UNIVERSITIY OF CALIFORNIA SAN DIEGO

Potential tradeoff between developmental time and lifespan in the intertidal copepod, *Tigriopus californicus*

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by

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The Thesis of Rebecca Pak is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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ABBREVIATIONS

SD	San Diego
SCN	Santa Cruz
SD x SCN	San Diego (female) x Santa Cruz (male) hybrid
SCN x SD	Santa Cruz (female) x San Diego (female) hybrid

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ABSTRACT OF THE THESIS

POTENTIAL TRADEOFF BETWEEN DEVELOPMENTAL RATE AND LIFESPAN IN THE INTERTIDAL COPEPOD, *TIGRIOPUS CALIFORNICUS*

by

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In eukaryotes, both mitochondrial and nuclear genes encode subunits of electron transport system (ETS) enzymes that must favorably interact (mitonuclear interactions) to produce metabolic energy. Therefore, mitochondrial and nuclear genes coevolve within populations such that hybrids bred from populations in different geographic locations may result in mitonuclear incompatibilities, leading to hybrid breakdown in numerous phenotypic traits. Current hybrid studies of *T. californicus* reveal the effects

of mitonuclear interactions in the breakdown of developmental times, ATP production, and survival; however, the linkages amongst numerous phenotypic traits are still largely unknown. To determine the extent of breakdown of phenotypic traits and their linkages to one another, we recorded developmental times and monitored longevity after categorizing copepods into fast-developing and slow-developing groups. Contrary to previous studies, we found that mitonuclear incompatibilities did not greatly affect the developmental times in hybrids. Additionally, individuals from the slow-developing groups for each of the crosses showed higher survivorship to day 89 than the fast-developing group; i.e., there was an apparent tradeoff between early life stage developmental rates and longevity where fast-developing individuals displayed shorter lifespans compared to slow-developing individuals. Future studies should examine the effects of different rates of development on longevity and potentially when the tradeoff starts between fast early life stage development and a shorter lifespan. Other potential future directions include measuring different life history traits alongside longevity such as lifetime egg production in females.

INTRODUCTION

Mitonuclear Interactions in the Electron Transport System

Mitochondria are the powerhouses of the cell, producing metabolic energy for cellular functioning. Within the mitochondria, the electron transport system (ETS) in concert with the Kreb's cycle generates most of the cell's energy in the form of adenosine triphosphate (ATP) via the process of oxidative phosphorylation (OXPHOS). Four of the five OXPHOS complexes are comprised of subunits that are encoded by both the mitochondrial and nuclear genomes, whereby the subunits must favorably interact to comprise functional complexes capable of producing metabolic energy (Blier et al., 2001; Grossman et al., 2004; Rand et al., 2004). While Complex II of the ETS is comprised of only nuclear-encoded subunits, both mitochondrial- and nuclear-encoded subunits comprise Complexes I, III, IV, and ATP Synthase (Complex V) (Saraste, 1999). Mitochondrial DNA only encodes 13 protein subunits while nuclear DNA codes for 73 OXPHOS protein subunits for eukaryotes that utilize aerobic metabolism (Blier et al. 2001; Rand et al. 2004; Alston et al., 2017). Compatible mitochondrial and nuclear genes are evolutionarily coadapted within populations to properly form the enzyme complexes necessary for generating ATP (Edmands and Burton, 1999; Blier et al., 2001). On the other hand, mitonuclear incompatibilities can result when the translated subunits interact unfavorably and produce abnormally functioning complexes, potentially resulting in decreased ATP production rates (Ellison and Burton, 2006) and other breakdown effects in measured phenotypic traits. Mitonuclear incompatibilities can have far reaching effects. MacFarlane et al. (2016) concluded that

the lack of ficedula flycatchers F_2 hybrids was clear evidence of mitonuclear incompatibilities. This conclusion was based in part from evidence of postzygotic isolation in F_1 hybrids of collared flycatchers and pied flycatchers, which are two species that recently underwent speciation (Kawakami et al., 2014). Although mitonuclear interactions play a significant role in energy production, we have yet to find the direct effects of altered metabolic energy production on longevity in relation to early development.

