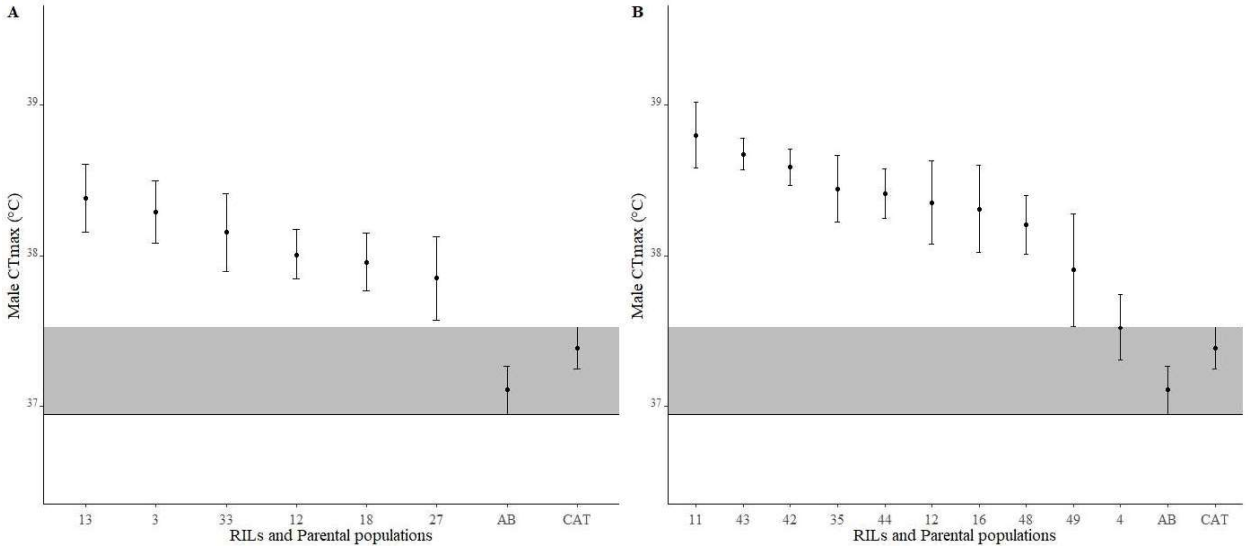

Figure A3-1. The CTmax and standard error for males from the CAT♀xAB♂ (A) and AB♀xCAT♀ (B) RILs compared to the CTmax of the parental populations AB (Abalone Cove) and CAT (Catalina Island). The numbers on the x-axis indicate the individual RILs and letters represent the parental population crossed to create the RILs. The grey band indicates the parental CTmax range, values that are above or below the grey band indicated positive or negative transgression respectively (n=16 for each).

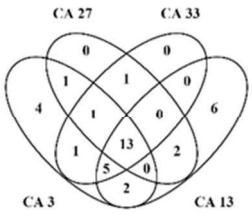

Figure A3-2. Venn diagram of the overlap in differential expression of the CAT♀xAB♂ RILs.

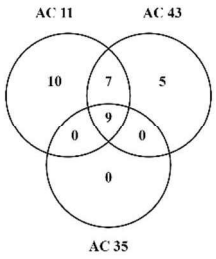

Figure A3-3. Venn diagram of the overlap in differential expression of the AB♀xCAT♂ RILs.

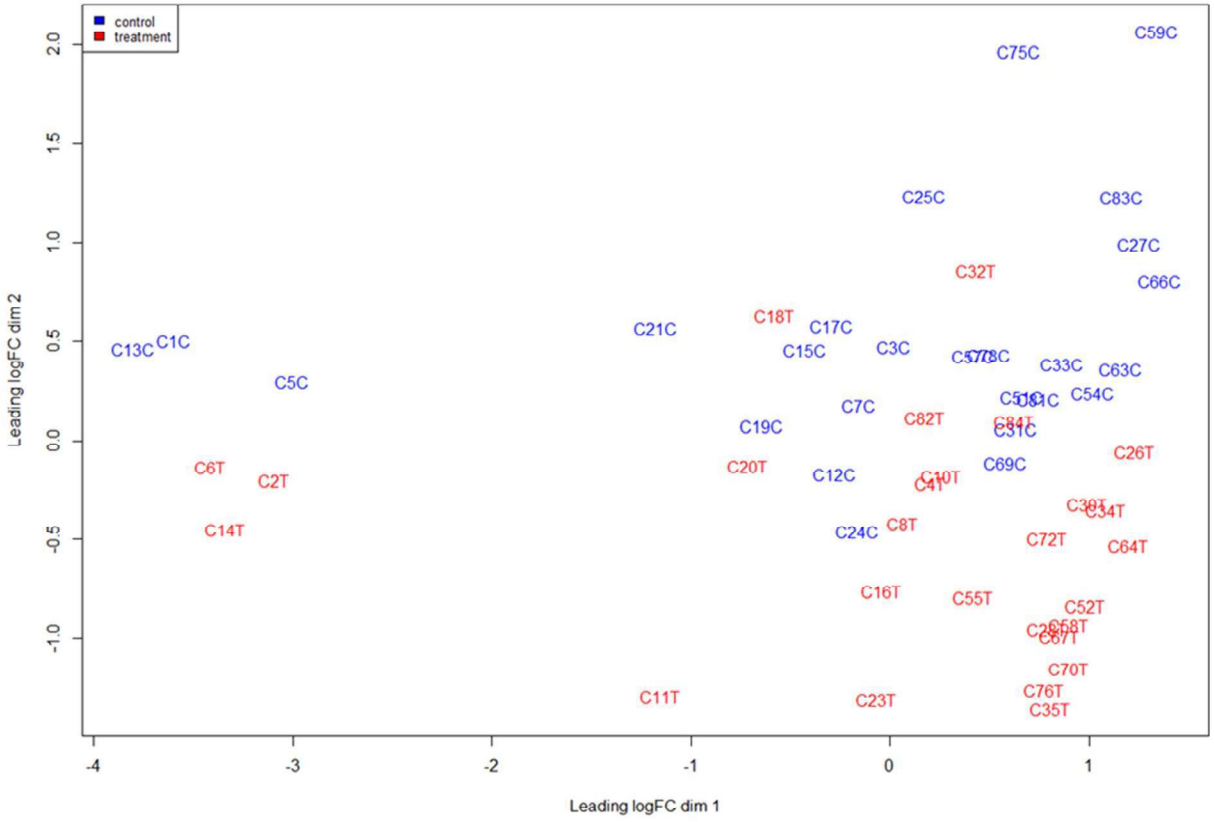

Figure A3-4. A multidimensional scaling plot of samples separated by treatment.

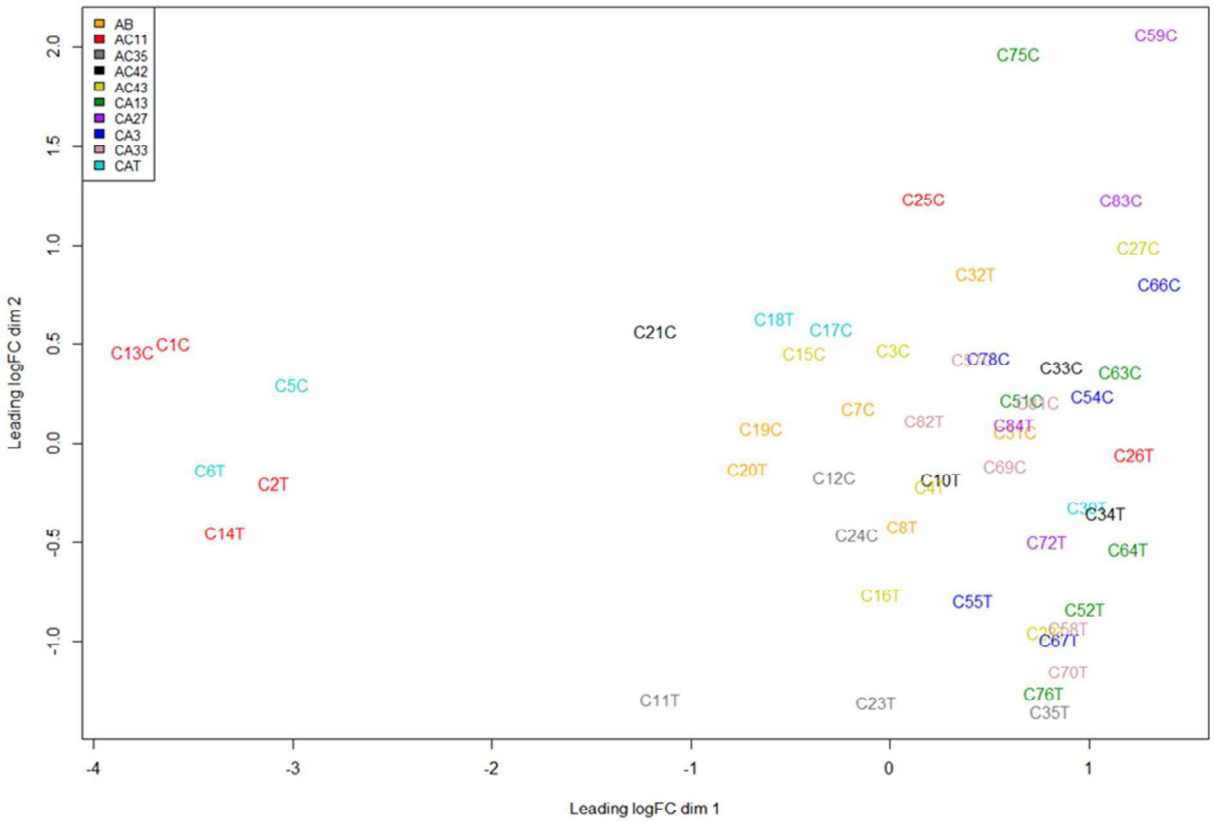

Figure A3-5. A multidimensional scaling plot of samples separated by population or line.

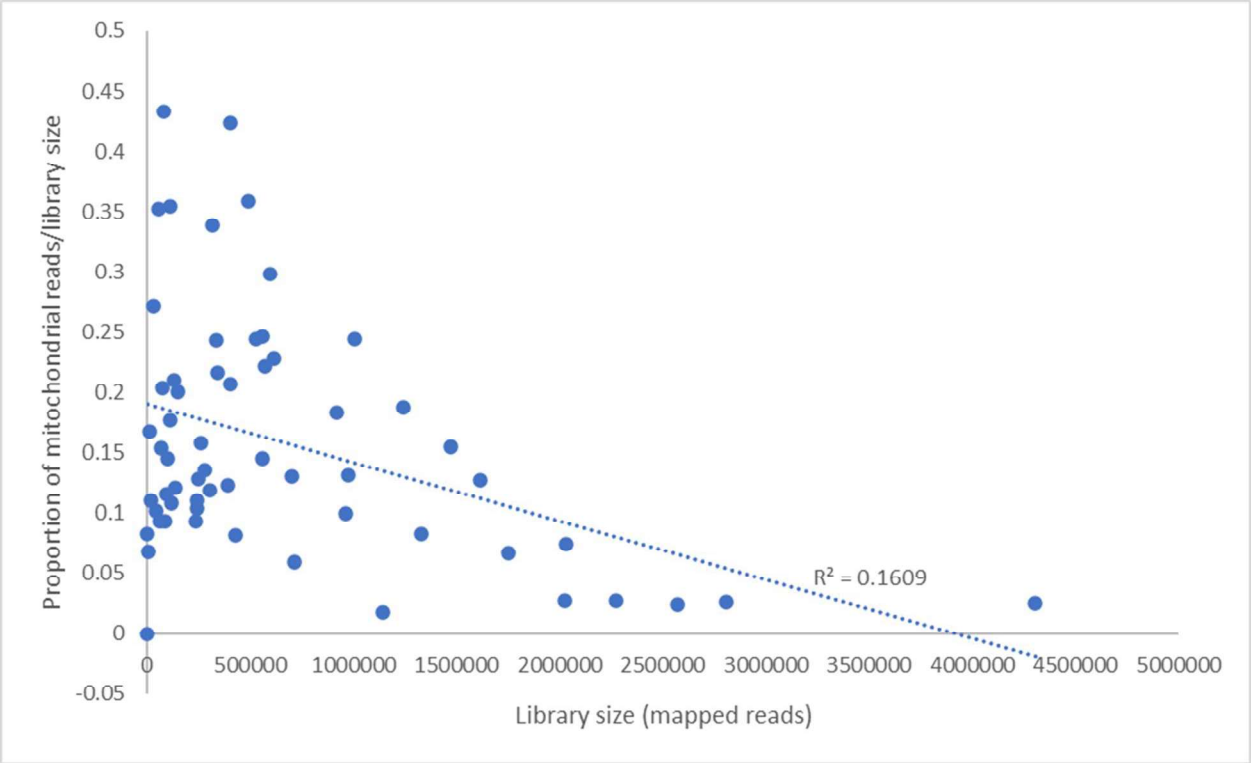

Figure A3-6. The relationship of the proportion of mitochondrial reads compared to the size of libraries for each sample.

Table A3-1. The number and percent of reads mapped to coding sequence of the hybrid reference genome.

