UNIVERSITY OF CALIFORNIA, SAN DIEGO

# Fine-scale local adaptation and genetic differentiation: case study of an intertidal copepod

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

Lori Hiroko Luers

Committee in charge:

Professor Ronald S. Burton, Chair
Professor Joshua Kohn, Co-Chair
Professor David Holway

2015

The Thesis of Lori Hiroko Luers is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California, San Diego

2015

# DEDICATION

In recognition of all the people who have given me strength, direction, and faith in my project; this thesis is dedicated to the whole Burton lab, my professors, and my family and friends. An infinite amount of thanks to Ron Burton; he gave me the opportunity for this research project and to dive into the realm of Marine Biology, with loads of patience and hummus. Although I used a light saber as my pointer for my first presentation, he did not kick me out of the lab. Thank you to Ricardo Pereira, who trained me and provided me with foundation for falling in love with copepods. I cannot forget to thank Felipe for his advice and bellowing laughter that echoes down the halls, and Alice and Mado for telling me that being weird is OK. Thank you Sasaki-san for sharing our love for phylogenetics, copepods, and also for sharing our office! I am grateful for the opportunity to exchange with great new members of our lab such as Thiago, who is sure to keep the lab in good spirits. A big thank you to the rest of the Burton lab current and past members: Lani, Tessa, Reggie, Por, Ana, Chase, Summer, Marina, Kate, Martha, Matt, Manoela, Amanda, Brittany, and Elise. Thank you for being patient, listening to my questions, and simply being pleasure to spend time with. I hope my chalkboard drawings of copepods will instill an everlasting affection for our harpacticoid friends as well as the ability to recall nerdy alliteration for every day of the week.

I would also like to dedicate this thesis to my family and friends, who have always made me feel like a super-scientist although I know I am far from it. I would like to recognize my father, Richard P. Luers, for assisting me through my undergrad and graduate degree and allowing me to easily pursue a higher education. I can’t believe I have a parent so prepared to pay for my tuition bills! This is for you, dad.

Finally, thank you for those that attended my defense---there is no better feeling than having a room full of people that support and care about you.

EPIGRAPH

It is not the strongest of the species that survives, nor the most intelligent, but the one most responsive to change.

*Charles Darwin*

The more clearly we can focus our attention on the wonders and realities of the universe about us, the less taste we shall have for destruction.

*Rachel Carson*

Freedom lies in being bold.

*Robert Frost*

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ABSTRACT OF THE THESIS

Fine-scale local adaptation and genetic differentiation: case study of an intertidal copepod

by

Lori Hiroko Luers

Master of Science in Biology

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Professor Ronald S. Burton, Chair

Professor Joshua R. Kohn, Co-Chair

Divergence of conspecific populations can occur by mutation, natural selection, and genetic drift. Such divergence may result in local adaptation to the environment as well as reproductive incompatibility. These processes can ultimately lead to speciation; to what extent they contribute to speciation is an

area of interest. Past studies of the marine copepod, *Tigriopus californicus*, show F1 hybrids of populations 8 km apart (Bird Rock and San Diego) undergo hybrid breakdown and are genetically divergent (10.4% on *CYTB*). Although the BRxSD F1 hybrids have reduced fitness, they also exhibit transgressive segregation in thermal tolerance, implying different mechanisms of adaptation. Thermal adaptation has been measured for other *T. californicus* populations but only at larger scales. At what scale such reproductive incompatibilities and differences in thermal tolerance occur has yet to be investigated. This study looks into micro-scale differentiation and determines its consequences. Every inhabited outcrop was sampled from La Jolla (LJ) to Bird Rock (BR) (San Diego County, CA) to determine genetic differentiation and population differences in thermal tolerance. To determine consequences of such divergence, several hybrid crosses were produced to find the spatial scale at which genetic incompatibilities and transgressive segregation may occur. This study found fine-scale genetic differentiation and significant differences in thermal tolerance at a new spatial scale. Despite these differences, hybrid breakdown was not observed at a smaller scale; however, evidence for hybrid breakdown was observed in the LJ and SD (~13 km distant). Our preliminary results suggest that differences in thermal tolerance occur at the fine scale before reproductive incompatibilities arise. The finding of such differences in thermal tolerance at this scale has significance for fine-scale studies and implications for future work in understanding if there are effects of the microclimates.

## Introduction

Patterns of differentiation have been studied in many marine organisms in an effort to shed light on mechanisms of speciation. There are several modes of speciation distinguished by the various roles of evolutionary processes. Some of the ways these mechanisms can drive differentiation include genetic drift and selection for adaptive variation (local adaptation) (Schluter, 2001). Local adaptation involves a balance of selection and gene flow (Sanford and Kelly, 2010) and can drive differentiation of populations (Lamichhaney et. al., 2012). Differentiation via local adaptation can even occur in the presence of gene flow, given there is strong selection (Le Corre and Kremer, 2012). Thus understanding the scale of differentiation provides insights into evolutionary processes and population dynamics; it has been observed in marine invertebrates over many scales and across taxa with a range of life histories (Sanford and Kelly, 2010). Resolving the scale of such differentiation can aid in understanding community ecology and effects of climate change.

Fine-scale studies have elucidated complex patterns. Microhabitat dependent differentiation was found to occur on the scale of 10's of meters to 100's of kilometers in barnacles living in heterogeneous intertidal environments (Rand et. al., 2002); Adaptation to microenvironments has also been found for *Littorina saxatilis* populations a few meters distant (Johannesson and Johannesson, 1996). Differential thermal tolerance has been found for varying vertical distributions of the same beach in species of

intertidal shrimp (Madeira et. al, 2015). Even highly vagile species like killer whales have been found to be highly differentiated in response to resource specialization (Moura et. al., 2014). Other fine-scale studies have been performed in in environments without sharp clines: thermal adaptation has been found to occur in nesting sea turtles of beaches a few kilometers distant (Weber et. al., 2012). Overall, micro-scale studies can yield surprising results and provide better understanding of a system that would be lost on a larger scale. Additionally, such differentiation can have important consequences and, in some cases, may lead to reproductive incompatibility.

A type of reproductive incompatibility is postzygotic isolation; Postzygotic isolation may be due to consequences of local adaptation or intrinsic selection, or both (Wolf 2010). As populations evolve adaptations to different habitats, mutations can accumulate which could affect traits relevant to reproductive isolation (Coyne and Orr, 2004). In this way, reproductive isolation can be manifested as an indirect consequence of local adaptation rather than a direct result of intrinsic selection (Funk et. al. 2002, Coyne and Orr 2004, Westneat 2010). There has been a debate as to whether genes underlying ecological adaptations also play a role in hybrid incompatibilities (Unckless and Orr, 2009, Muller, 1942) as well as what the contribution of selection and drift is to those incompatibilities (Cutter, 2012).

The reduced fitness of hybrids due to genetic incompatibilities among parental lineages is known as hybrid breakdown. The deleterious interactions

between parental genotypes are believed to be multilocus interactions (Dobzhansky-Muller incompatibilities) (Wu and Ting, 2004). These epistatic interactions have been found in multiple species, including *Arabidopsis,* nematodes, and *Drosophila* strains. Consequences of the interactions are, respectively, recessive embryo lethality (Bikard et. al., 2009), F1 and F2 inviability and sterility as well as embryonic arrest (Dey et. al., 2014), and reduced lifespan (Dickman and Moehring, 2014). Hybrid breakdown can manifest in multiple ways and be studied to explain processes of early stages of speciation based on genetic and ecological differentiation. Major players of these interactions are likely co-adapted gene interaction systems, such as cytonuclear and mitonuclear genes that code for proteins that interact for vital processes. Additionally, the higher rate of evolution of mtDNA also enforces such coevolution with nuclear DNA (Burton et. al., 2013). This has been well demonstrated for *T. californicus* (Ellison and Burton, 2008). Reduced fitness can be observed in a variety of characteristics, and traced to the genetic level.

Not all genetic interactions in hybrids are deleterious; hybridization has been seen as a potential mechanism for speciation by natural selection (Rieseberg et. al., 1999) as it may provide a source of variation for adaptation (Lewontin and Birch, 1966). It can produce novel phenotypes, as described by the terminology: transgressive segregation. Transgressive segregation is defined by the phenotypic novelty that is produced when hybrids of two populations with similar phenotypes possess traits beyond the range of the parental populations. Due to the ability to exhibit transgression, hybridization

has been thought to influence adaptive radiation and evolvability (Parsons et. al., 2011). Whether these transgressive traits confer an advantage over parentals requires investigation; however, transgressive segregation in hybrids may be a mechanism of speciation.

The intertidal copepod, *Tigriopus californicus*, is of particular interest when studying evolutionary mechanisms of speciation due to its wide ecological distribution, high genetic divergence among populations, and ease of culturing. *Tigriopus* have a generation time of approximately 3-4 weeks with visible stages of development: six naupliar larval stages, five juvenile copepodid stages, and the adults. Adult males use their antenna to clasp virgin females until females reach reproductive maturity, so it is easy to separate males and females for experimental hybridization (Burton, 1985). This harpacticoid copepod inhabits rocky tide pools in the splash zone ranging from Baja, Mexico to Alaska (Burton and Feldman, 1981) with little gene flow (Burton and Lee, 1994). Accordingly, they inhabit a range of climates and show differential thermal tolerances (Willett, 2010; Kelly et. al., 2012) as well as exhibit high genetic divergence (Burton, 1998, Edmands, 2001, Willett and Ladner, 2009, Willett, 2012a). Encompassing all these features of *T. californicus*, a multitude of experiments have investigated population differentiation, hybrid breakdown, and even transgressive segregation.