Inducing Mitonuclear Incompatibilities in Hybrids

Mitonuclear incompatibilities can resemble the mismatches that cause certain mitochondrial diseases and metabolic disorders. One method of inducing viable mitonuclear incompatibilities for study is through creating hybrid lines from crossing one maternal population with a different paternal population from the same species (Burton et al., 2006; Ellison and Burton, 2008, 2010). In other hybrid studies, 2nd generation (F_2) interpopulation hybrid lines were generated by interbreeding the offspring of two different parental populations, creating individuals that possessed the mitochondrial genome from the maternal population and the nuclear genomes from both the paternal and maternal populations (Ellison and Burton, 2006; Pritchard et al., 2011; Healy and Burton, 2020). F_2 hybrids are known to exhibit hybrid breakdown revealed through phenotypic disadvantages and broad phenotypic variation such as decreased fecundity, increased oxidative damage levels, decreased lifespan, and decreased ATP and enzyme complex activity, potentially due to these mitonuclear incompatibilities (Edmands, 1999; Rand et al., 2001; Ellison and Burton, 2006, 2010; Barreto and

Burton, 2013; Burton et al., 2013; Meiklejohn et al., 2013; Zhu et al., 2014; Healy and Burton, 2020). Hybrid breakdown describes the phenotypic breakdown of hybrids compared to the phenotype of parental populations.

Previous hybrid studies have been conducted on the intertidal copepod, *Tigriopus californicus*, to determine the effects of mitonuclear incompatibilities on ATP production, enzyme activity, and the relationship between cytochrome c (CYC) and cytochrome c oxidase (COX) (Rawson and Burton, 2002; Ellison and Burton, 2006; Healy and Burton, 2020). Ellison and Burton (2006) found that hybrid crosses displayed breakdown through a significant decrease in ATP production and enzyme activity (except for Complex II, which only contains nuclear-encoded subunits) compared to their respective parental populations. Additionally, the phenotypic traits of hatching number of nauplii (larvae) from the egg sacs, survivorship fraction, and metamorphosis fraction were tracked and compared to ATP production rates to identify any linkages amongst these phenotypes. The relationship between survivorship fraction and ATP production was the only one that was found to be significantly correlated with a positive relationship (Ellison and Burton, 2006).

Subsequently, Burton et al. (2006) found F_2 hybrid breakdown in *T. californicus* in the traits of the number of larvae produced in the first brood, ATP production, and the relationships between ATP production and metamorphosis, larval production, and survivorship (at 14 days). The breakdown of these phenotypic traits in F_2 hybrids compared to parental populations was concluded to most likely be attributed to mitonuclear incompatibilities and the more frequent occurrence of recessive homozygous genotypes in the F_2 generation (Burton et al., 2006). Healy and Burton

(2020) similarly found hybrid breakdown in the trait of developmental time to metamorphosis due to mitonuclear incompatibilities. Barreto and Burton (2013) found an increase in oxidative damage of more than 30% in recombinant inbred lines (F_9^+) in comparison to the parental populations, which was found to be correlated with decreased fecundity. These effects can most likely be attributed to negative mitonuclear effects as Ellison and Burton (2008) provide data on transcript levels of hypoosmotic stressed intertidal copepods to support that interpopulation hybridization disrupts the coadapted mitochondrial transcription regulation system, which comprises the mitochondrial transcription of parental populations that have evolved to maintain compatible mitochondrial RNA Polymerases (mtRPOL) and mitochondrial promoters. This could contribute to the decreased capability to deal with stress and a lower fitness than their respective parent populations.

Ellison and Burton (2008) determined that mitonuclear incompatibilities caused hybrid breakdown through recovering the low fitness of F_2 and F_3 hybrid copepods through the introduction of a maternal backcross. In this experiment, F_2 and F_3 hybrid copepods displayed lower fitness in traits such as fecundity, survivorship, metamorphosis fraction, and ATP production rate while F_1 hybrids had a similar or higher fitness compared to parental populations in these traits (Ellison and Burton, 2008). When F_2 and F_3 hybrid females were crossed with paternal population males (paternal backcross), no recovery in fitness of these traits were observed. However, F_2 and F_3 hybrid females crossed with maternal population males (maternal backcross) nearly, if not fully, recovered fitness to that of the parental copepods. Ellison and Burton (2008) concluded that compatible mitonuclear interactions are responsible for

the recovery in fitness as a maternal backcross would restore matching mitochondrial and nuclear genes, whereas a paternal backcross would result in mismatched mitochondrial and nuclear genes.