Sample ID	Population	Treatment	Trimmed reads	Number of reads mapped	Percent mapped
C19C	AB	control	4414933	382463	69.02
C20T	AB	treatment	1120116	127479	44.08
C31C	AB	control	6180377	461090	64.61
C32T	AB	treatment	533147	54513	62.14
C7C	AB	control	4232168	494785	72.78
C8T	AB	treatment	2845046	226294	45.99
C13C	AC11	control	8255974	1217554	84.43
C14T	AC11	treatment	7600761	678636	45.16
C1C	AC11	control	4679186	719767	84.84
C2T	AC11	treatment	5566807	695596	69.09
C25C	AC11	control	2813649	406572	45.63
C26T	AC11	treatment	1402757	214213	51.46
C11T	AC35	treatment	6095139	784057	75.99
C12C	AC35	control	3671075	495282	71.46
C23T	AC35	treatment	5663453	793526	76.33
C24C	AC35	control	3240521	550415	67.50
C35T	AC35	treatment	2776683	438673	67.76
C36C	AC35	control	235	75	46.38
C9C	AC42	control	107644	21325	44.58
C10T	AC42	treatment	2324835	316086	44.14
C21C	AC42	control	3892707	764450	44.37
C22T	AC42	treatment	25634	7376	49.12
C33C	AC42	control	3601066	532940	64.13
C34T	AC42	treatment	2069503	262741	60.48
C15C	AC43	control	1858706	216880	43.06
C16T	AC43	treatment	1782781	272301	60.52
C27C	AC43	control	896297	101084	40.16
C28T	AC43	treatment	5318698	777266	65.05
C3C	AC43	control	2220522	308720	54.27
C4T	AC43	treatment	3066581	424959	53.10
C51C	CA13	control	4262601	558942	61.60
C52T	CA13	treatment	2845440	351437	58.99
C63C	CA13	control	2677663	286203	61.46
C64T	CA13	treatment	4309553	479584	57.96
C75C	CA13	control	440285	84320	62.45
C76T	CA13	treatment	4916073	914443	72.02
C59C	CA27	control	698415	85159	34.62
C60T	CA27	treatment	845906	95072	29.80
C71C	CA27	control	40933	6878	34.85
C72T	CA27	treatment	4858977	797476	60.02
C83C	CA27	control	1778367	188704	31.65
C84T	CA27	treatment	3593350	513856	54.31
C54C	CA3	control	2797944	315907	63.65
C55T	CA3	treatment	1172435	198309	62.16
C66C	CA3	control	979480	163650	51.26
C67T	CA3	treatment	4800611	770298	71.61
C78C	CA3	control	2807658	459832	61.12
C79T	CA3	treatment	73557	15681	63.23
C57C	CA33	control	4619487	401063	42.97
C58T	CA33	treatment	3460835	511191	67.53
C69C	CA33	control	3853656	607058	70.36
C70T	CA33	treatment	4681785	700869	66.13
C81C	CA33	control	1874517	419889	66.29
C82T	CA33	treatment	853776	155326	68.57
C5C	CAT	control	2321027	54596	81.56
C6T	CAT	treatment	5880763	176885	83.50
C17C	CAT	control	1902506	282036	51.86
C18T	CAT	treatment	1648257	199225	46.10
C29C	CAT	control	677761	85959	54.29
C30T	CAT	treatment	4001475	419420	57.17

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Chapter 4

Genomic composition of transgressive segregants with increased thermal limit in *Tigriopus californicus*

Introduction

Hybridization is a prevalent and important evolutionary mechanism that can create new genetic and phenotypic variation (Abbott et al. 2016) or reduce variation by homogenizing populations (Rhymer and Simberloff 1996). In general, hybridization is the crossing of two genetically divergent taxa that produces viable offspring (Barton 2001; Burke and Arnold 2001; Mallet 2005). Interactions between parental genomes in hybrids can result in a wide variation of fitness (Taylor and Larson 2019). Although parental level fitness is the most common outcome, a reduction or increase in hybrid fitness is frequent (Arnold and Hodges 1995). In some cases hybrid fitness is so extreme it is outside the range of both parents in a positive or negative direction. The hybrids exhibiting extreme phenotypes are known transgressive segregants (Rieseberg et al.1999; Rieseberg et al. 2003; Soltis 2013). Evidence of transgressive segregation is commonly found in plants and is starting to become recognized as an important evolutionary process in animals (Mallet et al. 2015; Mackay et al. 2020). Genomes of transgressive segregants may be highly admixed (Lexer et al. 2003; Rieseberg 2003) or more similar to adaptive introgression where just a few loci are present from one parent (Mavárez et al. 2006).

The first generation hybrids contain a full set of coadapted gene complexes from each parent allowing systems to function properly. Because of this, the F1 hybrids typically show fitness similar to parentals. Over successive generations, parental genomic blocks are broken down by recombination. Recombination can disrupt intrinsic coadapted gene complexes resulting in either incompatiblities and reduced fitness or novel beneficial combinations that increase fitness (Rieseberg et al. 1999). The novel transgressive phenotypes are primarily the result of complementary gene action in hybrids. To a lesser degree, epistasis and overdominance are mechanisms that contribute to transgressive phenotypes (Rieseberg et al. 2003).

Rieseberg described the general genetic architecture of transgressive segregation as common in natural and domesticated populations (Rieseberg et al. 2003). Transgression is typically the result of recombination in hybrids of antagonistic quantitiative trait loci (QTL) from the parental populations. Selection in isolation is an important step for transgression to be present in hybrids. If the populations are experiencing contrasting direction of selection and isolated then they will diverge for different trait profiles and the alleles favorable for a trait will be population specific. If the direction of selection is the same for both populations and they are isolated then the favorable alleles will be dispersed between the populations. These are general rules, transgressive segregant genomes will not always contain only alleles for increasing the trait value. In a high oil maize long term experiment 50 QTLs are known for this trait, the high producing lines were found to be fixed at 1/5 of the sites for the decreasing allele (Laurie et al. 2004). Interogating the genomic composition of transgressive hybrids is still an important step in understanding the underlying mechanism of hybrid fitness.

The copepod *Tigriopus californicus* is a unique system to study the effects of hybridization. Populations live in ephemeral intertidal tidepools habitats from Baja California, Mexico to the southern tip of Alaska (Edmands 2001; Willett and Ladner 2009; Peterson et al. 2013). Populations may experience high mortality when conditions remain outside of optimal for long periods of time (Dethier 1980; Dybdahl 1994; Burton 1997; Altermatt et al. 2012). There is a negative correlation between temperature tolerance and latitude with southern populations surviving higher temperatures (Willett 2010; Pereira et al. 2017; Healy et al. 2019). Geographically close populations produce similar thermal phenotypes but have evolved differing gene expression patterns (Lima and Willett 2017). Within population variation is low compared to between population variation indicating low migration rates (Burton 1986; Edmands 2001;

Burton et al. 2007; Willett and Ladner 2009). Divergence between populations can be as high as 20% between mitochondrial genomes (Barreto et al. 2018). Even with such high interpopulation divergence, *T. californicus* populations from California and further north hybridize easily in the lab.

Fitness measurements in hybrids are easily quantified revealing heterosis and outbreeding depression in a wide range of *T. californicus* crosses (Edmands 1999). Most crosses produce F1 generation hybrids with parental level fitness. The second generation hybrids often display reduced fitness attributed to the disruption of coadapted gene complexes (Burton et al. 1999; Burton et al. 2006; Ellison and Burton 2006). Later generation hybrids also display differences in fitness. Novel (transgressive) morphometric characters in late stage hybrids occurs in different combinations of population crosses (Pritchard et al. 2012; Hwang et al. 2016). Gene expression measurements of reduced fitness F3 hybrids showed transgressive gene expression (Barreto et al. 2015), where upregulation of antioxidant pathways may compensate for observed increases in oxidative stress in hybrids (Barreto and Burton 2013). Hybridization between two southern California populations resulted in some later generation hybrids with greater thermal tolerance than either parental population. Transgressive thermal tolerance was shown to occur between ecologically similar populations (i.e., with similar thermal tolerances) but not between ecologically dissimilar populations (Pereira et al. 2014).

To investigate the genomic composition of transgressive segregants for increased thermal limit, I created recombinant inbred lines (RILs) between two populations (Catalina Island and Abalone Cove) with similar thermal limits. Using 3' RNA-seq on individual copepods, I compared gene expression in RILs following thermal stress (Chapter 3). Because high levels of sequence divergence between parental populations (Barreto et al. 2011; Pereira et al. 2016), the

RNAseq data also permits genotyping of the individual hybrid copepods within and between RILs based on SNPs in the transcriptome reads. This method allowed me to assess both the patterns of gene expression and the genomic composition of individual copepods within and between RILs. Here I present genotypic data on copepods from eight RILs displaying transgressive thermal phenotypes. These results provide a foundation for the understanding of the genomic composition of increased thermal tolerance of transgressive segregants in *Tigriopus californicus*.

Methods

***Tigriopus californicus* collection**

Copepods were collected from high intertidal rock pools at two locations in southern California separated by ~ 34 km of ocean. The populations are from Abalone Cove (33.736857, -118.373817) from the California coast and Catalina Island (33.446687, -118.484823) from the Channel Islands. Populations were maintained in multiple 400 ml beakers with ~250 ml 0.2um filter seawater at 34 ppt and fed dried *Spirulina* powder ab libitum. Both populations completed at least two generations in the (approx. 60 days) to remove environmental acclimation before performing any experiments. Water changes were conducted monthly by removing 1/3 volume of seawater and detritus. During water changes, beakers containing the same population were mixed to maintain similar genetic composition. The beakers were maintained in constant 20 ℃ incubators with 12-hour light and 12-hour dark photoperiod.

Tigriopus californicus copepods are sexually dimorphic in adult morphology. Females have thin antennae and dark greenish to black striping indicating gonadal maturation, while males are identified by their geniculate antennae which they use to clasp immature virgin females (Vittor 1971; Burton 1987). Copulation occurs after the female matures and the male

releases the female after fertilization. The female will mate only once allowing the development of crosses between genetically distinct populations in which mitochondrial heritage can be tracked (Egloff 1966, Burton 1985).

Recombinant inbred line creation

Hybrid copepod lines were created reciprocally, and mitochondrial heritage was tracked for all generations of hybrids. Recombinant inbred lines (RILs) were labelled by the maternal population crossed with paternal population, i.e. CATxAB = CAT♂xAB♀ and ABxCAT = AB♂xCAT♀. To produce each reciprocal cross, 20 males from one population and 20 virgin females from the other were placed into a petri dish with ~ 30 ml of filtered seawater and dried Spirulina. This step was repeated for a second petri dish for a total of 40 males and 40 females for each cross. Petri dishes were visually inspected daily for pairing, once gravid females appeared non paired males were removed. The females were maintained in the petri dish until F1 offspring were visible (copepodite stage). The F1 copepods were allowed to mature to adult and when clasped pairs were formed, the pairs were teased apart and placed into new dishes with the opposite sex from the second F1 dish. The hybrids were maintained in Petri dish mass culture with discrete non-overlapping generations until F3 generation. When the F3 females became gravid, each female was isolated in a well of a 6 well tissue culture dish (Corning Costar non treated) with ~ 10 ml of seawater and ground Spirulina. This process was repeated for both reciprocal crosses to produce 50 iso-female lines for each cross. In wells that the female produced less than 5 nauplii or the egg sac did not hatch, the female was replaced with another gravid female from that cross. Wells were inspected daily, once nauplii hatched the female was removed and the well was monitored for the appearance of breeding pairs. The first breeding pair that appeared in a well was moved to a new well to create the next generation by full sib mating.

Selecting the first pair selects for the fastest developers and likely the healthiest individuals from each clutch. Controlled inbreeding was conducted until the F7 generation (i.e., 3 generations of inbreeding), at this stage copepods were moved to 400 ml beakers with 200 ml of seawater and dried Spirulina to allow the recombinant inbred line to expand in numbers with overlapping generations.

CTmax

The thermal limit of each parental population and recombinant inbred line was measured with sixteen male copepods. The upper thermal limit was the point at which copepods stopped swimming and responding to external stimuli (Harada et al. 2019; Healy et al. 2019). Prior to testing, copepods were moved into 6 well tissue culture dishes with fresh filtered seawater and no food for 24 hrs. to clear their guts. The day of the experiment, the seawater was removed and replaced with fresh sweater. To start the test, copepods were moved in 100 ul aliquots of fresh seawater into a 200 ul microcentrifuge tube. Each tube contained a single copepod. Tubes were placed into a SimpliAmp Thermal Cycler (Thermo Fisher Scientific, Waltham, MA) with the lids open to allow oxygen exchange. The temperature of the thermal cycler was held constant at 20℃ for 5 minutes followed by an increase in temperature of +0.5 ℃/min from 20.1℃ to 32 ℃, followed by +0.1 ℃/min from 32.1℃ to 45 ℃. Individuals were monitored every 60 seconds for movement, when no movement was detected, a jet of water from a micropipette was used to check responsiveness. In the event the copepod did not respond, the temperature was recorded as the thermal limit.