Populations of *T. californicus* along the coast of North America experience various temperatures and have different thermal tolerances, consistent with adaptation. Lower latitude populations have greater

survivorship at higher temperatures than higher latitudes (Willett, 2010) and are shown to have different responses to heat stress (Schoville et. al., 2012). Even after multiple generations of artificial selection, higher latitude populations could not achieve the same heat tolerance as low latitude populations (Kelly et. al., 2012). Additionally, differences in thermal tolerance have been found between southern populations (LJS and SD) at a scale of ~11.8 km (Willett, 2010). These results indicate complexity at the fine-scale that has yet to be investigated.

Many studies have shown that hybrid breakdown can be observed between populations of *T. californicus*. F2 hybrids have been shown to exhibit slower development (Burton, 1990) and reduction in fecundity and survivorship (Edmands, 1999, Ellison and Burton, 2008) at levels of 21%-23% divergence of the cytochrome oxidase subunit one (COI) gene. Reduction of ATP synthesis and oxidative stress has been observed as well (Ellison and Burton, 2008, Barretto and Burton, 2013). Surprisingly, the 8 km distance between BR and SD produces breakdown in naupliar survivorship in F1 hybrids but not in the F2 generation (Pereira et. al., 2014). This may be a result of dominance of some BR alleles, yet more crosses are necessary to test this hypothesis. Additionally, recombinant inbred lines (RILs) produced for the same cross exhibited breakdown in the form of delayed male development (Krick, 2014). It has been demonstrated in *T. californicus* that hybrid inviabilities are caused by genetic incompatibilities between nuclear and mitochondrial genes that are differentially coadapted (Ellison and Burton 2008, 2006, Foley et. al., 2013,

Willett and Burton 2004,). There is also evidence that nuclear-nuclear epistasis plays a role in hybrid breakdown (Burton et al., 1999, Edmands and Burton, 1999, Edmands et. al., 1999).

Although reduced fitness is typically observed in the first few generations of hybridization, increased hybrid performance has also been observed in the form of transgressive segregation. Pereira et. al. (2014) found that crosses between two heat-tolerant populations (BRxSD) produced transgressive hybrids that show significantly greater survival to heat stress (Pereira et. al., 2014). A further study expanded the number of RILs and found the frequency of these extreme phenotypes to be quite high; SDxBR RILs (9 lines) had up to 41% transgressive phenotypes, and BRxSD RILs (5 lines) to have up to 28% (Krick, 2014). It can therefore be speculated that BR and SD have evolved different mechanisms for thermal tolerance; some hybrid offspring appear to combine these mechanisms so that they can tolerate temperatures well outside the range of the parental populations. It would be interesting to investigate the scale of population divergence such that hybridization of similarly adapted populations could lead to transgression.

In light of the results described, this project focuses on the following question: At what scale are populations of *T. californicus* genetically differentiated? At what scale do populations differ in thermal tolerance? At what scale does differentiation result in hybrid breakdown or transgression? To address these questions, a series of experiments were performed. The study region spanned La Jolla, California (LJ) to 5 km south, Bird Rock,

California (BR). Every potential habitat (rocky outcrop) was searched for *T. californicus* populations. Building on previous work using cytochrome *b* (*CYTB*) as a marker for population differentiation (Willett and Ladner, 2009), individuals were collected and genotyped for *CYTB*. After determining *CYTB* based differentiation, differentiated populations were collected to study their respective survivorship to heat stresses. In the context of such divergence, controlled hybridization was then performed to examine any breakdown in life history traits. Lastly, F5+ RILs were produced between the most distant populations of the study area (LJ and BR) to test for transgressive segregation in thermal tolerance and breakdown in male developmental time. Conclusions of these experiments help to understand the scale of hybrid breakdown and transgressive segregation in the context of geography and differentiation.

## Materials and Methods

*Collection*

Approximately 5 km of coastline between La Jolla Cove and Bird Rock (San Diego County, CA) was examined for tide pools inhabited by *Tigriopus californicus*. Previously the phylogeography of this area was examined with six sites by Willett and Ladner (2009) (see Figure 1). In this study, a total of 27 study sites were identified (see Figure 2). Live cultures were initiated from 4 of those sites and genetic samples with a minimum of 20 adults were collected for each site and preserved in ethanol. Populations were kept in beakers and acclimated at 20 °C for 3 months to ensure experimental animals were lab reared. In the interest of time, gravid females were transferred to a beaker and removed after 1-2 weeks to separate individuals reared in the lab rather than waiting a 3-month acclimation period.

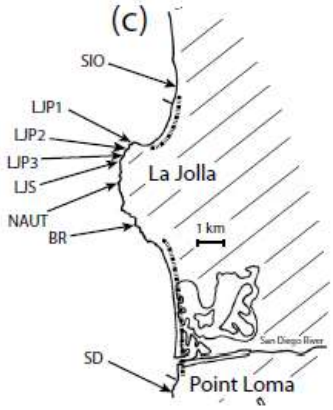


**Figure 1**: Map from Willett and Ladner (2009). Modified from Figure I, this shows localities which were sequenced for *CYTB* previously. It should be noted that SIO is not sampled in this study. Willett's LJP1 is the same as this study's LJ, as is Willett's LJP2 to our LJ7, LJP3 to CBP8, LJS to an area near CBP10, NAUT to V2-3, and BR is the same location.

*Genetic Differentiation*

To determine fine-scale genetic differentiation, cytochrome *b* (*CYTB*) was sequenced from individuals. To extract DNA, animals were crushed and heated in 14 µl of water at 95°C for 20 minutes. The PCR protocol and primer design were the same as that of Willett and Ladner (2009). Chromatograms of sequences were examined, trimmed, and aligned with Geneious©. Non-synonymous substitutions were also examined in Geneious©. A median-joining network was constructed using PopART (accessible for download via http://popart.otago.ac.nz) as recommended for intra-specific studies (Bandelt et. al., 1999). Relevant Willett and Ladner (2009) sequences were included in this analysis.

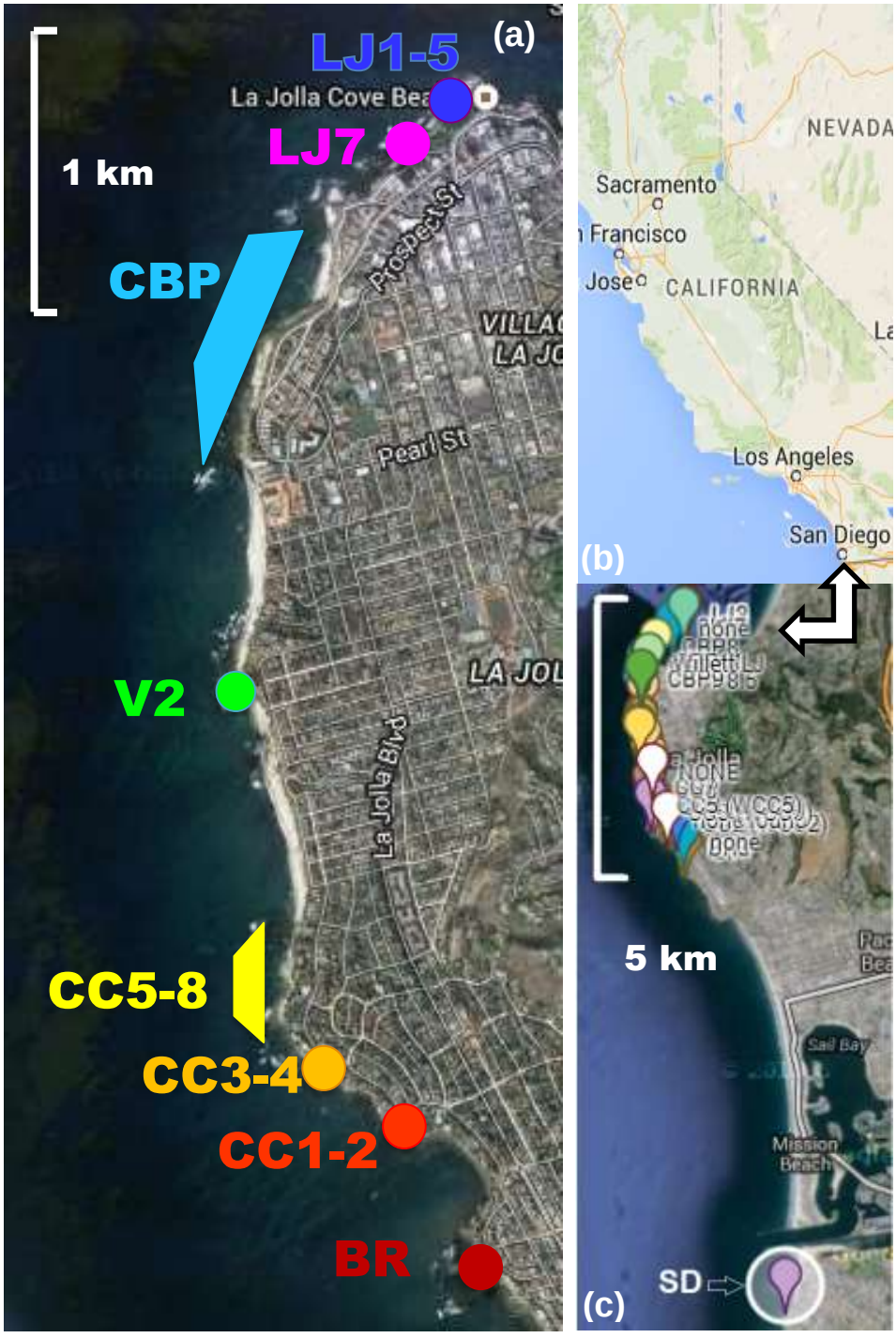


**Figure 2**: Map of collecting sites**.** (a) Map of La Jolla, California to Bird Rock, California spanning a 5 km coastal distance. A total of 27 sites were collected; LJ1-5 (LJ), LJ6-7 (LJ7), CBP8-11, including CBPP, and including V1 (CBP), V2-V3 (V2), CC5-8 including CC5.1-5.2 (CC5-8), CC3-4, CC1-2, and BR1-3 (BR). Population samples included LJ, LJ7, CBP, and BR. (b) Map of California for reference. (c) Study region relative to San Diego, California.