Effects of Mitochondrial Health on Lifespan

Mitochondria are known to affect aging and longevity, making the study of mitochondrial functioning essential to understand the molecular basis of certain diseases associated with aging. Aon et al. (2021) were able to correlate increased intrinsic aerobic capacity to increased longevity after testing high-capacity running rats and low-capacity running rats for various mitochondrial functions in cardiac myocytes of female rats of varying ages. The high-capacity running rats were found to have better mitochondrial health than the low-capacity running rats, displayed by their results of increased respiratory reserves of palmitate in 6 months old (young) and 17 months old (middle-aged) rats and increased respiratory reserves of glucose + palmitate in rats 6 months, 17 months, and 24 months (old) of age (Aon et al., 2021).

Mitochondrial Dysfunction and Aging

Some evidence suggests that mitonuclear mismatches can drive aging (Lane, 2011). According to the 'free radical' hypothesis of aging, an increased production of reactive oxygen species (ROS) resulting from a higher resting metabolic rate can accelerate aging (Harman, 1956). In other words, ROS production has a negative correlation with longevity. Although support has been provided for the 'free radical' hypothesis of aging, showing that increased ROS production is associated with aging

(Balaban et al., 2005), more recent theories explaining how the mitochondria contribute to aging departs from this theory. The 'uncoupling to survive' hypothesis suggests that increased metabolism results in a longer lifespan (Brand, 2000). Due to mitochondrial uncoupling of respiration from ATP production via uncoupling proteins, ROS production is decreased and, therefore, decreases oxidative damage (Brand, 2000; Speakman et al., 2004). In other words, metabolism has a positive correlation with longevity. Further evidence for the 'free radical' hypothesis include the genetic variation of uncoupling proteins (UCPs) in humans affecting longevity (Rose et al., 2011) and UCP1 allowing energy from the proton gradient to be dissipated as heat, which reduces ROS production in the brown adipose tissue of mice (Jastroch, 2017).

Disturbances in various mitochondrial elements are correlated with longevity. Miwa et al. (2014) suggests that the incomplete assembly of Complex I's matrix arm subunits in mice mitochondria contribute to an increase in ROS production, and therefore, a decrease in longevity. This is because the presence of more incomplete or not assembled Complex I's matrix arm subunits caused by decreased degradation or excessive production of these subunits results in subunits that can use substrates and produce ROS but are unable to pump protons or participate in electron transport to form ATP. This relative increase in Complex I's matrix arm subunits was found in shorter-living mice (Miwa et al., 2014). Long-lived mice typically showed a lower abundance of Complex I matrix arm subunits when they were younger and an increased abundance of ETC complex proteins when they became older (Miwa et al., 2014).

The stress-response could extend the lifespan of other model organisms. One example is the RNAi of ETS complexes and ATP synthase in *Caenorhabditis elegans*,

which reduced metabolic activity, and resulted in decreased body size, decreased rates of behaviors, and extended lifespans (Dillin et al., 2002). The RNAi was performed on larvae, but RNAi performed on adults did not elicit the same phenotypic changes of producing new feeding rates nor living longer like the larval ETS complex activity reduction had induced. Caloric restriction was also found to increase longevity, but their previous finding suggests that this is mediated through a separate mechanism. Therefore, Dillin et al. (2002) concluded that mitochondrial activity during development is possibly what determines respiration rates for the animal's whole life.