Single copepod gene expression

Lines from each reciprocal cross that on average displayed thermal tolerances above those of the parents (i.e., transgressive segregants) were selected for gene expression

measurements and genotyping. Within each RIL, 16 male copepods were retested to select those with highest thermal limits in each group using the same CTmax assay design as above. Roughly six months, or five to seven generations worth of time passed between the initial CTmax measurements (Figure 1) and the CTmax gene expression (Figure 2). Copepods were removed from thermal cycler at their thermal limit and isolated into a single well of a 24 well tissue culture dish to recover for three days. The third day, six copepods were selected from each RIL. Three males for each line were assayed for gene expression response in a ramping thermal assay following the same protocol as the CTmax assay but with a 1 hour hold at 34°C. The other three males were maintained in an 20°C for the duration of the temperature stress. After heat treatment, copepods were moved to 1.5 ml microcentrifuge tubes and the seawater was removed. Immediately 250 ul of QIAzol and ~60 ul of 100 um silica and zirconium beads was added. The sample was homogenized and stored at -80 °C. The control copepods were removed from the 20 °C incubator and processed the same as the heat-treated samples. Total RNA was extracted following manufactures standard the protocol using a miRNeasy Micro Kit (Qiagen Inc.). Genomic DNA was removed from samples with Turbo DNA-free kit (Thermo Fisher Scientific) using standard protocol. Total RNA was fluorometric quantified with a Qubit 2.0 (Thermo Fisher Scientific).

Single copepod 3' mRNA transcriptome libraries

The QIAseq UPX 3' Transcriptome libraries (Qiagen Inc.) were prepared with 5 ng of total copepod RNA from each individual copepod according to manufacturer's handbook. There were three biological replicate control samples and three biological treatment samples for every RIL. Briefly, each sample (individual copepod) was tagged with a unique identifier and all RNA molecules were tagged with a unique molecular indices (UMI). The UMI allows the confident

removal of duplicate reads from PCR amplifications and therefore less sequencing depth to fully capture the transcriptome (Jarvis et al. 2020). The samples were reverse transcribed to cDNA and individually tagged samples were pooled into six libraries. The final libraries were quantified using the QIAseq Library Quant kit (Qiagen Inc.) and quality control was performed by capillary electrophoresis on a TapeStation System (Agilent Technologies) using a High Sensitivity D5000 Screen Tape. The 6 libraries were sequenced with a custom asymmetric run (read 1 = 100 bp and read 2 = 27 bp) on 2 lanes of a NovaSeq 6000 (Illumina) conducted at the IGM Genomics Center, University of California, San Diego, La Jolla, CA.

Read Mapping

Libraries were demultiplexed and trimmed with standard Qiagen UPX 3' workflow in CLC Genomics Workbench 20.0.3 (www.qiagenbioinformatics.com/) modified to replace human genome with the *Tigriopus californicus* reference genome. A high-quality reference genome for *Tigriopus californicus* (~190 Mb) is available for transcriptome mapping and includes ~ 15600 annotated protein coding genes (Barreto et al. 2018). Because there is a high degree of divergence between *Tigriopus californicus* populations (Burton 1997; Edmands 2001), population specific references were created for read mapping (Pereira et al. 2016; Barreto et al. 2018). Population reference genomes were equalized with a custom python script to control of any differences in assemblies to reduce mapping bias (Lima and Willett 2017; Healy and Burton 2020).

The hybrid (RILs) copepod reads were aligned with STAR (Dobin et al. 2013) to a custom reference genome built from merged equalized genomes of Catalina Island and Abalone Cove populations. The BAM file of uniquely mapped reads was filtered for a MAPQ score > 20

and read depth was calculated for all genomic positions. The read depth file was imported into R for allele frequency analysis (R software team).

Reads were filtered to retain only reads with population specific SNPs, any SNP with less than five reads was removed. There are 1590361 diagnostic SNPs scattered across the genome differentiating the Catalina Island and Abalone Cove populations. The twelve *Tigriopus californicus* chromosomes were divided into 1 Mb genomic windows resulting in 188 windows. Allele frequency of the maternal side of the cross was calculated for every window, a minimum of five SNPs to retain a window. There were three genotypes possibilties for each window, homozygous AB, homozygous CAT, or heterozygous. A SNP was called fixed for maternal population ≥ 0.95 and paternal population ≤ 0.05. All values in between were called as heterozygous. The consensus genotype in each RIL was called if all individuals carried the same genotype at a genomic window. Windows where no genotype was called were not used to calculate consensus in a RIL.

Results

CTmax

The CTmax of all RILs except ABxCAT 4 were outside of the parental range indicating positive transgression for thermal limit (Figure 4-1A & 4-1B). No lines displayed a CTmax below the parental range (negative transgression). The Catalina Island (CAT) population had a higher thermal range than the Abalone Cove (AB) population.

I selected four postive transgressive lines from each reciprocal cross to produce transcriptomes. The RILs selected for gene expression were ABxCAT RILs 11, 35, 42, 43, and CATxAB RILs 3, 13, 27, 33. Each RIL was retested for CTmax and we selected the six copepods with the highest thermal limit in each. I saw a slight increase in average CTmax of the

six copepods selected for gene expression in each RIL with the exception of CATxAB RIL 27 (Figure 4-1 & 4-2). The largest increase, ~1 ℃, was found in the CATxAB RIL 33 copepods (39.16 ℃ vs 38.15 ℃, Table A4-3 & A4-4). The persistence of the increased thermal limit phenotype over six months in the retested RILs indicates the phenotype was heritably stable in each RIL.

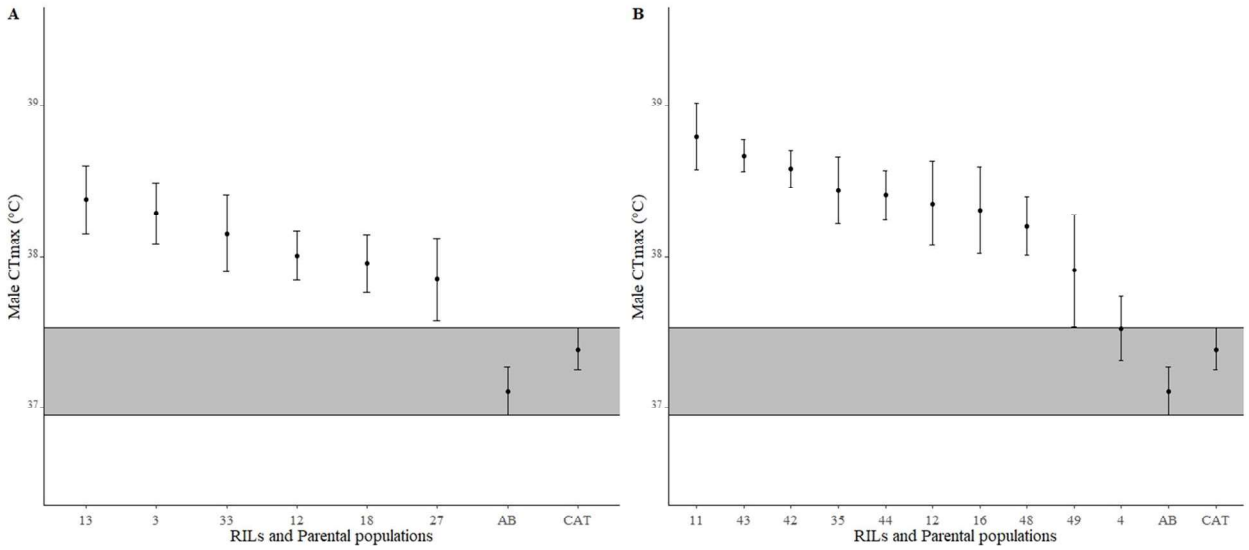

Figure 4-1. The CTmax and standard error for males from the CAT♀ x AB♂ (A) and AB♀ x CAT♂ (B) RILs compared to the CTmax of the parental populations AB (Abalone Cove) and CAT (Catalina Island). The numbers on the x-axis indicated the individual RILs and the letters respresent the parental population crossed to create the RILs. The grey band indicates the parnetal CTmax range, values above or below the grey band indicated positive or negative transgression respectively (n = 16 for each).

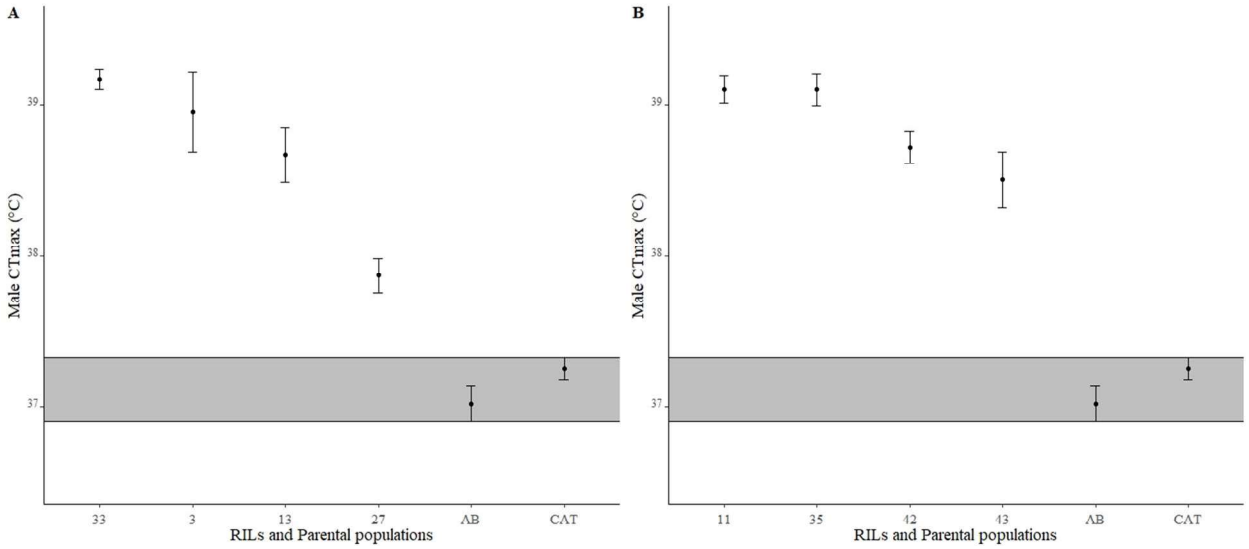

Figure 4-2. The CTmax and standard error for males from the CAT♀ x AB♂ (A) and AB♀ x CAT♂ (B) RILs compared to the CTmax of the parental populations AB (Abalone Cove) and CAT (Catalina Island). The numbers on the x-axis indicated the individual RILs and the letters respresent the parental population crossed to create the RILs. The grey band indicates the parnetal CTmax range, values above or below the grey band indicated positive or negative transgression respectively (n = 6 for each).

Genomic composition - allele frequencies

To investigate the genomic composition of transgressive segregants displaying increased thermal limit I generated 48 3' mRNAseq transcriptomes. Each transcriptome was created from a single copepod. Sequence depth per sample was variable and ranged from a low of 425 UMI reads to greater than 22 million UMI reads before trimming and filtering (Table A4-1). Similar to sequencing depth, the trimmed and filtered reads, mapped reads, and number of populations specific SNPs identified per sample were greatly variable between samples. All samples were retained for analysis of hybrid genome composition regardless of the depth of reads. Not all windows were retained in each individual due to low number of SNPs in a window. Sample ABxCAT 35 C3 of 425 UMI reads identified no SNPs and the sample ABxCAT 11 C2 of > 22 million UMI reads identified > 68k SNPs (Supplementary table 1). To genotype a single

individual across the entire genome, samples yielding > 15k diagnostic SNPs were effective (Table A4-1).

ABxCAT RILs

The ABxCAT RIL 11 individuals were primarily composed of the CAT genotype. The AB genotype was primarily fixed on the ends of chromosome 9 and 7. The CAT genotype was consistently fixed on the majority of chromosome 6,11,12, the end of chromosome 2,4, and middle of chromosome 1 (consensus Figure 4-3A). Excluding samples with < 98% of windows genotyped (Sample C3 and T3, missing 2.1% and 2.7% of windows respetively) the ABxCAT RIL 11 averaged 13.7 % AB, 50.7 % CAT, 35.7 % heterozygous regions (Table A4-2).
The ABxCAT RIL 35 individuals primarily composed of the CAT genotype. The AB genotype was fixed on the middle of chromosome 2, 9, the beginning and ends of chromosome 3. The CAT genotype was fixed at the beginning of chromosome 7,8, middle of chromosome 10,11, the end of chromosome 12, and ~ 2/3 of chromosome 4 (consensus Figure 4-3B). Sample C3 did not provide any SNPs in our analysis due to low read counts for this sample. Excluding sample C3 the ABxCAT RIL 35 averaged 20.1 % AB, 43.4 % CAT, and 36.5% heterozygous across the genome (Table A4-2).

The ABxCAT RIL 42 five of six samples yielded < 15.5k SNPs which resulted in only one sample (C3) having coverage over the entire genome (Figure 4-3C, Table A4-1). For all chomosomes except chromosome 5 and chromosome 8, one to two windows were heterozygous across samples. A single window was fixed for CAT genotype at the beginning of chromosome 3 (consensus Figure 4-3C). Excluding samples < 98% windows genotyped (T2, C1, C2) the ABxCAT RIL 42 averaged 16.1% AB, 28.4% CAT, and 55.1% heterozygous regions (Table A4-2).

The ABxCAT RIL 43 individuals were primarily heterozygous across the genome. The AB genotype was fixed on end of chromsome 2, 5, and beginning of chromsome 3. The CAT genotype was fixed on beginning of chromosome 12, 8, 11, and the middle of chromsome 2,11 (consensus Figure 4-3D). Not including samples genotyped at < 98% of windows (C1, C2, C3) the ABxCAT RIL 43 averaged 20.9 % AB, 27.8% CAT, and 50.9% heterozygous across the genome (Table A4-2).