*Ecological Differentiation*

To determine if populations differ in thermal tolerance, ten males and ten females of each population (CBP, LJ, LJ7, and BR) were transferred to tubes with 10 ml of water and allowed one hour of acclimation. Heat stress was performed by moving the tube to a water bath for one hour at 35.5°C – 36.5°C. During the stress the spatial arrangement of tubes was maintained and randomized for each replicate. Afterwards the tubes were immersed in 20°C beakers and allowed to cool down for one hour, then the animals in each tube were transferred to a well in 6 well plates and survivorship was counted three days later.

*Hybrid Breakdown*

Hybrids were produced between LJ and BR, and LJ and SD; reciprocal crosses were performed to represent both maternal (mitochondrial) lineages (LJxBR, BRxLJ, LJxSD, and SDxLJ). Clasped pairs of males and females from each population were dissected apart using a fine needle under a microscope; immature females obtained in this way have not mated and can be combined with males from another population to mate. When females became gravid they were transferred into new plates to release clutches. To ensure no inbreeding in F1 and F2 generations, the gravid females were transferred to 2 separate plates such that pairs of each plate could be outcrossed. Once copepodids were visible, adult females were transferred to new plates to keep generations separate and females were allowed to produce offspring that were transferred to the appropriate plate.

Life history traits were measured for F1 and F2 hybrid generations and compared to parental values to determine breakdown. Three traits were measured for F1 and F2 hybrids: fecundity, percent copepodids on day 14, and survivorship on day 14. Fecundity was measured as the number of eggs in the first clutch. Fourteen days from the hatching of the egg sac, number of nauplii and copepodids were counted allowing for calculation of percent copepodids on day 14 and percent survivorship day 14.

*Recombinant Inbred Lines (RILs)*

RILs were produced by isolating pairs from a beaker of F3+ hybrids and transferring them to 6 well plates. Males were removed once a gravid female was seen. Each well represented the start of a RIL; copepodids from all clutches were from a given well and transferred into petri plates to allow inbreeding. Two generations were inbred before testing for thermal tolerance. For control, inbred lines were created with parental populations, of which initial pairs were from population beakers and then inbred 2 generations. Only LJ PILs were available for testing; BR PILs did not produce enough animals for experimentation. LJxBR started with 66 F3+pairs, ultimately producing 41 replicates for F5+ RILs; BRxLJ started with 36 F3+ pairs, ultimately producing 27 F5+ RILs. Notably, seven replicates from LJxBR F3+ generation were terminated when clasped pairs were found to consist of two males; such apparent mistakes in male choice are very rare in parental populations.

*RILs: Transgressive segregation*

To determine differential adaptation of populations, the thermal tolerance of RILs was measured relative to that of parentals. First, parental population sub-lethal temperature had to be determined via experimentation with temperatures resulting in 0-30% survivorship. Once determined, parentals, PILs, and RILs were heat stressed as described previously.

*RILs: Breakdown in male developmental time*

To examine breakdown in other life history traits, developmental time to first adult male was measured. Gravid females were selected from RILs and allowed to release their first clutch, then removed. Once copepodids were visible, replicates were monitored on a daily basis for adult males.

## Results

*Genetic Differentiation*

A haplotype network of *CYTB* was constructed to elucidate fine-scale population differentiation (see Figure 3). A total of 116 sequences were obtained for 27 sites, including sequences from Willett and Ladner (2009). A minimum of 2 individuals were sequenced for each site, however after preliminary results more individuals were sequenced for BR and LJ7 due to the strong divergence found.

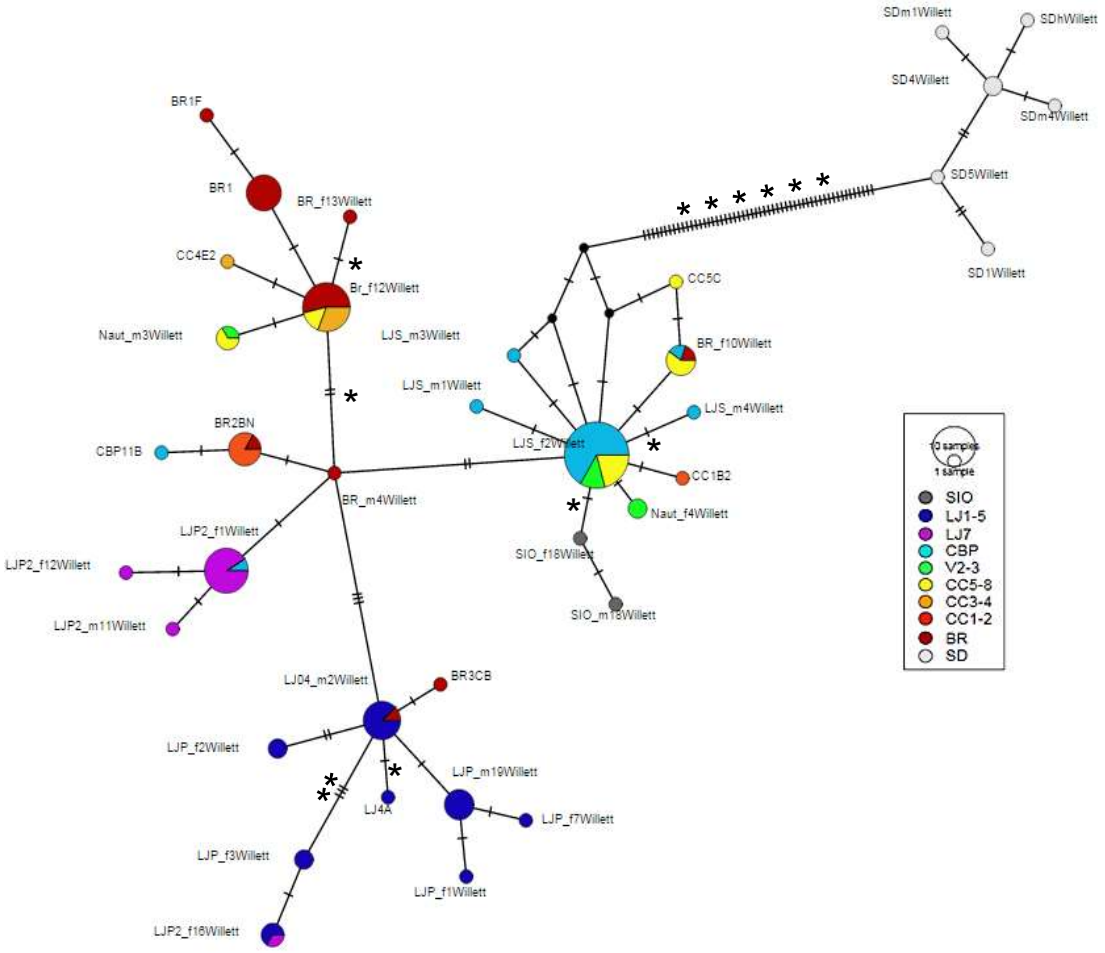


**Figure 3**: *CYTB* haplotype network. A median-joining network was produced with PopART (http://popart.otago.ac.nz) including sequences from Willett and Ladner (2009) and this study. Hatch marks represent single substitutions and asterisks denote non-synonymous substitutions. It was built with 116 sequences which were aligned and examined in Geneious© to 661 nucleotides. Due to a shorter sequence it was truncated to 617 nucleotides in PopART. Non-synonymous substitutions that are not included due to trimming include: LJ7-1D, LJS_f2Willett, SD1 and SD5. Legend is ordered north to south. BR(deep red/brown) included 21 sequences, CBP (turquoise): 23, V2(green):6, CC5-8(yellow): 13, CC3-4(light orange):5, CC1-2(red/orange):6, LJ1-5(dark blue):21, LJ7(purple):13, SD(white):7, and SIO(grey):2. See Figure 2 for map with color coded regions. Nucleotide diversity: pi = 0.0192851, number of segregating sites: 81, number of parsimony-informative sites: 69, Tajima's D statistic: D = -0.903739, (D >= -0.903739) = 0.807.

Non-synonymous substitutions were also examined; one non-synonymous substitution defines the group of BR haplotypes and a subgroup in LJ is defined by two non-synonymous substitutions. Otherwise, single substitutions were found similar Willett and Ladner (2009). Two substitutions

were not detected in the network due to an ambiguity code of Y (T or C base) in LJ5B which still results in an amino acid change; these two substitutions are in CBPPB and LJP_f8Willett, and resulted in amino acid changes. SD has at least 6 private non-synonymous substitutions. Including synonymous substitutions, it is shown that BR and LJ individuals (the most distant) can have up to 1.8% divergence on *CYTB* (11 substitutions of 617 nucleotides).