Comparing consensus regions between the four ABxCAT RILs yielded no similar regions between them (consensus Figure 4-3E). There was a similar pattern between all the ABxCAT RILs though, the AB genotype was fixed at ~ 21% or less of the genomic composition (Table A4-2). Other similarites were more apparent when comparing 3 RILs only. There are overlapping CAT genotypes for RILs 11, 35, and 43 on chromosome 11 and 12.

CATxAB RILs

The CATxAB RIL 3 individuals were primarily heterozygous across their genomes. The CAT genotype was fixed in the middle of chromosome 5 and roughly the first half of chromosome 6. The AB genotype was fixed on the beginning of chromosome 2, 8, 10, the ends of chromosome 3, and 10 (consensus Figure 4-4A). Excluding sample T3 for having < 98% of windows genotyped, the CATxAB RIL 3 averaged 28.4% AB, 16.4% CAT, and 54.9% heterozygous (Table A4-2).

The CATxAB RIL 13 copepods were fixed for the AB genotype for most of the genome (Figure 4-4B). The CAT genotype was fixed for a windows in the middle of chromosome 5, and near the end of chromome 4. The AB genotype was fixed on the ends of chromosome 3,10, the beginning of chromosome 2, 8, 10, and the middle of chromosome 1 (consensus Figure 4-4B).

All copepods were genotyped at > 98% of the genome. The average genomic composition for CATxAB RIL 13 was 42.6% AB, 18.3 % CAT, and 38.6% heterozygous (Table A4-2).

The CATxAB RIL 27 individual genomes were primarily heterozygous. The CAT genotype was fixed near the middle of chromsome 5, 7, 12, the beginning of chromosome 10, and the last window of chromosome 9. The AB genotype was fixed in the middle of chromosome 4, beginning of chromosome 2, and near the middle region of chromosome 9. There were two (T2 and T3) of the six samples that could be genotyped at > 98 % of the genome (Figure 4-4C). The average genome composition of the two individuals from CATxAB RIL 27 was 25% AB, 28.2 % CAT, and 46.8% heterozygous (Table A4-2).

The CATxAB RIL 33 copepods were primarily heterozygous across their genomes. The CAT genotype was only found to be fixed on the first half of chromsome 6. The AB genotype was fixed on the first windows of chromosome 1, the beginning region of chromosome 2, 3, 10, the end of chromosome 3 and 10 (consensus Figure 4-4D). There were 2 individuals (C1 and T3) genotyped at < 98% of genomic windows. The CATxAB RIL 33 averaged 31% AB, 20.1% CAT, and 48.4% of the genome was heterozygous (Table A4-2).

All of the CATxAB RILs were fixed for the AB genotype in window 3 (200k-300k bp) Chromosome 2 (consensus Figure 4-4E). Interestingly CATxAB RIL 3,13, and 33 were fixed for AB genotype at the beginning and end of chromosome 10 and again at the end of chromosome 3. These three RILs were also displayed a higher themal limit than RIL 27 (Figure 4-2). RIL 3 and 33 were the most similar in genomic compostion, including the regions mentioned above, they were fixed for CAT genotype on roughly the first half of chromosome 6 and a region for the AB genotype near the beginning of chromosome 3.

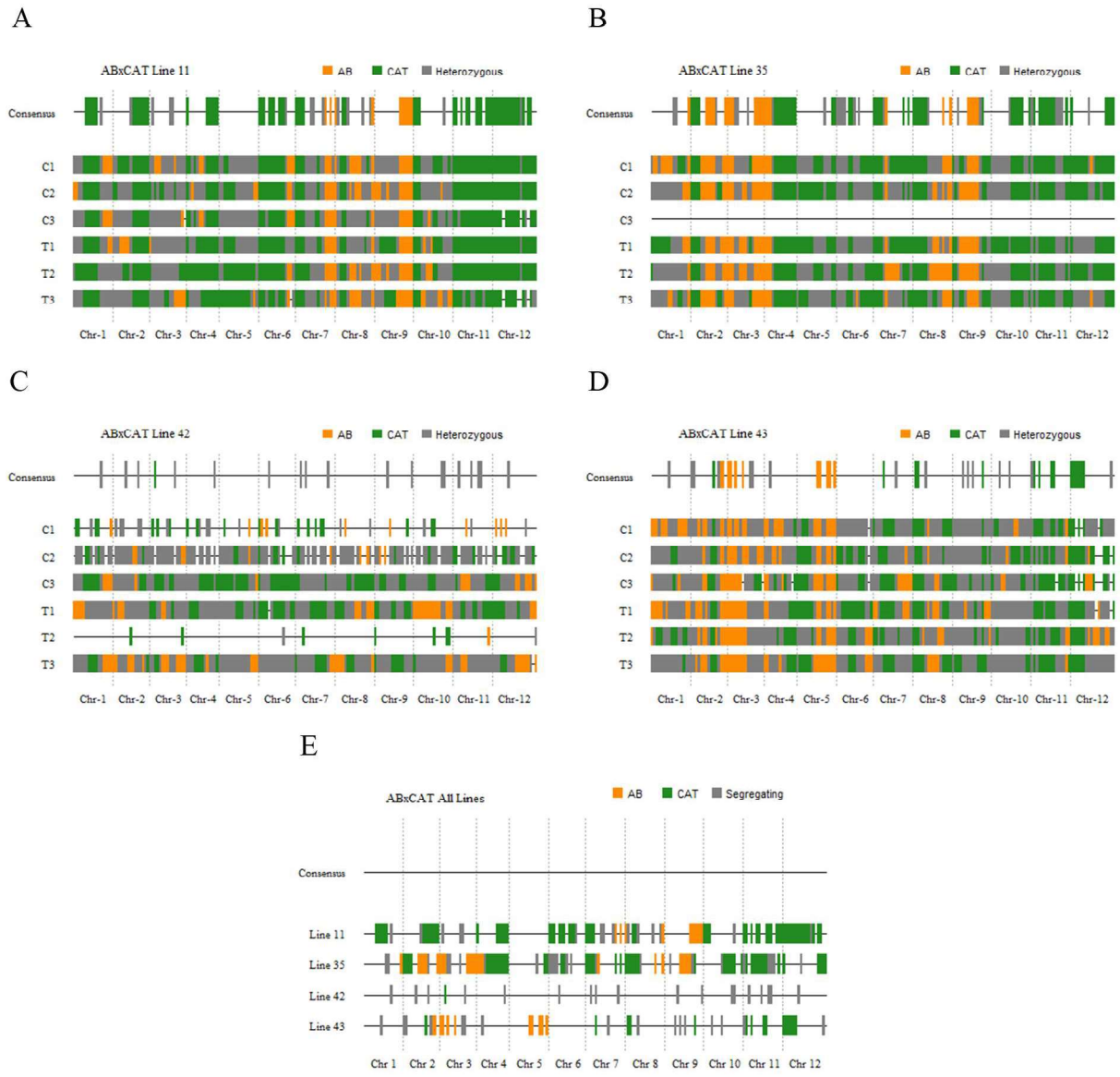

Figure 4-3. Allele frequency plotted by chromosome for control (C1, C2, C3) and treatment (T1, T2, T3) individuals in each recombinant inbred line (RIL) and consensus regions. A region is considered homozygous if the allele frequency in a window is > 0.95 or < 0.05, all values between are heterozygous. The green and orange colors indicate regions that are homozygous for the Catalina Island (CAT) or Abalone Cove (AB) parental populations. Heterozygous regions are indicated by gray color and regions of no information are white. (A) Allele frequency per individual from ABxCAT RIL 11. (B) Allele frequency per individual from ABxCAT RIL 35. (C) Allele frequency per individual from ABxCAT RIL 42. (D) Allele frequency per individual from ABxCAT RIL 43. (E) Allele frequency plot comparing consensus region within a RIL to consensus regions of other RILs.

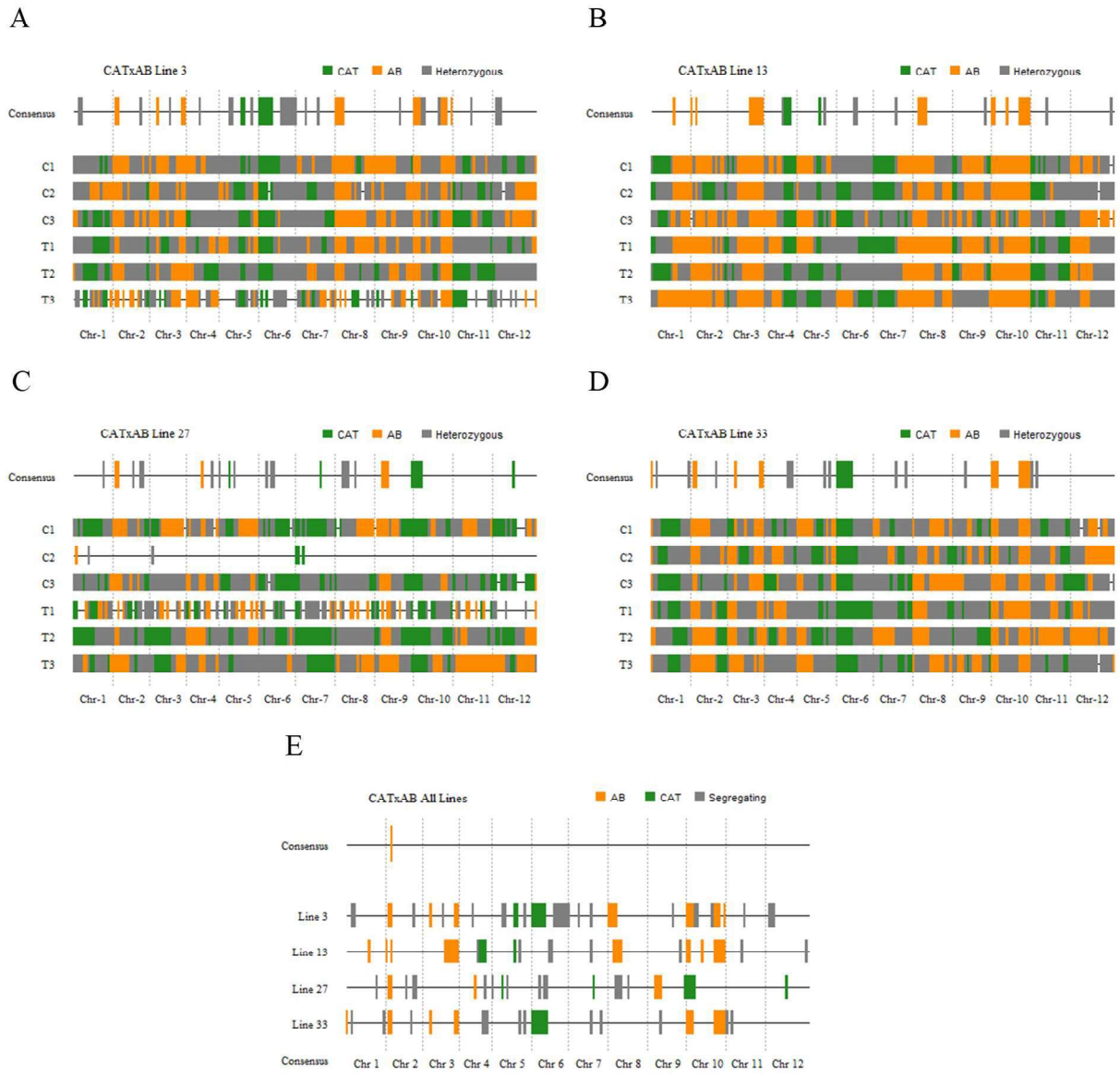

Figure 4-4. Allele frequency plotted by chromosome for each individually sequenced control (C1, C2, C3) and treatment (T1, T2, T3) copepod in each recombinant inbred line (RIL) and regions of consensus among them. A region is considered homozygous if the allele frequency in a window is > 0.95 or < 0.05, all other values between this range are heterozygous. The green and orange colors indicate regions that are homozygous for the Catalina Island (CAT) or Abalone Cove (AB) parental populations. Heterozygous regions are indicated by gray color and regions of no information are white. (A) Allele frequency per individual from CATxAB RIL 3. (B) Allele frequency per individual from CATxAB RIL 13. (C) Allele frequency per individual from CATxAB RIL 27. (D) Allele frequency per individual from CATxAB RIL 33. (E) Allele frequency plot comparing consensus region within a RIL to consensus regions among other RILs.

Discussion

Crosses between genetically differentiated conspecific populations can produce a broad range of phenotypes. Here were examined sets of hybrid lines that gave rise to positive transgressive thermal phenotypes. Because of the genetic divergence between populations, I was able to identify population diagnostic SNPs across the transcriptome and map the SNPs to the 12 chromosomes in the *T. californicus* draft genome. A minimum of ~15500 SNPs was required to effectively genotype the nuclear genome. By examining these lines, I found the genetic basis of increased thermal limit in transgressive segregants of *T. californicus* hybrids appears to be quite complex. The genomes of the transgressive segregants were found to be highly admixed, consisting of multiple parental loci across the genome (Lexer et al. 2003; Rieseberg 2003). These results are not unexpected, environmentally relevant phenotypes such as thermal limit are highly polygenic and allopatric populations of *Tigriopus californicus* with similar thermal phenotypes have been previously been shown to differ in gene expression, indicating multiple paths exist to achieve the same result (Lima and Willett 2017).

As expected, there is more similarity between individuals within a RIL than between RILs. There were no consensus regions between all the ABxCAT RILs. The Catalina genotype was found to be predominately homozygous on chromosome 11 and 12 in the two most thermal tolerant ABxCAT RILs (11 and 35). Surprisingly, all CATxAB RILs were homozygous for Abalone Cove genotype in window three on chromosome 2. This section (window) of chromosome 2 contains several heat stress responsive gene that were found to be differentially expressed. The Hsc70-4 (TCALIF_04517) was found to be expressed at > 4 log2 fold change in all RILs and parental populations (Chapter 3 Table 3-1). The small chaperone protein, Stress Inducible Protein 1 (SIP1, TCALIF_10081), was found to expressed in the ABxCAT RIL 11 and

CATxAB RILs 3,13, and 33. Barreto et al. (2015) knocked down expression of SIP1 in the San Diego population using RNAi greatly reducing the survivorship from acute heat stress.

Tigriopus californicus recombination rate is reduced by half because only the male sex recombines (Ar-rushdi 1962; Burton and Feldman 1981). Over the course of 3 generations of controlled crossing and 4 generations of inbreeding I would expect most locations in the genome to be segregating in large parental blocks. There was a large amount of heterozygosity, all individuals were > 35% heterozygous and half of RILs were > 50% heterozygous. Barring selection to maintain compatible genetic combinations (coadapted gene complexes), after three rounds of inbreeding I would expect ~ 87% of the genome to be homozygous. The large of amount of heterozygosity in this study could be related to the fitness in the paternal populations, there could be a significant amount of genetic load within parental populations (Harrison and Edmands 2006; Xiong and Mallet 2021). *T. californicus* populations have been shown to contain low amounts of heterozygosity (Burton and Lee 1994) most likely from frequent population crashes from the nature of the emphemeral nature of tide pools in the spash zone (Dethier 1980; Dybdahl 1994).

The genomic blocks of the parents in late stage hybrids could be the stochastic result of genetic drift (Ungerer et al. 2006; Buerkle and Rieseberg 2008). Recombination will break up gene complexes and if complexes are unlinked, advantageous epistatic interaction will rapidly disossociate reducing persistence of increased hybrid fitness. The transgressive phenotype in our study was heritably stable and in some lines the trait mean increased in the six-month time window between tests. The *Tigriopus californicus* life cycle takes ~ month in lab conditions of constant 20 ℃ and 12-hour light dark schedule. The typical male life span of a southern California *T. californicus* copepod is between 65-120 days at 15 ℃ and 40-63 days at 25℃

(Foley et al. 2019). In my study I did not separate and track generations after F7 and the temperature was maintained at 20 ℃. A time frame of ~ 6 months between CTmax assessment for each RIL and the gene expression thermal assay. Assuming an average male life span ~60 days in our study, I would expect ~2-3 generations of males between thermal assays. These results demonstrate the heritability of transgressive phenotypes and the unique parental combinations observed in this set of population crosses.

Some population hybrids in this species have been shown to produce less ATP and develop slower due to incompatible nuclear and mitochondrial loci (Healy and Burton 2020). Hybrids with compatible nuclear and mitochondrial electron chain proteins produced more than twice the amount of ATP and developed faster; as such, our RILs are probably not truly a random sample of the parental genomes. Selecting the first clasped pair selects for copepods that develop faster and I more than likely reduced the possibility of selecting individuals with incompatible genomic combinations.

Conclusion

Rapid changes in the global climate are occuring, witnessed by more frequent and extreme weather events of precipitation, storm surge, and temperature (IPCC 2018). To escape harm from these events, organisms will relocate or adapt to survive. The speed at which organisms are able to adapt or move will limit survival. Contact between divergent taxa may become more frequent increasing chances of hybridization. Hybridization may promote adaptive evolution, in some cases hybrids may evolve adaptive traits faster than natural populations (Mitchell et al. 2019). Functional novelty may be a common outcome of these hybridization events, especially in traits that are highly polygenic. Similar to morphological concept of many to one mapping (MTOM), which is considered a type of functional redundancy; the heat shock

system is composed of many different sizes of chaperone proteins and several isoforms are upregulated in stress response with some amount of functional redundancy (Parnell et al. 2008; Rosenzweig et al. 2019). The results presented here further our understanding of the genomic composition of increased extrinisic fitness.

Acknowledgements

Funding for the research in this dissertation was provided by grants from the National Science Foundation (DEB 1551466 and IOS 1754347) to Dr. Ronald Burton and the Graduate Research Fellowship Program from the National Science Foundation.

Chapter 4, in part is currently being prepared for submission for publication of the material. Blackwell, Reginald C.; Healy, Timothy; Burton, Ronald. The dissertation author was the primary investigator and author of this material.

Appendix

Table A4-1. The UMI read count for each sample, trimmed reads, and the number of reads mapped to custom hybrid genome with STAR. The number of population specific SNPs found within mapped 3' RNAseq reads.

RIL	Sample	UMI reads	Trimmed reads	Mapped reads	SNPs
ABxCAT11	C1	9009417	4679186	719767	42907
ABxCAT11	C2	22009995	8255974	1217554	68712
ABxCAT11	C3	6272247	2813649	406572	5901
ABxCAT11	T1	10954637	5566807	695596	46838
ABxCAT11	T2	17656441	7600761	678636	40699
ABxCAT11	T3	3044387	1402757	214213	7163
ABxCAT35	C1	6708278	3671075	495282	37411
ABxCAT35	C2	6743454	3240521	550415	25943
ABxCAT35	C3	425	235	75	0
ABxCAT35	T1	11867105	6095139	784057	46121
ABxCAT35	T2	12196954	5663453	793526	50464
ABxCAT35	T3	1391249	2776683	438673	27778
ABxCAT42	C1	190802	107644	21325	588
ABxCAT42	C2	7959286	3892707	764450	2411
ABxCAT42	C3	8766129	3601066	532940	16708
ABxCAT42	T1	4233904	2324835	316086	11352
ABxCAT42	T2	43517	25634	7376	135
ABxCAT42	T3	4454772	2069503	262741	12470
ABxCAT43	C1	4134747	2220522	308720	7073
ABxCAT43	C2	3963379	1858706	162127	6421
ABxCAT43	C3	1860259	896297	101084	4007
ABxCAT43	T1	5942550	3066581	424959	11410
ABxCAT43	T2	3659472	1782781	272301	19829
ABxCAT43	T3	13416286	5318698	777266	44016
CATxAB13	C1	10534287	4262601	558942	16351
CATxAB13	C2	5784023	2677663	286203	18037
CATxAB13	C3	1069763	440285	84320	6711
CATxAB13	T1	6989873	2845440	351437	23410
CATxAB13	T2	9463920	4309553	479584	22844
CATxAB13	T3	14090105	4916073	914443	44610
CATxAB27	C1	1731713	698415	85159	4052
CATxAB27	C2	103963	40933	6878	141
CATxAB27	C3	5631637	1778367	188704	8639
CATxAB27	T1	2058260	845906	95072	1498
CATxAB27	T2	10706952	4858977	797476	30076
CATxAB27	T3	9770592	3593350	513856	18691
CATxAB33	C1	11901916	4619487	401063	19689
CATxAB33	C2	8553169	3853656	607058	34274
CATxAB33	C3	4820161	1874517	419889	20246
CATxAB33	T1	8587983	3460835	511191	34736
CATxAB33	T2	10407861	4681785	700869	37727
CATxAB33	T3	2139941	853776	155326	14123
CATxAB3	C1	6588030	2797944	315907	20264
CATxAB3	C2	2094595	979480	163650	7310
CATxAB3	C3	7141971	2807658	459832	25185
CATxAB3	T1	2556670	1172435	198309	15578
CATxAB3	T2	11360672	4800611	770298	45245
CATxAB3	T3	183918	73557	15681	1374

Table A4-2. The percent of genomic windows (188 in total) with a genotype call or no information. The windows are classified as AB = homozygous Abalone Cove, CAT = homozygous Catalina Island, Het = heterozygous AB & CAT, NA = no call due to missing information or removed with quality filter.

RIL	Sample	AB	CAT	Het	NA
ABxCAT11	C1	15.43	47.87	36.70	0
ABxCAT11	C2	14.89	46.28	38.83	0
ABxCAT11	C3	12.23	40.43	45.21	2.13
ABxCAT11	T1	11.17	51.60	37.23	0
ABxCAT11	T2	13.30	56.91	29.79	0
ABxCAT11	T3	15.96	45.21	36.17	2.66
ABxCAT35	C1	20.21	47.34	32.45	0
ABxCAT35	C2	20.21	41.49	38.30	0
ABxCAT35	C3	0	0	0	100
ABxCAT35	T1	19.68	48.40	31.91	0
ABxCAT35	T2	22.87	39.36	37.77	0
ABxCAT35	T3	17.55	40.43	42.02	0
ABxCAT42	C1	5.32	10.64	9.04	74.47
ABxCAT42	C2	5.32	11.70	60.64	22.34
ABxCAT42	C3	8.51	39.36	52.13	0
ABxCAT42	T1	18.09	29.26	52.13	0.53
ABxCAT42	T2	0.53	3.72	1.06	94.68
ABxCAT42	T3	21.81	16.49	61.17	0.53
ABxCAT43	C1	19.15	19.68	57.98	2.13
ABxCAT43	C2	13.83	30.85	52.66	2.66
ABxCAT43	C3	19.68	32.45	42.02	5.85
ABxCAT43	T1	23.4	29.79	45.74	1.06
ABxCAT43	T2	20.21	25.00	54.79	0
ABxCAT43	T3	19.15	28.72	52.13	0
CATxAB13	C1	48.40	18.09	32.98	0.53
CATxAB13	C2	32.45	19.68	47.34	0.53
CATxAB13	C3	38.83	15.96	43.62	1.60
CATxAB13	T1	49.47	20.74	29.26	0
CATxAB13	T2	38.30	19.15	42.55	0
CATxAB13	T3	48.40	15.96	35.64	0
CATxAB27	C1	29.26	31.38	35.11	4.26
CATxAB27	C2	0.53	1.60	1.06	96.81
CATxAB27	C3	13.30	28.19	55.85	2.66
CATxAB27	T1	19.15	14.36	27.66	38.30
CATxAB27	T2	14.89	38.30	46.81	0
CATxAB27	T3	35.11	18.09	46.81	0
CATxAB33	C1	27.66	18.09	51.60	2.66
CATxAB33	C2	30.85	19.68	49.47	0
CATxAB33	C3	27.66	20.74	50.00	1.60
CATxAB33	T1	25.00	21.28	53.19	0.53
CATxAB33	T2	40.43	18.62	40.96	0
CATxAB33	T3	27.13	15.43	54.79	2.66
CATxAB3	C1	32.45	13.30	54.26	0
CATxAB3	C2	29.26	11.17	57.98	1.60
CATxAB3	C3	34.57	20.74	44.68	0
CATxAB3	T1	25.00	14.36	60.64	0
CATxAB3	T2	20.74	22.34	56.91	0
CATxAB3	T3	22.34	13.83	27.13	36.70

Table A4-3. The CTmax of RILs and parental populations.

Line (RIL or Parental)	Cross	N	Temperature (°C)	sd	se	ci
11	ABxCAT	16	38.79	0.89	0.22	0.47
12	ABxCAT	16	38.35	1.11	0.28	0.59
16	ABxCAT	16	38.31	1.14	0.28	0.61
35	ABxCAT	16	38.44	0.89	0.22	0.48
4	ABxCAT	16	37.53	0.87	0.22	0.47
42	ABxCAT	16	38.58	0.49	0.12	0.26
43	ABxCAT	16	38.67	0.42	0.10	0.22
44	ABxCAT	16	38.41	0.65	0.16	0.35
48	ABxCAT	16	38.20	0.77	0.19	0.41
49	ABxCAT	16	37.91	1.49	0.37	0.80
AB	AB	16	37.11	0.64	0.16	0.34
CAT	CAT	16	37.39	0.56	0.14	0.30
12	CATxAB	16	38.01	0.66	0.17	0.35
13	CATxAB	16	38.38	0.89	0.22	0.47
18	CATxAB	16	37.96	0.76	0.19	0.41
27	CATxAB	16	37.85	1.09	0.27	0.58
3	CATxAB	16	38.29	0.82	0.20	0.44
33	CATxAB	16	38.15	1.02	0.26	0.54

Table A4-4. The CTmax of copepods for gene expression in RILs and parental populations.