Similar to past findings, the differentiation on *CYTB* is consistent with limited gene flow between several rocky outcrops. Four major groups of haplotypes are produced: one dominated by northern LJ haplotypes(dark blue in Figures 2-3), another just south of LJ on a rock represented by LJ7 (purple), one dominated by mid-region haplotypes spanning 3.4 km, referred to as CBP (turquoise), and lastly the southern region containing sites CC1-4 and BR (orange, red, and dark red) . Differentiation and mixing varies across the localities. CBP appears to be an area of extensive gene flow with less *CYTB* variation, and BR has higher polymorphism with haplotypes shared in every other major grouping (with exception of LJ7, to which it has a closely related haplotype). LJ7 is particularly differentiated although only 270 m from LJ, split by a sandy beach. LJ is also more differentiated on *CYTB* relative to CBP.

The network reveals sharing of haplotypes in this region which may reflect some gene flow or polymorphism. Haplotypes are shared across 3.4 km with CBP, V2, and CC5-8. Potential barriers to gene flow would include the two sandy beaches (~0.8 km each, see Figure 2 for map) separating V2 from mid-region (CBP) and southern (CC and BR). Sites CC5-8 (yellow) is an

area that can be considered temporary habitat; after revisiting this area about a year later, no copepods were visible in any of those sites. However, copepods were still abundant at CC4 and at two new sites (CC5.1 and 5.2, between CC5 and CC6). It was found that the newly sampled “temporary” sites shared *CYTB* haplotypes not only with the original CC5-8 sites, but also with both the north and south regions: specifically Willett’s Naut (same as V2 here), CBP, CC3-4, and BR.

*Ecological Differentiation*

Significantly different thermal tolerances were found at each temperature of 35.5°C, 36.0°C, and 36.5°C (see Figures 4a-4c as follows). No significant differences were found between sexes of populations at all temperatures using pairwise Wilcoxon test with fdr adjustment. The most distant populations of the study region, LJ and BR, proved to be significantly different at 35.5°C and 36.5°C. The highest temperature, 36.5°C was lethal to BR whereas LJ maintained an average of 30.00 ± 15.59% (two standard errors) survivorship. LJ7 had an average of 8.57 ± 6.35%, whereas CBP exhibited the highest survivorship at 68.33 ± 18.72%.

At 36.0°C, averages were as follows: BR with 37.5 ± 21.0%, LJ with 35.0 ± 13.6%, LJ7 with 41.3 ± 24.9%, and CBP with 75.0 ± 8.5% survivorship. CBP retains the highest survivorship and the other populations become indistinguishable in their response to heat stress. At 35.5°C, BR has 98.8 ± 2.5%, LJ has 57.5 ± 11.2%, LJ7 has 80.0 ± 10.7%, and CBP has 81.3 ± 8.0% survivorship. At these lower temperatures which are not the sub-lethal

temperature for any of the populations, patterns are harder to distinguish; however significance was still detected in all population comparisons with the exception of CBP and LJ7 at both 36.0°C and 35.5°C, and BR and LJ at 36.0°C.

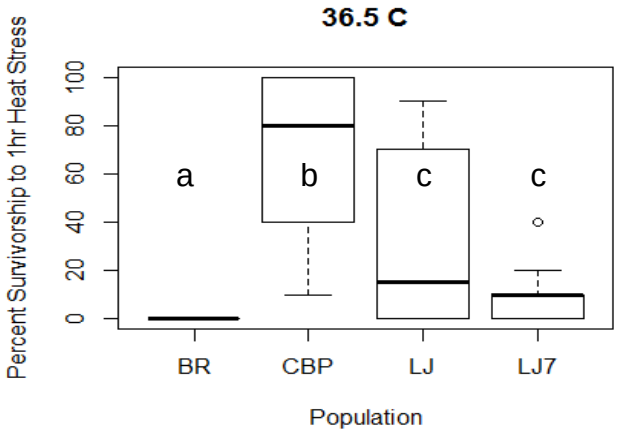


**Figure 4a:** Percent survivorship to 36.5$^0$C heat stress. Boxplot was made in R; top and bottom of boxes denote the first and third quartile while the line represents the median. Pairwise Wilcoxon rank sum test with fdr adjustment was utilized to determine statistical significance. Different letters indicate p-value less than 0.05. CBP and LJ are significantly different with p=0.0039, CBP and LJ7 at p=0.00018, CBP and BR at p=9.32e-06, LJ and BR at p=0.00034, LJ7 and BR at p=0.00135.

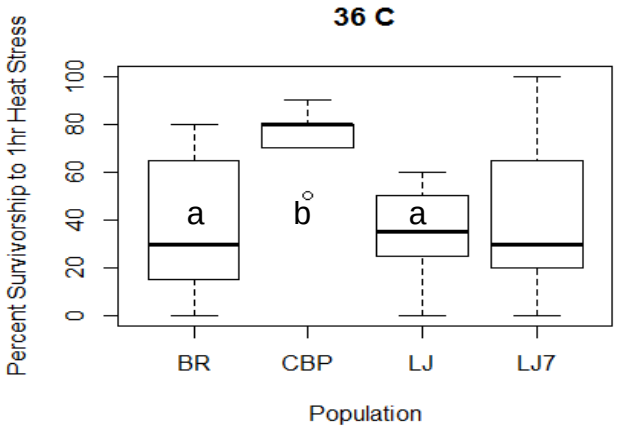


**Figure 4b**: Percent survivorship to 36.0$^0$C heat stress. Boxplot was made in R; top and bottom of boxes denote the first and third quartile while the line represents the median. Different letters indicate p-value less than 0.05. Pairwise Wilcoson with fdr adjustment was used for analysis. BR and CBP are significantly different with p=0.036 and CBP and LJ are significantly different with p=0.01.

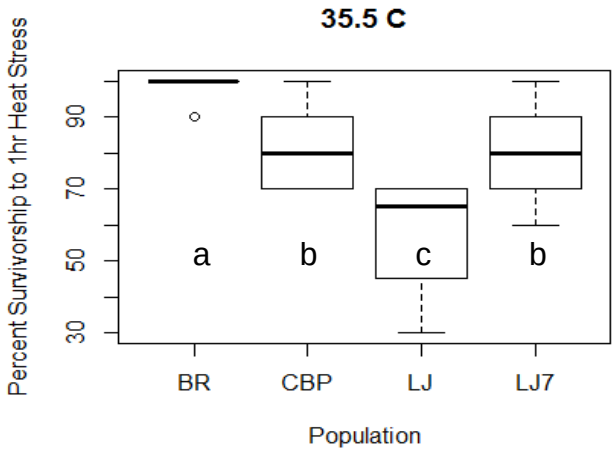


**Figure 4c**: Percent survivorship to 35.5$^0$C heat stress. Boxplot was made in R; top and bottom of boxes denote the first and third quartile while the line represents the median. Different letters indicate p-value less than 0.05, determined via pairwise Wilcoxon test with fdr adjustment. For CBP and LJ, p=0.0102, for CBP and BR p=0.0099, for LJ and LJ7 p=0.0193, for LJ and BR p=0.003, and for LJ7 and BR p=0.0128.

*Life history traits: BR and LJ hybrids*

For F1 and F2 generations, no significant differences were detected from both parentals for fecundity, percent survivorship on day 14, and percent of copepodids on day 14. No breakdown was observed in either reciprocal cross. See Figures 5-7 as follows for life history traits of crosses between BR and LJ.

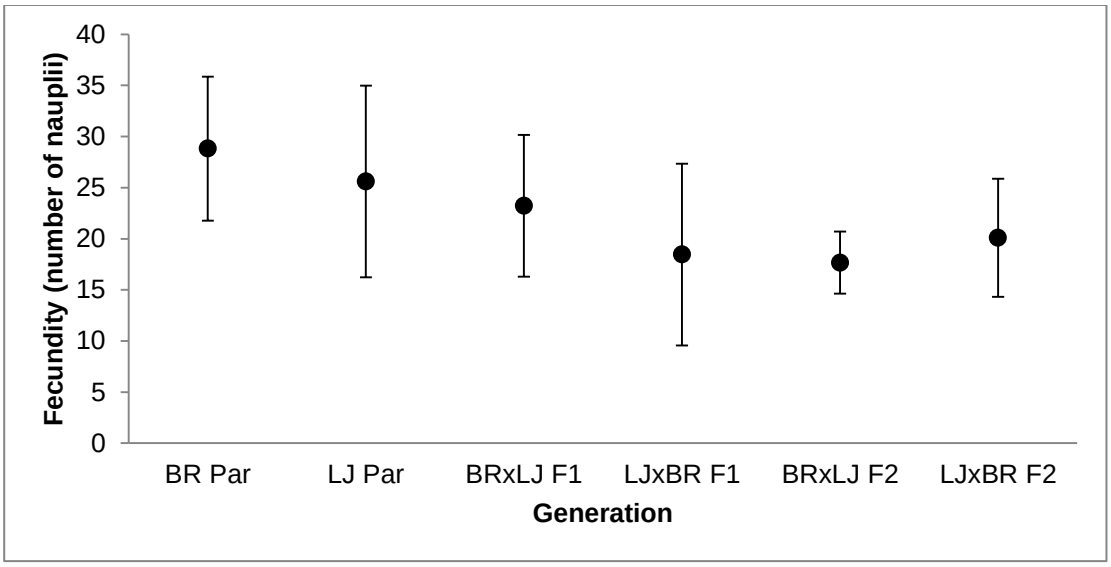


**Figure 5**: Fecundity of F1 and F2 hybrids of BR and LJ crosses. Mean fecundity is represented for parental and hybrid generations. Error bars represent two standard errors. LJ parental fecundity violated normality, thus pairwise Wilcoxon test with fdr adjustment was used to determine statistical significance. No hybrid generations have significantly different fecundity than both the parentals.

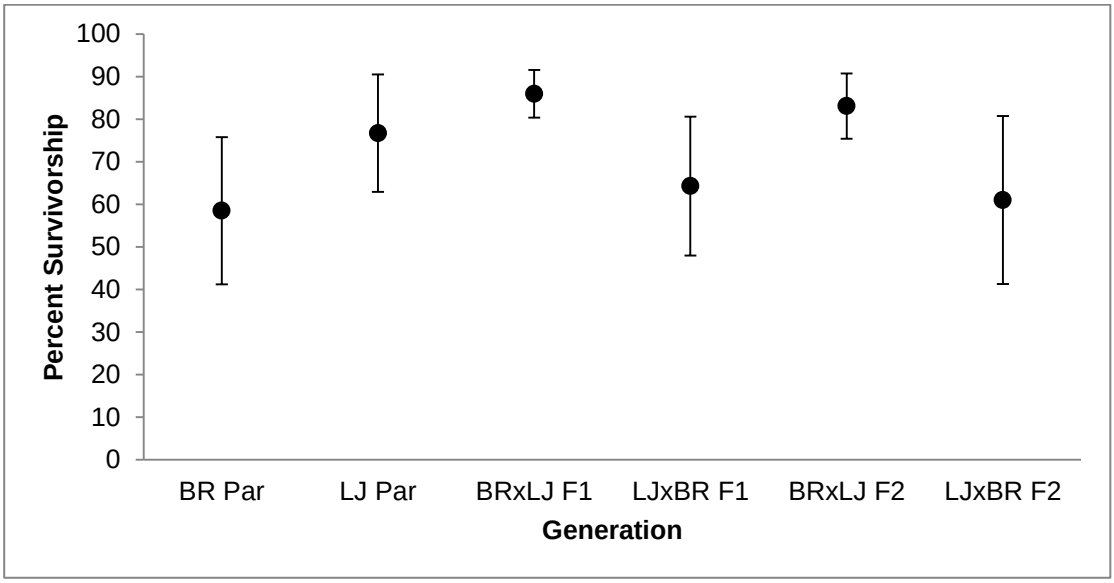


**Figure 6**: Survivorship on day 14 of F1 and F2 hybrids of BR and LJ. Mean survivorship from 2 weeks of hatching is represented for parental and hybrid generations. Error bars show two standard errors. Data was normal and homoscedastic; ANOVA was used for statistical analysis. No hybrid generations were significantly different than both parentals.

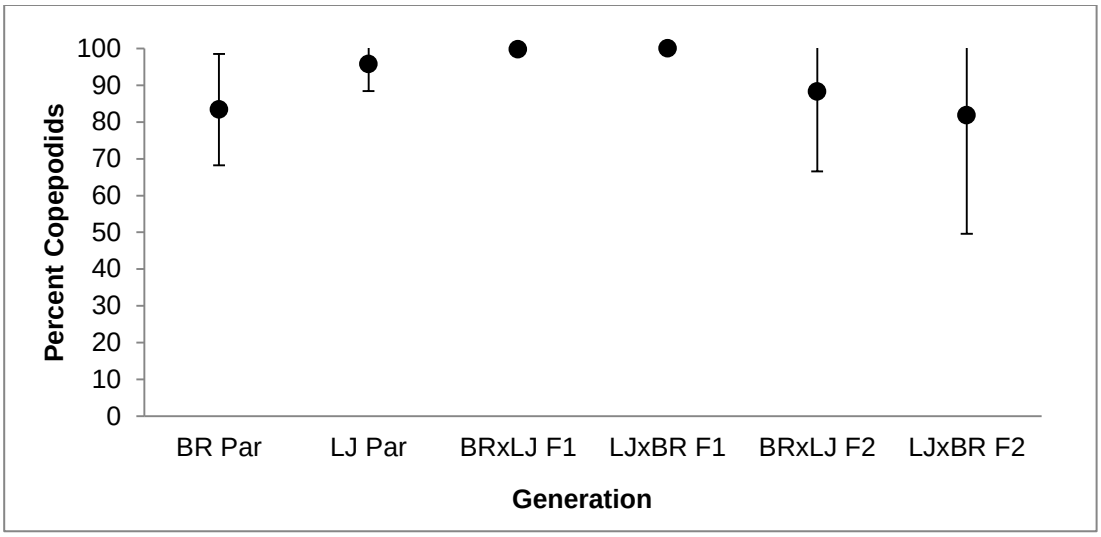


**Figure 7**: Percent copepodids on day 14 of F1 and F2 hybrids of BR and LJ crosses. Mean percent copepodids on day 14 is represented for parental and hybrid generations. Due to non-normal data, pairwise Wilcoxon test with fdr adjustment was performed. No hybrid generations were significantly different than both parentals. Error bars represent two standard errors. F1 generation had no error as all values were 100%.

*Life history traits: LJ and SD hybrids*

Of the three fitness traits measured, only percent copepodids on day 14 yielded significant differences between hybrids and either parent (see Figures 8-11 as follows). No significant reduction in fecundity was detected for F1 and F2 hybrids of both reciprocal crosses, but it can be noted that the frequency of clutches of less than 10 nauplii were higher for LJxSD F1; five out of thirteen replicates fell in this category relative to the one of seventeen for LJ, and three of sixteen for SD. No significant reduction of survivorship on day 14 was detected. Breakdown in percent copepodids on day 14 was observed as SDxLJ F2 exhibited a significant reduction, indicating delayed development.

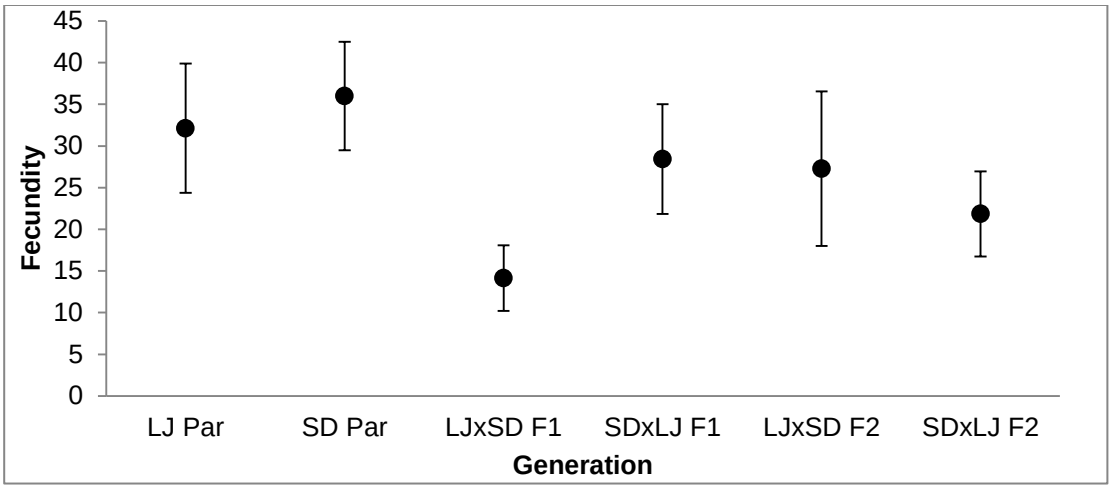


**Figure 8**: Fecundity of F1 and F2 hybrids of LJ and SD crosses. Mean fecundity is represented for parental and hybrid generations. Error bars represent two standard errors. Data was homoscedastic and normal, thus ANOVA statistical test was performed. LJxSD F1 with a p=0.0026 was significantly different from LJ, and p=0.62 with SD; no hybrid generations had significantly reduced fecundity from both parentals.

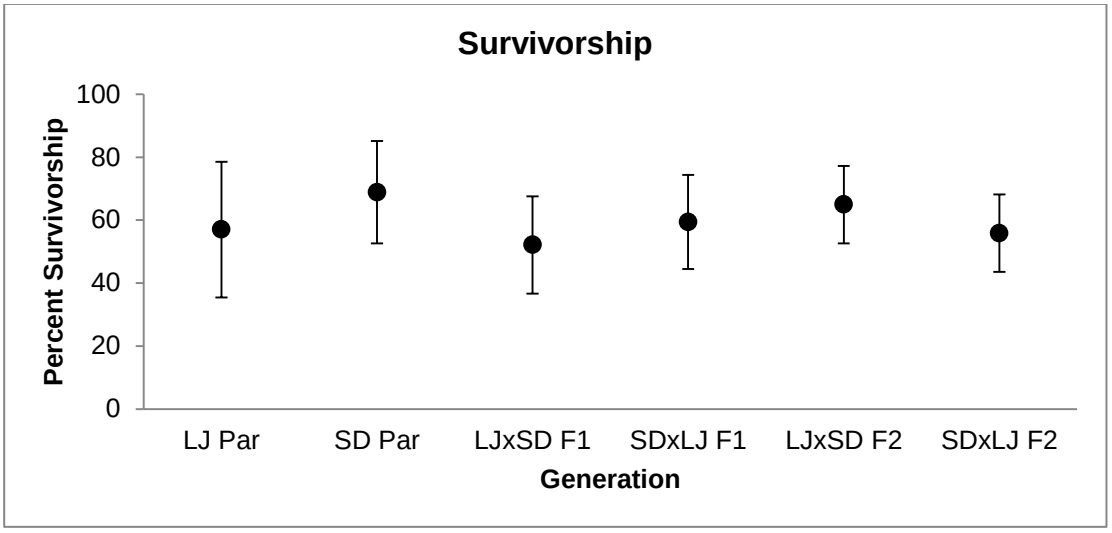


**Figure 9**: Survivorship on day 14 of F1 and F2 hybrids of LJ and SD crosses. Mean survivorship from 2 weeks of hatching is represented for parental and hybrid generations. Error bars show two standard errors. Pairwise Wilcoxon test with fdr adjustment was used for statistical analysis. No hybrid generations were significantly different than both parentals.