Line (RIL or Parental)	Cross	N	Temperature (°C)	sd	se	ci
11	ABxCAT	6	39.10	0.22	0.09	0.23
35	ABxCAT	6	39.10	0.25	0.10	0.27
42	ABxCAT	6	38.72	0.26	0.11	0.28
43	ABxCAT	6	38.50	0.45	0.18	0.47
AB	AB	6	37.02	0.29	0.12	0.30
CAT	CAT	6	37.25	0.18	0.07	0.18
13	CATxAB	6	38.67	0.44	0.18	0.46
27	CATxAB	6	37.87	0.27	0.11	0.29
3	CATxAB	6	38.95	0.65	0.26	0.68
33	CATxAB	6	39.17	0.16	0.07	0.17

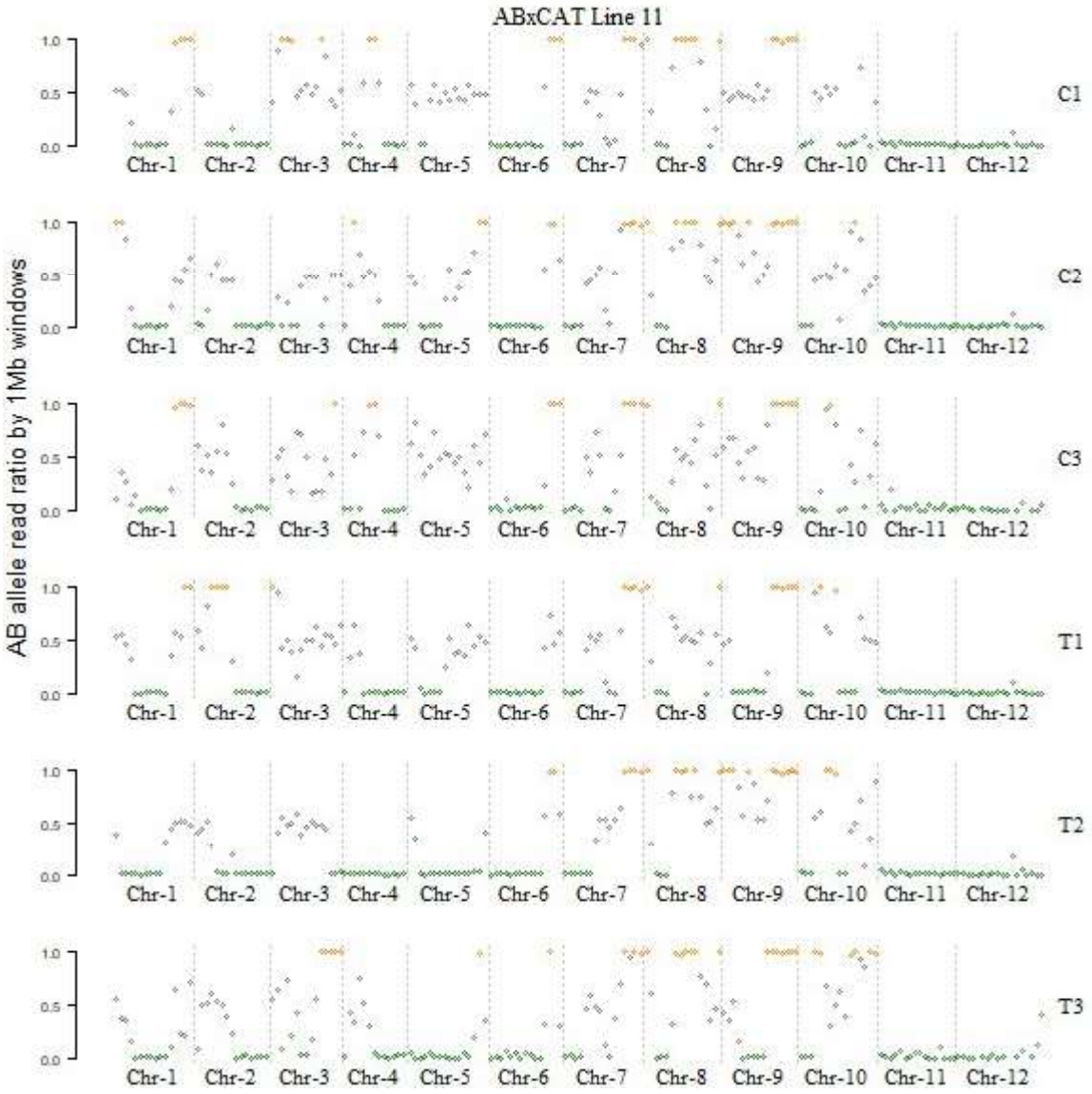

Figure A4-1. The genotype frequency calls from mapped 3' mRNA seq transcriptome reads the ABxCAT RIL 11 control (C1, C2, C3) and treatment (T1, T2, T3) individuals across the twelve Tigriopus californicus chromosomes. The dots represent the ratio of the AB alleles at each window. Values > 0.95 are homozygous for the AB allele (orange), values < 0.05 are homozygous for the CAT allele (green), and any values in between are heterozygous regions (gray).

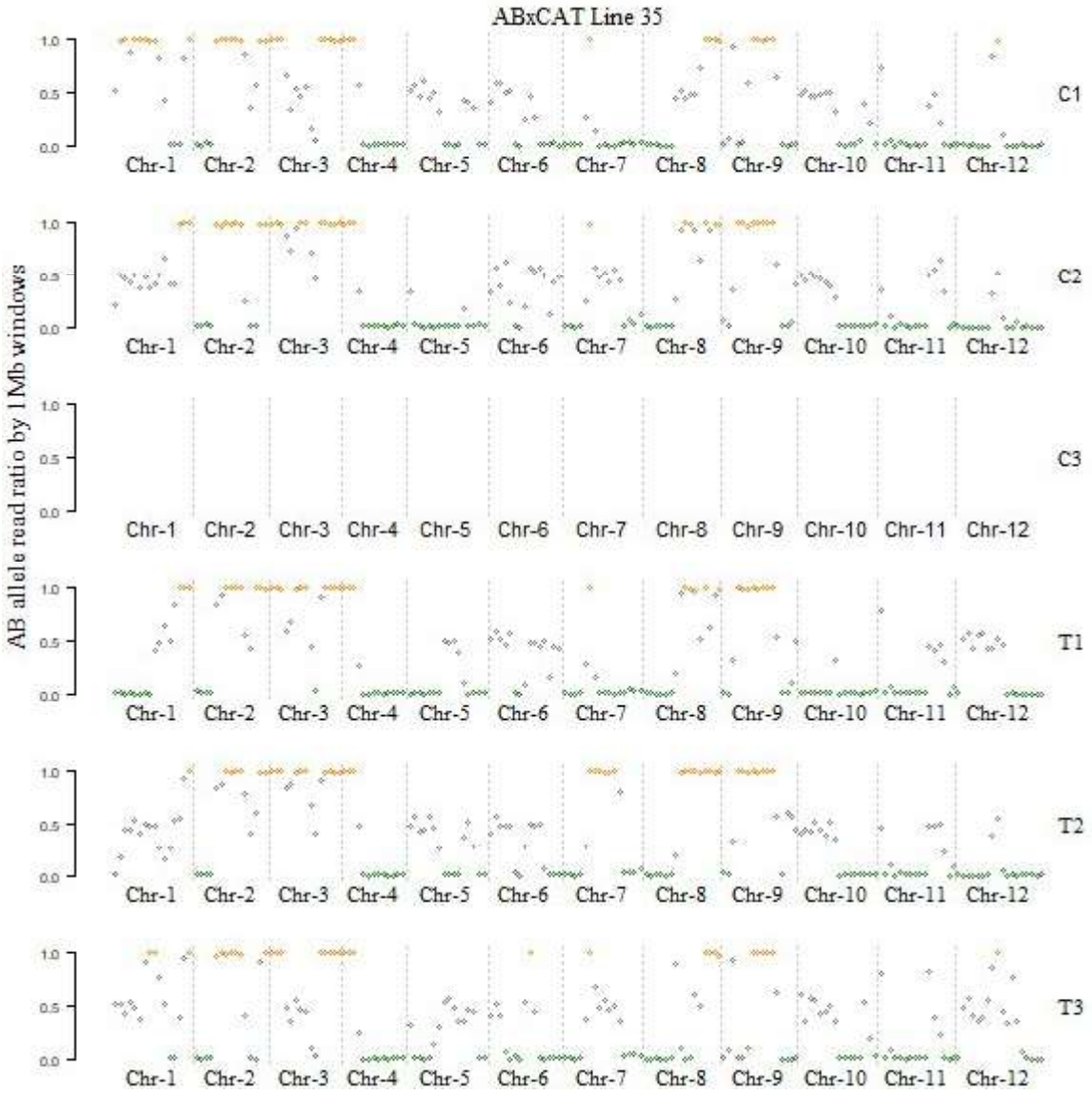

Figure A4-2. The genotype frequency calls from mapped 3' mRNA seq transcriptome reads the ABxCAT RIL 35 control (C1, C2, C3) and treatment (T1, T2, T3) individuals across the twelve Tigriopus californicus chromosomes. The dots represent the ratio of the AB alleles at each window. Values > 0.95 are homozygous for the AB allele (orange), values < 0.05 are homozygous for the CAT allele (green), and any values in between are heterozygous regions (gray).

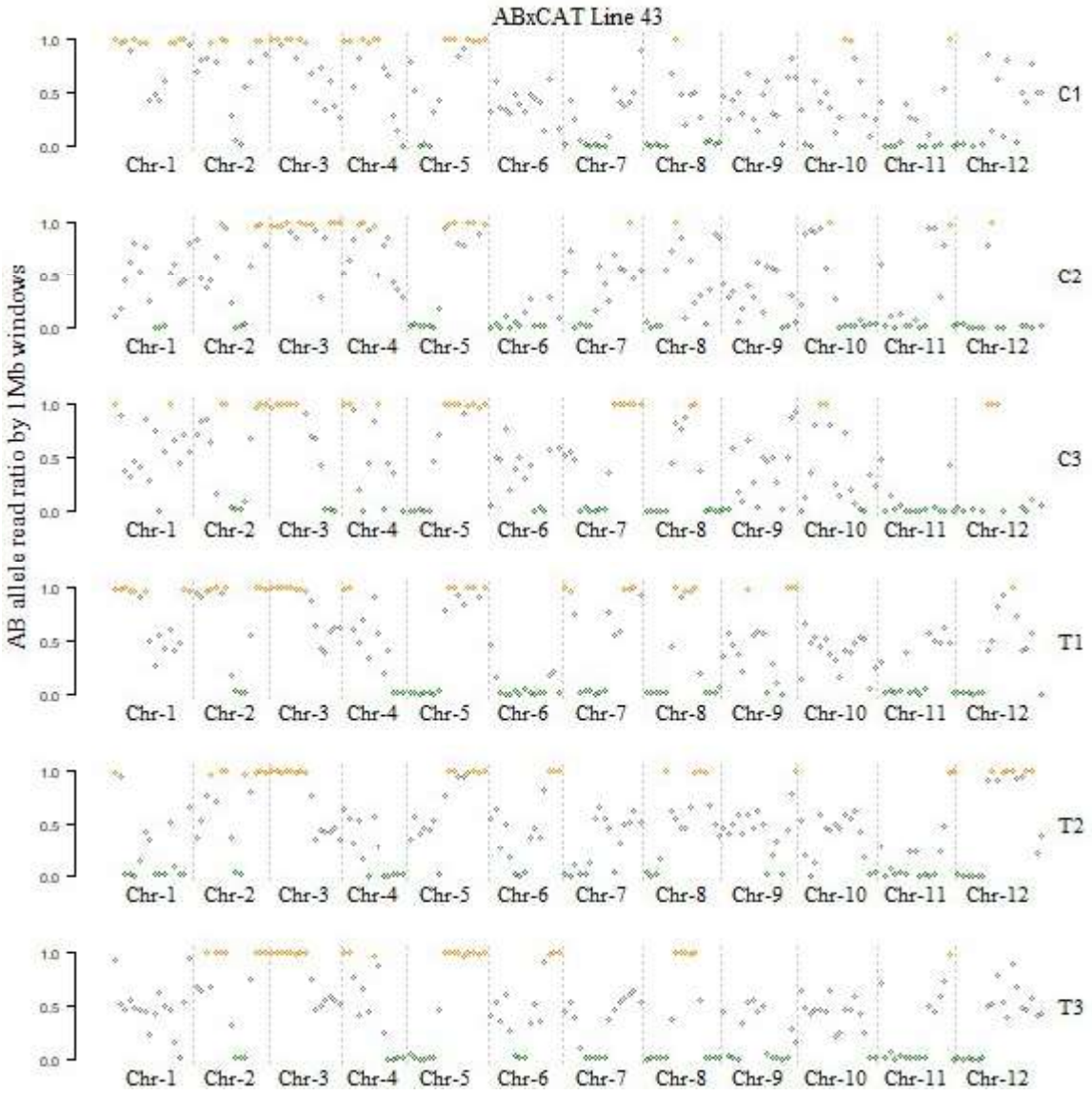

Figure A4-3. The genotype frequency calls from mapped 3' mRNA seq transcriptome reads the ABxCAT RIL 43 control (C1, C2, C3) and treatment (T1, T2, T3) individuals across the twelve Tigriopus californicus chromosomes. The dots represent the ratio of the AB alleles at each window. Values > 0.95 are homozygous for the AB allele (orange), values < 0.05 are homozygous for the CAT allele (green), and any values in between are heterozygous regions (gray).