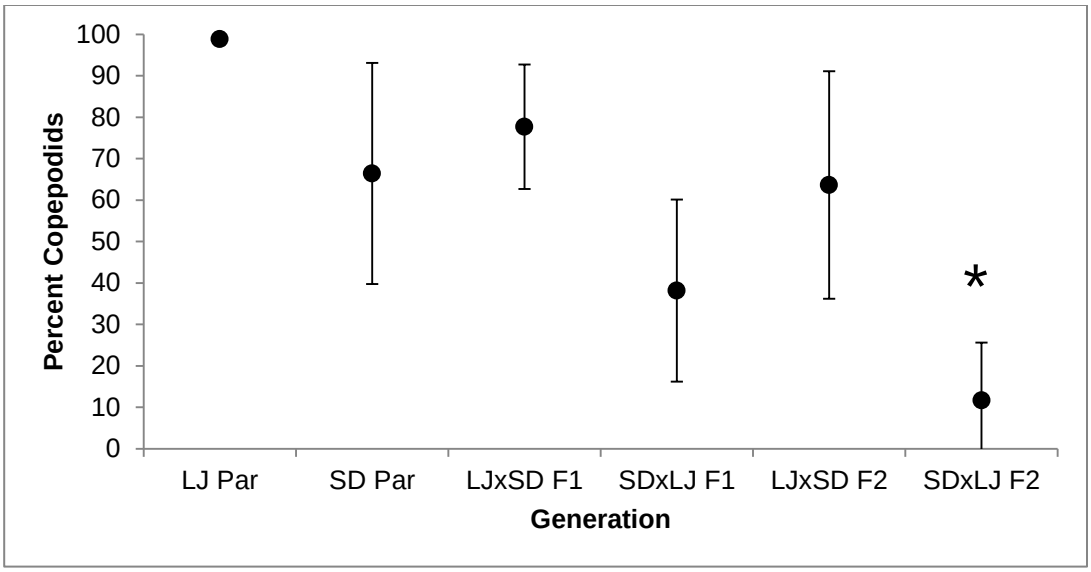


**Figure 10**: Percent copepodids on day 14 of F1 and F2 hybrids of LJ and SD crosses. Mean values are represented for parental and hybrid generations. Error bars represent two standard errors. Pairwise Wilcoxon test with fdr adjustment was used. SDxLJ F2 percent copepodids is significantly different from both parentals: from LJ with p=0.00093, and p=0.01352, with SD. LJxSD F2 was also significantly different from LJ at p=0.04096 and SDxLJ F1 was significantly different from LJ at p=0.00158.

*Testing for transgressive segregation*

The sub-lethal temperature was determined to be 36.5°C for LJ, which was lethal for BR. Of the 20 BRxLJ RILs and 36 LJxBR RILs stressed at 36.5°C, zero exhibited novel phenotypes of transgressive thermal tolerance (see Figures 11a-b). Only three LJ PILs had enough animals for experimentation, and yielded similar survivorship to LJ parental population. Limitations included having enough replicates as RILs are more prone to extinction.

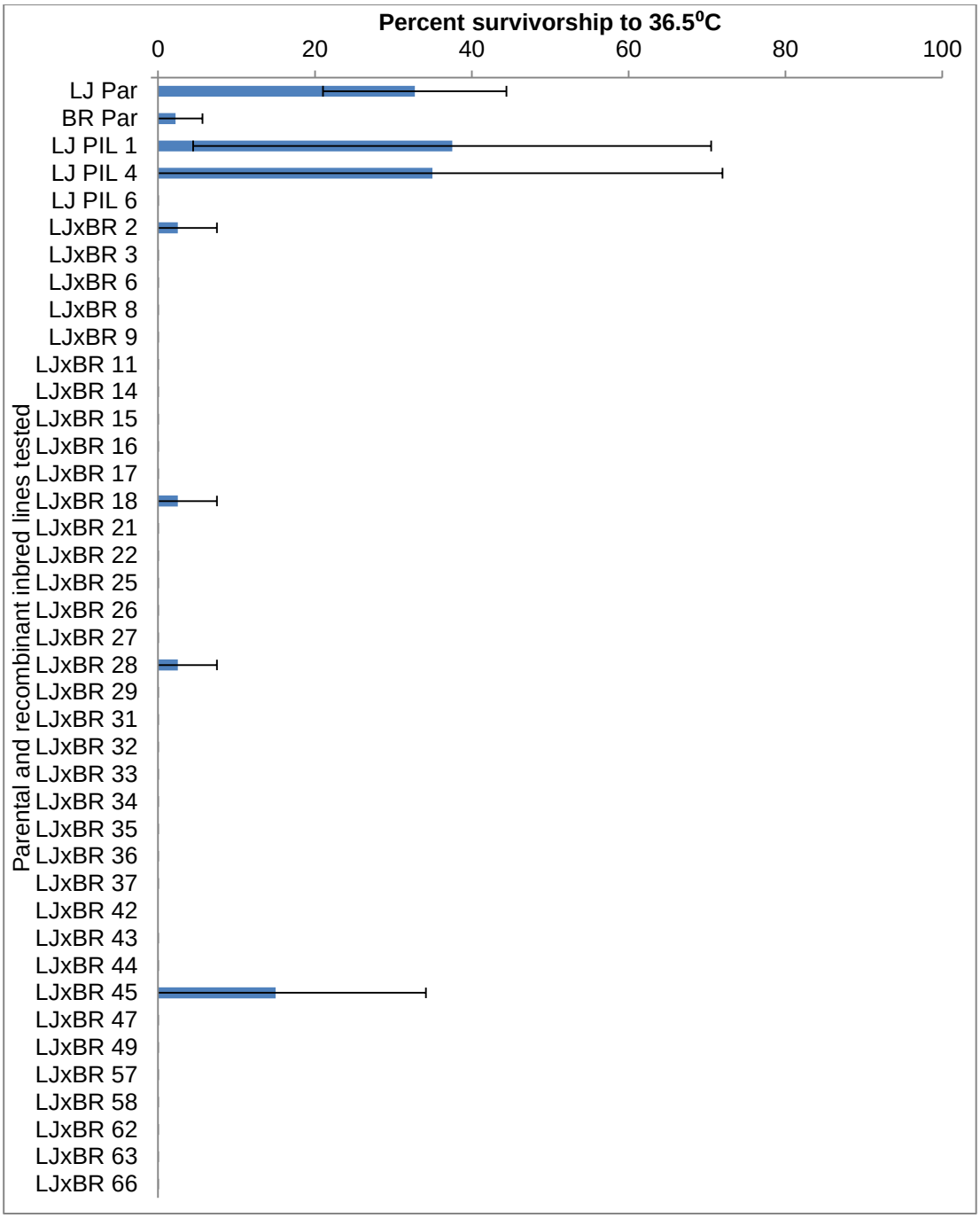


**Figure 11a**: Testing for transgressive segregation in LJxBR RILs. A total of 36 LJxBR RILs were tested with no significant differences. Error bars denote two standard errors. Minimum of 4 replicates for each PIL and RIL, LJxBR 22, 26, and 25 only had 2 replicates as the lines went extinct. Parentals of LJ and BR had n=22 and n=18, respectively. No significance detected other than between parentals LJ and BR; used pairwise Wilcoxon rank sum test with fdr adjustment.

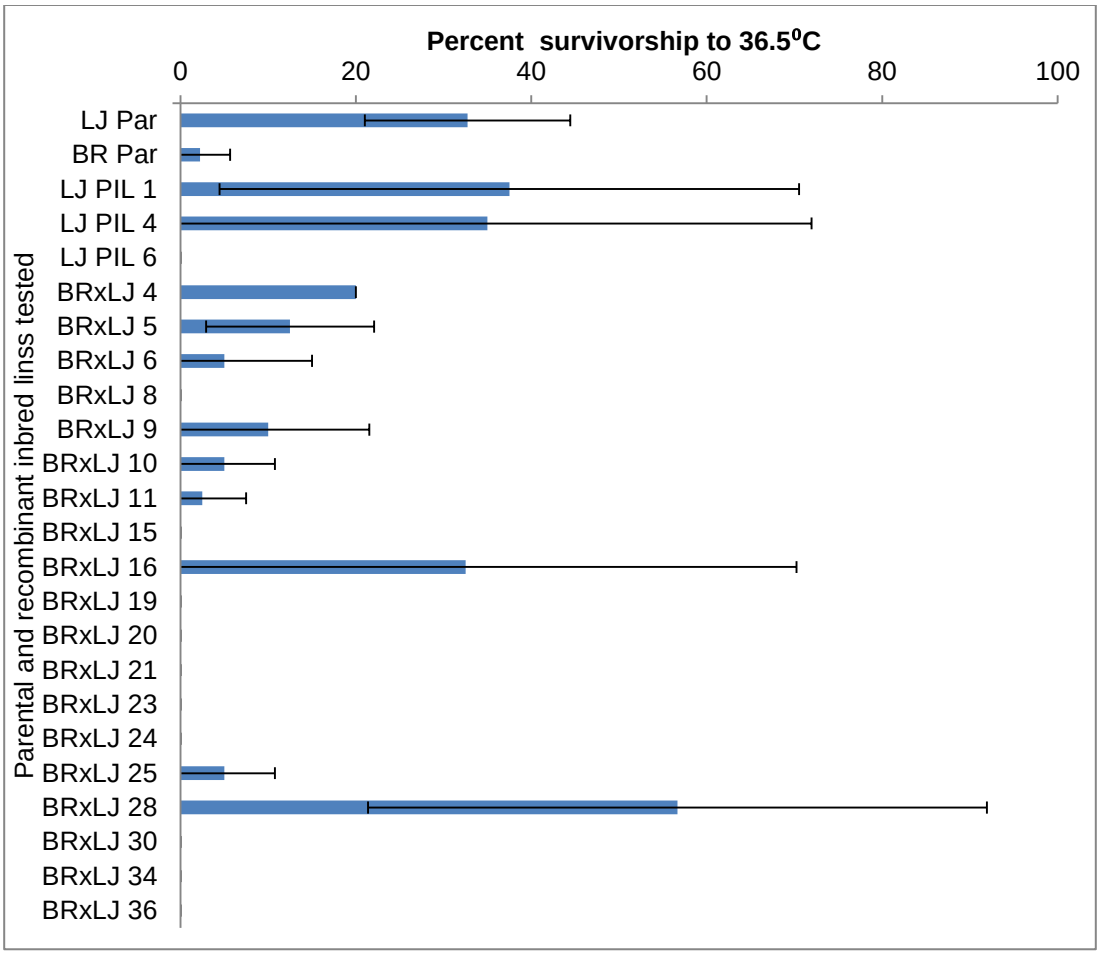


**Figure 11b**: Testing for transgressive segregation in BRxLJ RILs. A total of 20 BRxLJ RILs were tested. Error bars represent two standard errors. Due to extinction of lines, BRxLJ 4 had only one replicate, BRxLJ 15 had two replicates, and BRxLJ 28 had three replicates. All the others had at minimum four. No significance outside of LJ parental from BR parental was detected using pairwise Wilcoxon rank sum with fdr adjustment.

*Male Developmental Time of RILs*

Male developmental time was measured as the number of days it took for the first adult male to appear from the first clutch. Breakdown in male developmental time for BRxSD RILs had been found previously (Krick, 2014), but was not found for the cross of BR and LJ (see Figure 12).

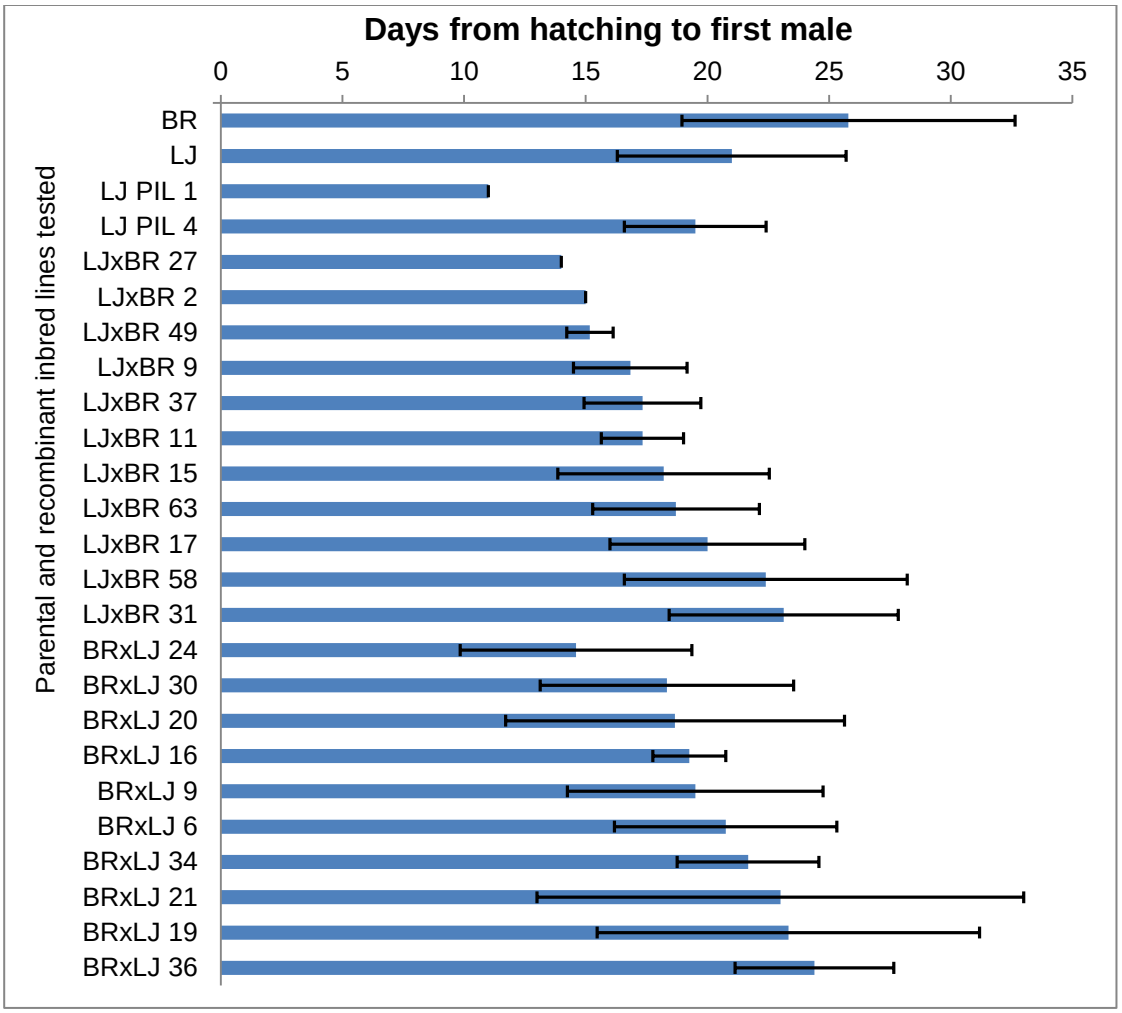


**Figure 12**: Male developmental time. Error bars show two standard errors. The aim was to have a minimum of four replicates; due to extinction LJ PIL1, LJxBR 2, only had one replicate, and BRxLJ 21 had 2 replicates, while LJxBR 37, LJxBR 17, BRxLJ 30, and BRxLJ20 had 3 replicates. No significance detected via pairwise Wilcoxon rank sum test with fdr adjustment.

## Discussion

Fine-scale studies can reveal complex patterns and insights into early stages of speciation. This study sought to address the degree of fine-scale differentiation among *T. californicus* populations and assess the consequences of such differentiation. Most studies have only looked at larger scales; two studies are similar to this scale: one found variation in enzyme encoding loci to be significantly different for populations a half-kilometer apart near San Francisco, California (Burton and Feldman, 1981). Another looked at genetic differentiation of six sites within this study region (Willett and Ladner, 2009). This study furthers this work by sampling all potential habitats and recording tolerance to heat stress on a scale not previously examined. Additionally, previous studies found BRxSD F1 hybrids exhibit reduced fitness (Pereira et. al., 2014) and BRxSD RILs exhibited a high frequency of transgressive thermal tolerance (Krick, 2014). This suggests the potential for incompatibilities and transgression to occur at even smaller spatial scales. Overall this study finds that differences in thermal tolerance can occur at an even finer scale, but that reproductive incompatibilities are not yet observed at a scale smaller than BR and SD.

*Fine-scale genetic differentiation*

The differentiation of *T. californicus* populations along the 5 km sector proved to vary with region of interest. In some areas, populations appeared to

be genetically distinct though only 270 m distant, while in others there are shared haplotypes along several kilometers. Although living in fragmented habitats of isolated populations, *T. californicus CYTB* differentiation patterns can be affected by past long-distance dispersal events, genetic drift, extinction and colonization events, and presence of polymorphism.

As well explained in Willett and Ladner (2009) copepod habitat has varying stability which is likely to explain the complex fine-scale differentiation. The habitat is composed of isolated pools in the splash zone of the rocky intertidal habitat prone to extinction events. Some may be more prone to desiccation due to exposure to sun and/or depth. In addition the number of pools on a single outcrop influences the differentiation on that single outcrop (Willett and Ladner, 2009). Populations living in less favorable habitats may maintain a small effective population size, increasing the potential role of drift in their divergence (Husemann et. al., 2015); drift likely plays a role in the differentiation as well due to founder effects. Furthermore, chance events such as a storm bringing in kelp to smother the pools may cause extinction, as it was observed during collection in this study. Types of algae and microbial diversity may certainly play a role in providing suitable habitat. Recolonization may occur by wave splashes, and vectors such as birds, crabs, and seaweed; such long-distance dispersal events are expected to be of low frequency but have shown to possibly influence broad-scale differentiation (Willett and Ladner, 2009).