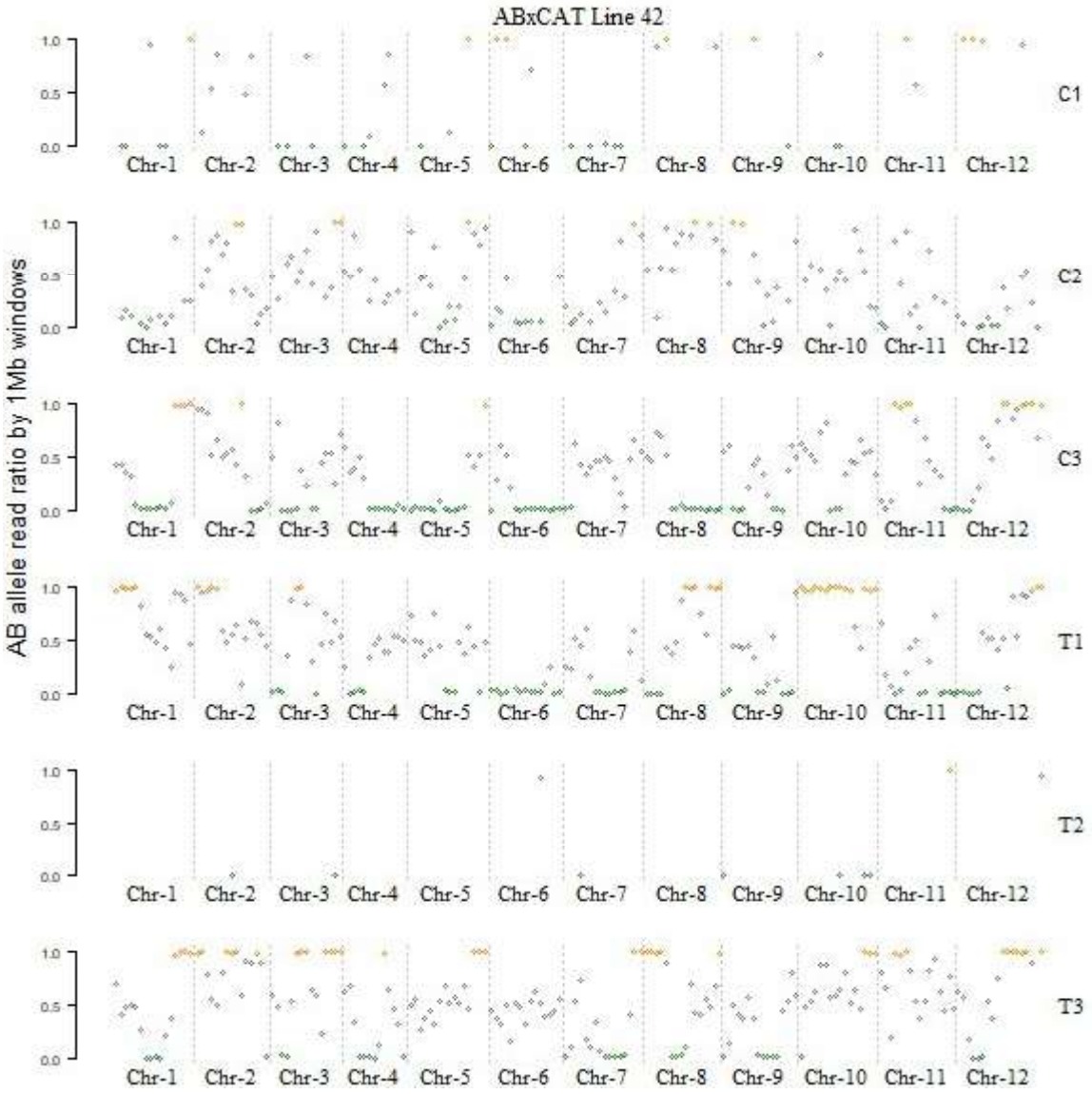

Figure A4-4. The genotype frequency calls from mapped 3' mRNA seq transcriptome reads the ABxCAT RIL 42 control (C1, C2, C3) and treatment (T1, T2, T3) individuals across the twelve Tigriopus californicus chromosomes. The dots represent the ratio of the AB alleles at each window. Values > 0.95 are homozygous for the AB allele (orange), values < 0.05 are homozygous for the CAT allele (green), and any values in between are heterozygous regions (gray).

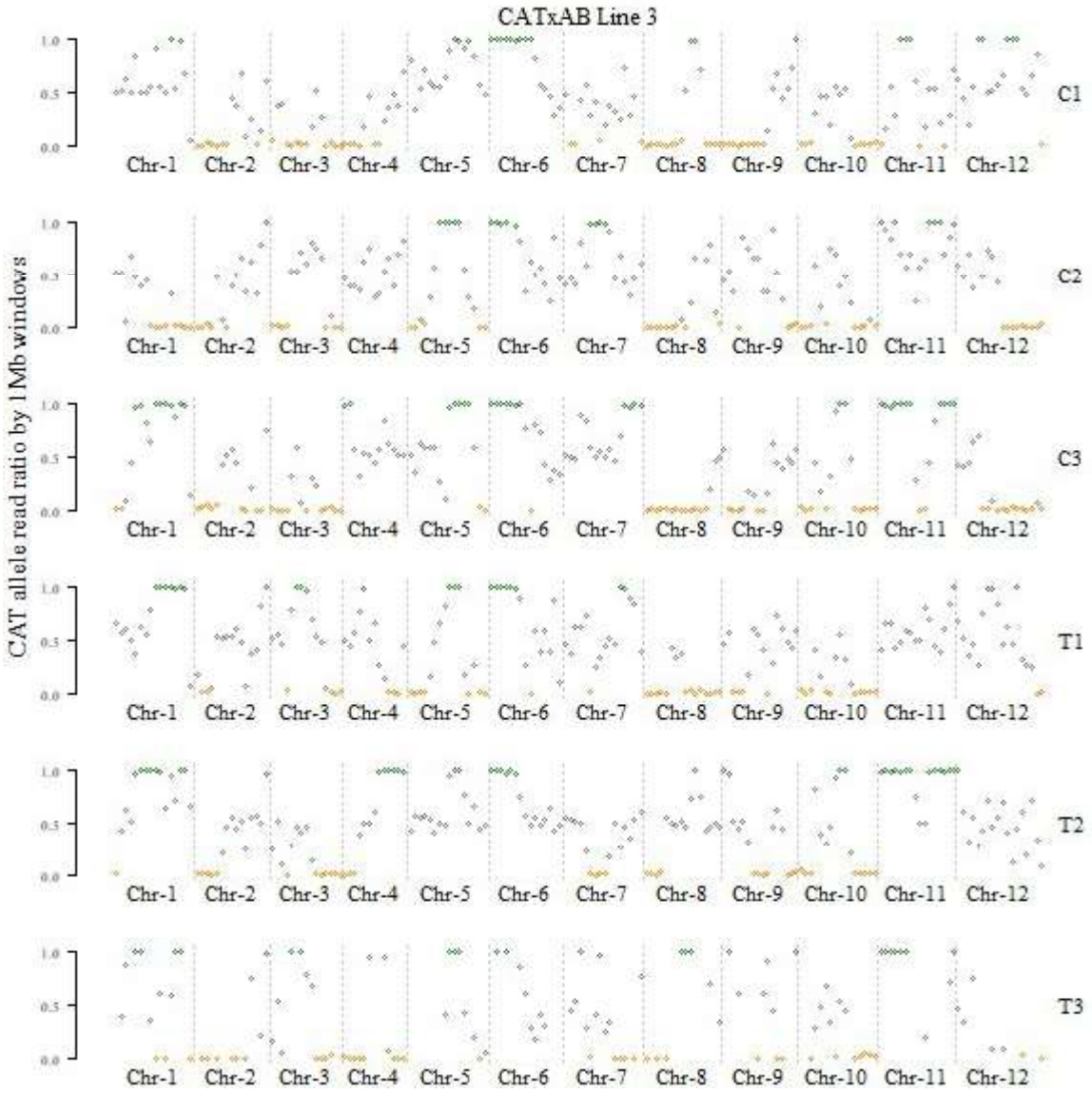

Figure A4-5. The genotype frequency calls from mapped 3' mRNA seq transcriptome reads the CATxAB RIL 3 control (C1, C2, C3) and treatment (T1, T2, T3) individuals across the twelve Tigriopus californicus chromosomes. The dots represent the ratio of the AB alleles at each window. Values > 0.95 are homozygous for the CAT allele (green), values < 0.05 are homozygous for the AB allele (orange), and any values in between are heterozygous regions (gray).

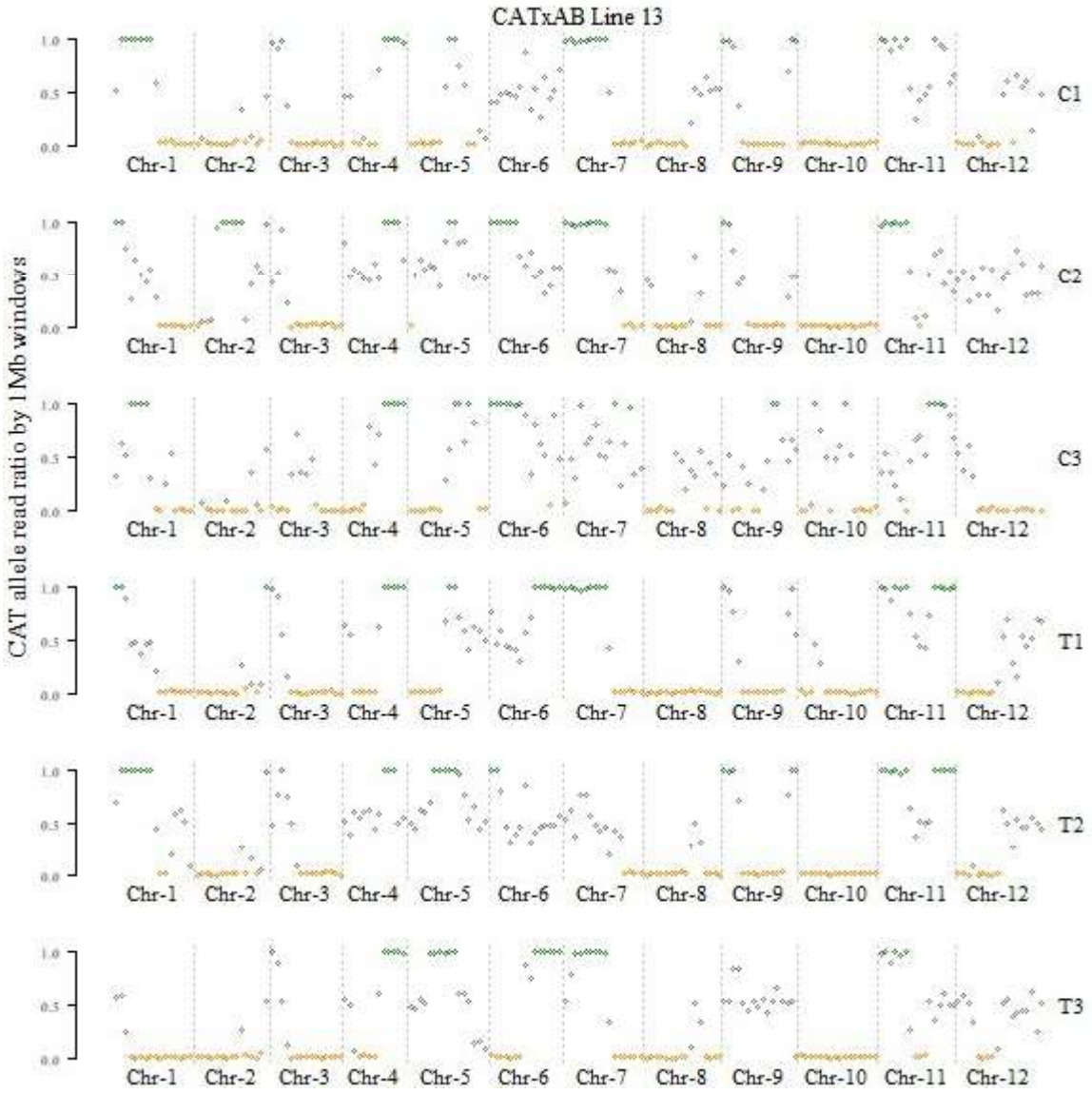

Figure A4-6. The genotype frequency calls from mapped 3' mRNA seq transcriptome reads the CATxAB RIL 13 control (C1, C2, C3) and treatment (T1, T2, T3) individuals across the twelve Tigriopus californicus chromosomes. The dots represent the ratio of the AB alleles at each window. Values > 0.95 are homozygous for the CAT allele (green), values < 0.05 are homozygous for the AB allele (orange), and any values in between are heterozygous regions (gray).