As found previously, the shared *CYTB* haplotypes from non-neighboring outcrops indicate long-distance dispersal may play a role in fine-scale differentiation. The scale of this dispersal was from BR to SIO, SIO being ~8 km coastal distance north of BR (see Figure 1). Such dispersal was found to have a great effect such that geographically distant populations have low levels of divergence and proximal populations can have higher divergence. Two possibilities were described: one that long-distance dispersal is rare and has little role in homogenizing outcrops and second, that the migrant copepod is rarely successful at colonizing new outcrops (Willett and Ladner, 2009). Thus the new outcrops may have less suitable habitat, and then be considered a temporary pool. Permanent pools could be on large outcrops with an ecosystem structure ideal for copepod, and be unaffected by such long-distance events. Overall the more permanent pools may then be expected to be more divergent due to random mutations and drift, than pools near outcrops with extensive gene flow.

The most interesting result of the *CYTB* network is related to the northernmost sites, LJ7, which encompasses LJ6-1, LJ7-1, and Willett's LJP2; it appears to be highly differentiated from its closest neighbor, LJ, and has two private substitutions. They are separated only by 270 m, and yet LJ7 is more closely related to the southern haplotypes (CC1-2, BR). The only shared haplotype with LJ7 is from site CBP8, which is the site closest to LJ7 on the south side indicating a potential chance event. CBP11 also appears to be closely related to BR and CC1-2 haplotypes, which are the closest *CYTB*

haplotypes to LJ7 and could also be a result of a chance event or polymorphism. LJ7 itself is highly divergent from its neighbor and is the best example of such fine-scale differentiation. It appears that the 250 m of sandy beach is indeed isolating it from LJ to an extent that allows for its genetic differentiation. Furthermore, LJ7 is composed of very few pools and has less variation at *CYTB* than LJ, consistent with recent colonization.

BR shares haplotypes in every major grouping and has high polymorphism. This implies that BR may have been a source or sink of colonists for other outcrops, or simply that BR has high polymorphism. Temporary populations were observed between CC5-8; these sites (CC5.1 and CC5.2) were not observed upon first sampling, and found upon revisiting the area (previously sampled sites CC5, 6, 7, and 8 were not observed). The individuals sequenced shared haplotypes with BR, CC3-4, V2-3, and CBP, potentially indicating mixing and colonization from outcrops both to the north and south. However, it should be noted that it is difficult to prove there was a complete extinction as nauplii are not visible to the naked eye; this could simply represent polymorphism that was already present at the site. Alternatively, there are many birds in the area of BR which may act as possible vectors for copepods via feet, carrying crabs, seaweed, etc. The sharing of these haplotypes and similarity of *CYTB* sequences imply mixing between BR and CBP, and potential past long-distance dispersal events. The sandy beaches flanking the sides of V2 (see Figure 2) may not serve as barriers to gene flow.

Supporting past results, CBP contains haplotypes shared along ~3.4 km, consistent with long-range colonization. There is less divergence in this region with only one substitution from a common haplotype for the majority of haplotypes. This may imply some homogenization via gene flow and multiple recolonization events, but greater sample size is necessary. Being about 1 km in coastal distance, CBP has many permanent pools, and one may expect a greater chance of migrants from this outcrop; V2/V3 sequenced in this experiment always shared haplotypes with CBP (immediately north), and Willett's sequences (NAUT) shared haplotypes with both CBP and CC5-8 (haplotypes more similar to BR), just to the south. This could indicate colonization or polymorphism, but great sample size is necessary for quantifying gene flow.

For LJ there are three private amino acid substitutions and there appears to be more genetic differentiation than CBP. LJ is a smaller outcrop (~60m) and there are fewer habitable pools than CBP. Like BR, LJ appears to have greater variation on *CYTB*, but all haplotypes are closely related indicating the persistence of a population. The shared haplotypes among LJ sites may indicate gene flow within the small outcrop, and drift may contribute to its differentiation from other outcrops. In addition one of Willett's LJP2 (LJ7) haplotypes is shared, possibly indicating a dispersal event in 2009.

Complex patterns of genetic differentiation have also been found for other systems. Similar to *T. californicus*, intertidal *Ligia* isopods have restricted dispersal abilities, are distributed on the Pacific rocky shores

between central California and Mexico, and experience high genetic divergence. Between the north and south of the Gulf of California, there can be up to 26.47% divergence on COI. Great differentiation was found among regional clades; within some clades shallow divergence was found across extensive distances that could indicate recent genetic exchange (Hurtado et. al., 2010). Such shared patterns can shed light on complex histories.

*Fine-scale ecological differentiation*

Significant differences in survivorship after heat stress were determined for a smaller spatial scale than found before for *T. californicus*. This holds several implications; for one, the importance of studying the microclimate. The differences may be completely random and a result of drift, or it could reflect adaptation to micro habitats. Such fine-scale adaptation (2.4 m) has been documented for barnacles, measured via allele frequencies and survival in response to selection by temperature (Schmidt and Rand, 2001). Adaptation has been found to occur on a scale of less than one kilometer also in amphipods, various gastropods, and in anthozoans (Sanford and Kelly, 2010). Long-term monitoring of the tide pools is necessary to determine if the *T. californicus* populations are experiencing different environmental conditions that could select for local adaptation. How the microclimates differ has yet to be quantified, and the differences in thermal tolerance may reflect ecological differentiation or drift.

The smallest scale at which significant differences in thermal tolerance have been detected in the past is 11.8 km (Willett, 2010) between LJS (near

CBP10) and SD. This study examines a 5 km region in which significant differences were also found; this determines the smallest spatial scale of differences in thermal tolerance known in *T. californicus* to be ~1.5 km. CBP had significantly different survivorship from both BR and LJ at all temperatures (from LJ ~1.5 km distant and from BR ~3.5 km distant), and with LJ7 at 36.5°C. BR and LJ had significantly different survivorship at 36.5°C and 35.5°C, indicating such phenotypic differences can also be observed at 5 km. LJ and LJ7 in the closest vicinity (270 m) yielded significant differences at only at 35.5°C. This sub-lethal temperature yielded the smallest p-values among populations, and may the best way to examine thermal tolerance.

It would also be interesting to investigate if V2, which shares haplotypes with both CBP and BR, has thermal tolerance more similar to one or the other. Extinction-colonization dynamics of the CC5-8 area are unfavorable to local adaptation (Hanski, 1999), therefore it would be interesting to investigate the survivorship to heat stress in the temporary pools of the area in tandem with the genetic composition.

*Scale of hybrid breakdown*

The results from this study indicate that although they are genetically distinct and have different thermal tolerances, BR and LJ do not exhibit genetic incompatibilities. Such differentiation on *CYTB* and their genetic differences in survivorship to heat stress do not result in hybrid inviabilities; fecundity, survivorship, and developmental rates in the F1 and F2 generations were not significantly reduced from parentals. Additionally, RILs did not

exhibit breakdown in male developmental time. Thus, the smallest scale known for hybrid breakdown remains at BR and SD, as previously determined. It might then be predicted that LJ and SD hybrids would also experience genetic incompatibilities.

The SDxLJ F2 hybrids showed breakdown in developmental time. This supports how hybrid incompatibility can manifest early in development. It is interesting that BRxSD F1 hybrids had reduced survivorship; the general expected pattern for hybrid fitness reduction is the F1 generation will have no reduction in fitness or hybrid vigor, and the F2 generation will exhibit breakdown (Burton, 1986, 1990; Edmands, 1999). This suggests dominant allele interactions in BRxSD F1 that were not detected in crosses of LJ and SD. It is proposed that there are different underlying genetic causes for incompatibility in BRxSD and SDxLJ; mapping of various loci in different generations would help to determine what genetic interactions are causing fitness breakdown.

Although previous experiments have shown that reciprocal crosses both exhibit breakdown (Ellison and Burton, 2008), the current finding of asymmetry confirms the expectation of the Dobzhansky-Muller incompatibilities; as mitochondrial DNA is uniparentally inherited , the asymmetry dependent on mitochondrial background has been observed in a variety of other organisms from nemotodes, *Nasonia* (wasps), and salamanders(Turelli and Moyle, 2007, Dey et. al., 2014, Clark et. al., 2010, Devitt et. al., 2011)

*Transgressive Segregation*

Transgressive segregation may be observed in hybrids of similarly adapted populations and has been shown to occur in *T. californicus*. Previously it was detected at the same scale as hybrid breakdown at ~8 km; a high frequency of RILs had significantly improved thermal tolerance than parental populations (Krick, 2014). The BR and LJ RILs did not exhibit transgressive segregation in tolerance to thermal stress. Transgression may not occur in these population crosses, or BR and LJ may be similarly adapted to thermal tolerance. Future research includes producing LJ and SD RILs to test for transgressive segregation; if a high frequency of greater thermal tolerance is seen, the cellular response could be investigated via transcriptomics.

## Conclusion

This study elucidates the importance of fine-scale studies for insight into processes of population differentiation and adaptation. Differences in thermal tolerance were discovered at new spatial scale (~1.5 km versus ~11.8 km). As opposed to the 8 km distance leading to 10.4% divergence between BR and SD, the 5 km separating BR and LJ resulted in up to 1.8% divergence on *CYTB.* Additionally the differences determined in this study did not result in genetic incompatibilities. The finest scale at which hybrid breakdown and transgressive segregation has been observed in *T. californicus* still remains at ~8 km, the distance between BR and SD populations. Such experimental hybridization allows for the study of genetic incompatibilities; it has implications for future research in finding genes responsible for breakdown in specific life history traits. Additionally it allows for studying transgression; future research includes testing LJxSD and SDxLJ RILs for transgressive segregation in order to help determine which genes are responsible for any novel thermal tolerance observed in hybrids.

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