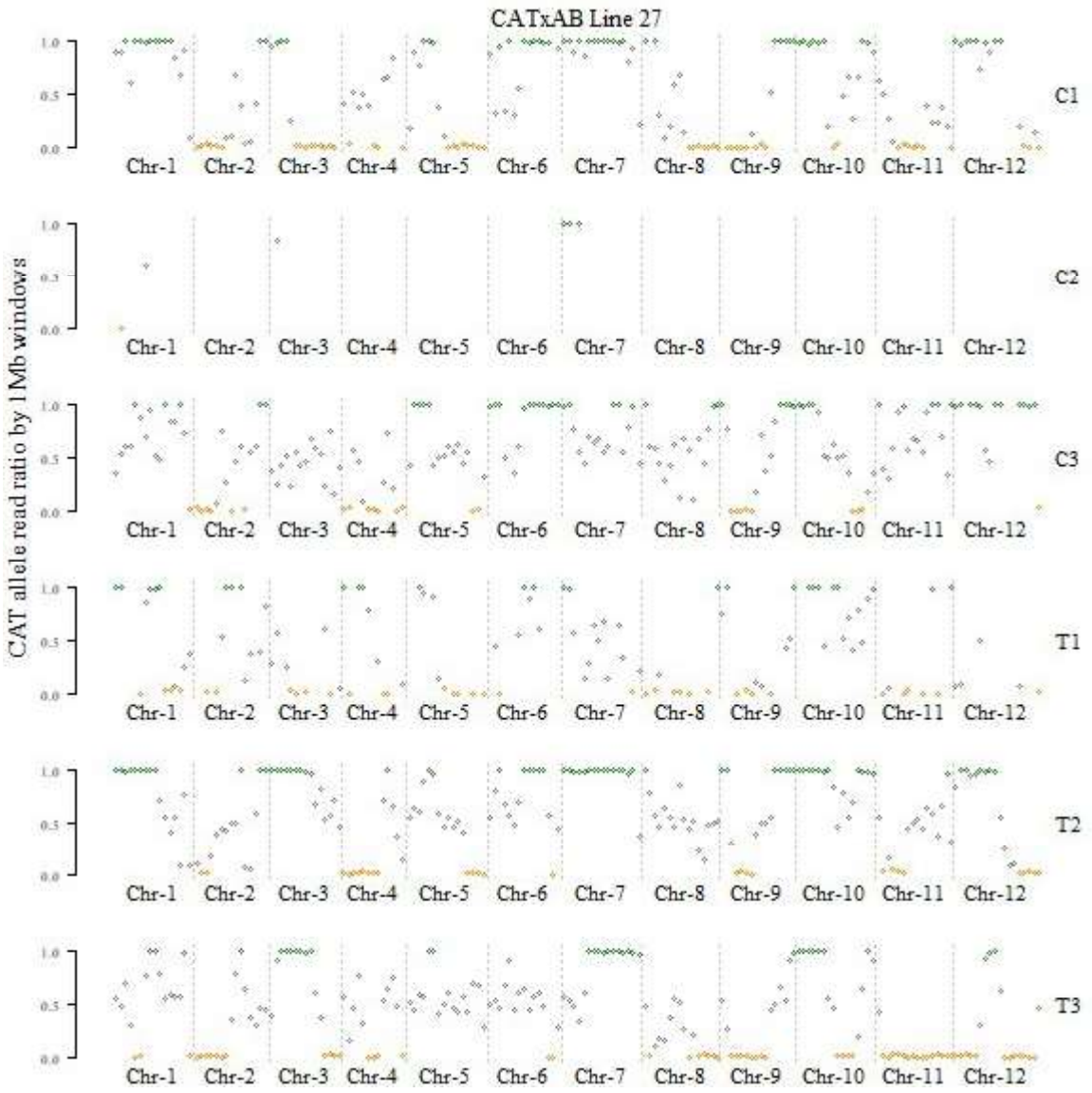

Figure A4-7. The genotype frequency calls from mapped 3' mRNA seq transcriptome reads the CATxAB RIL 27 control (C1, C2, C3) and treatment (T1, T2, T3) individuals across the twelve Tigriopus californicus chromosomes. The dots represent the ratio of the AB alleles at each window. Values > 0.95 are homozygous for the CAT allele (green), values < 0.05 are homozygous for the AB allele (orange), and any values in between are heterozygous regions (gray).

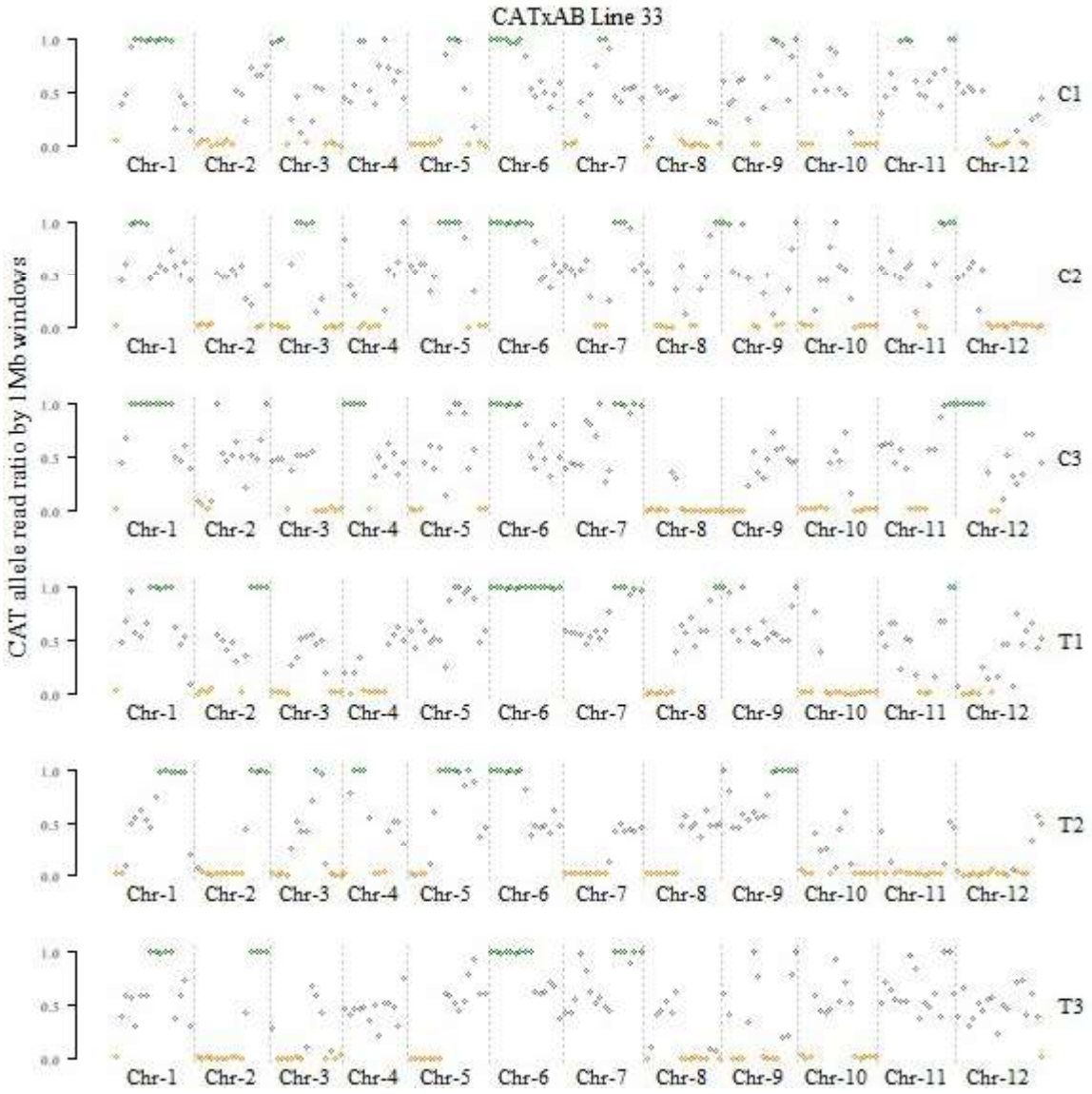

Figure A4-8. The genotype frequency calls from mapped 3' mRNA seq transcriptome reads the CATxAB RIL 33 control (C1, C2, C3) and treatment (T1, T2, T3) individuals across the twelve Tigriopus californicus chromosomes. The dots represent the ratio of the AB alleles at each window. Values > 0.95 are homozygous for the CAT allele (green), values < 0.05 are homozygous for the AB allele (orange), and any values in between are heterozygous regions (gray).

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Conclusion

Global temperatures are predicted to increase at a rapid pace possibly overtaking the capacity of species to adapt and evolve to the rate of change. Predicting the response of populations and species to rising temperatures is a difficult and complex problem in ecology and evolutionary biology. The capacity to adapt to rising temperatures is contingent on the ability to track changes in the environment. Organisms can migrate to favorable habitat, acclimate to new novel conditions by phenotypic plasticity, and/or adapt by natural selection based on the genetic diversity of the population. In general, the genetic variation for environmental response is unknown for most species and populations complicating predictions. Migrating organism may come into contact with other populations or species increasing the chance for hybridization to occur. Hybridization events will alter the trajectory of species by changing the adaptive capacity and the genetic diversity in a population which may lead to divergent ecological and evolutionary outcomes.

The results from this dissertation provide valuable information about the amount genetic diversity for thermal tolerance in a population of *Tigriopus californicus* from the southern edge of the species range. Further this dissertation expands our understanding of the basis of transgressive segregation for thermal tolerance in hybrid animals. Taken together, these results indicate the detrimental effects of rapidly rising temperatures maybe mitigated by either standing variation or hybridization between regionally close populations.

Genetic variation for thermal tolerance

In chapter 1, I demonstrate a population of *Tigriopus californicus* from the southern edge of its range does contain significant additive genetic variation for thermal limit. This result is somewhat unexpected because populations at the ends of a species range are generally not

genetically diverse. The heterogeneity of tidepools size, depth, rock type, and direction (i.e., southern facing) maybe maintaining genetic diversity in the population for thermal tolerance. I further show that the response to thermal stress (extrinsic stress) in these inbred lines does not appear to correlate with an increase in intrinsic stress from inbreeding. The results from this study are informative in predicting future response to climate change for this intertidal species and the importance of investigating intrapopulation adaptive genetic diversity to better forecast future population response. Future directions from this study can be expanded to determine if populations at the northern range limit and from the center of its range display similar genetic diversity for thermal stress response. Further, more research is needed to determine if this southern range population contains unique adaptive alleles for thermal response that are not found in other *Tigriopus californicus* populations.

Transgressive segregant thermal phenotypes

In Chapters 2,3, and 4 the objective was to explore the occurrence of transgressive segregants thermal phenotypes and identify the underlying genetic mechanisms and genomic composition of positive transgressive segregants. In Chapter 2, I found hybrids created from populations within the same biogeographic region produced positive and negative transgressive segregants but hybrids between different biogeographic regions displayed intermediate parental thermal stress phenotypes. The temperature profiles of the Bird Rock, San Diego, and Santa Cruz location tidepools demonstrates the similarities and differences in selection pressure from temperature between geographically close and geographically distant populations. Given the regularity of late-stage hybrids displaying transgressive thermal phenotypes, complementary gene action is the genetic mechanism that explains my results. To further increase our understanding of the transgressive thermal phenotypes it will be informative to perform similar

hybridization experiments between thermally sensitive populations from the northern range of this species.

Transgressive segregant gene expression

In chapter 3, I measured the gene expression response to thermal stress in individual copepods from multiple independent hybrid lines displaying positive transgressive thermal tolerance and the parental populations used to create the hybrids. The genetic response to thermal stress in the parental populations overlapped in the use of large and small heat shock genes. Several heat stress responsive genes were also found to be only used in one population or the other indicating different mechanisms in the parental populations. The response of the hybrids lines overlapped the shared response of the parental population but also incorporated population specific responses from both parental populations indicating possible complementary gene response as the mechanism. The response in each of the hybrid lines was found to overlap but also contain unique genes in the response. The *HspB1* gene was found to be upregulated in 3 of my lines but not differentially expressed in either or the parental populations. This result is possibly an indication of an increase of function in these lines. Results from this study provide an understanding of gene regulation contributing to increased thermal tolerance as a result of hybridization. Future research measuring the gene expression patterns of the negative transgressive thermal phenotypes in our study could be valuable in understanding decreased environmental fitness (extrinsic fitness) in hybrids while still maintaining intrinsic fitness of the stock population.

Transgressive segregation genetic composition

In chapter 4, I used the RNAseq data from chapter 3 and population specific SNPs to genotype the 12 chromosomes of *Tigriopus californicus* hybrid individuals. I found ~15500 SNPs was effective to identify the genomic composition of an individual hybrid copepod. Genotyping results show the genomic composition was more similar within a line than between lines. Because all lines were positive transgressive for themal phenotype this may indicate many possible pathways to produce transgressive thermal phenotypes. These results mirror the results of chapter 3 in which I saw overlap in the response to thermal stress but also genes that were regulated in a line-specfic manner (not used in other lines). Future research is needed to gain better resolution of the genetic mechanisms of positive trangressive thermal tolerance. To better understand the underlying mechanims, increasing the number generations of inbreeding will help to remove more of the heterozygosity I witnessed in each of my lines and may allow a clearer picture of the key components of the response.

Temperature is a key determinate of climate and sets limits on the physiological processes of all organisms. Interrogation of population genetic diversity for thermal stress provides greater understanding of the allelic diversity in the thermal stress response system of populations. Currently it is unknow how variable the thermal stress response system is in most of the populations across the range of *Tigriopus californicus*. This study provides a first look at one of the most thermal tolerant populations in the species range and shows high levels of genetic diversity in the heat stress response system. Other populations in the *T. californicus* range may harbor similar if not more genetic diversity in the thermal stress response system. Contact between these populations may become more frequent as extreme weather events such as precipitation and storm surge increases. Natural weather events that lead to hybridization

between geographically close populations may generate novel environmentally responsive phenotypes similar to those found in this dissertation. Novel thermal stress phenotypes from hybridization may promote adaptive evolution faster than natural selection alone.