Similarly, Copeland et al. (2009) performed RNAi of certain ETS genes encoding for complexes I and III which resulted in increased lifespan in *Drosophila*. However, this knockdown had to be moderate as higher knockdown levels caused mortality in younger fruit flies. Furthermore, the RNAi of two of these genes, *CG9172* and *CG17856*, (for complexes I and III, respectively) in adult *Drosophila* was sufficient to extend their longevity instead of inducing RNAi in larvae (Copeland et al., 2009). Copeland et al. (2009) attributed increased lifespan to other potential cell-signaling pathways, since they concluded that the observed longevity extension did not correlate with other measured physiological and energetic trade-offs.

Dilberger et al. (2019) found that inducing mitochondrial oxidative stress using paraquat caused the mitochondria to produce increased levels of ROS, and reduced stress resistance and longevity significantly. Nematodes were incubated in a 5mM paraquat solution for four hours, which resulted in mitochondrial dysfunction. However, ATP production did not significantly decrease until the concentration of paraquat was increased to a 10mM solution, although pyruvate and lactate quantities decreased

significantly under the 5mM paraquat condition (Dilberger et al., 2019). Through the findings of Wu et al. (2006), Dilberger et al. (2019) concluded that paraquat decreased the ability of *C. elegans* to detect their food (termed chemotaxis), and therefore directly decreased their longevity, creating a stronger linkage between mitochondrial dysfunction and longevity.

Testing mitonuclear interactions and their effects on cellular mechanisms affecting fitness and changes with aging were modeled in *Drosophila* of different mtDNA and nuDNA combinations in flies aged 15 days and 25 days (Pichaud et al., 2019). Pichaud et al. (2019) concluded that a mitochondrial mismatch from a mutated nuclear amino-acyl-tRNA synthetase caused an increase in ROS production and mtDNA, leading to mitochondrial dysfunction. They also found that this mitonuclear incompatibility resulted in decreased respiration rates and enzymatic activity of CI+CIII, which is consistent with previous studies which have found decreased enzymatic activities of CI, CIII, and CIV in *Drosophila* with the same mitochondrial and nuclear backgrounds ((*simw*^{501});*OreR* – mtDNA from *D. simulans* inserted into a wild-type nuclear background of *D. melanogaster*) (Meiklejohn et al., 2013; Holmbeck et al., 2015). Holmbeck et al. (2015) also showed this mitonuclear incompatibility to display decreased locomotor functioning (based on flight and climbing ability scores) and mitochondrial morphological changes, such as loose, swirled cristae and mitochondrial matrix gaps. However, whether the increase in ROS production plays a decisive role resulting in decreased longevity needs to be further studied since *Drosophila* in this experiment were able to survive in ideal lab conditions despite an

excess production of ROS (Pichaud et al., 2019). Further studies should encompass an older range of ages as well.

Longevity in intertidal copepods

Adult copepod longevity has been measured previously in multiple studies in relation to varying latitudes and different temperatures (Hong and Shurin, 2015; Foley et al., 2019). Hong and Shurin (2015) found that low latitudes and low temperatures correlated with increased adult copepod survival while high latitudes and high temperatures yielded decreased adult copepod survival. Foley et al. (2019) observed a similar phenomenon in which the adult copepods raised in 15 °C generally exhibited an increased survival probability over a longer time period than those copepods raised in 25 °C. Although studies have been performed on parental populations of intertidal copepods' longevity (Hong and Shurin, 2015; Foley et al., 2019), a study on how mitonuclear incompatibilities in hybrids affect longevity has yet to be conducted. Learning about the outcome of hybrids compared to parental populations and linkages between life-history traits can be helpful in determining the effects of changes to certain metabolic processes within the mitochondria.

Model System

In order to study mitonuclear incompatibilities and their effects, a model system with allopatric populations accruing independent genetic mutations would be ideal. For this experiment, an intertidal copepod species, *Tigriopus californicus*, was utilized for their genetic divergence, quick generation of offspring, and propensity for the

coevolution of mitochondrial and nuclear genomes (Blier et al., 2001; Barreto et al., 2018), which allows us to test the effects of mitonuclear interactions on F_2 hybrids. *T. californicus* live in high tidepools, typically distanced from regular interactions with ocean tides. Therefore, they do not have the opportunity to migrate to different tidepools and must adapt to local conditions with limited gene flow. These copepods are genetically divergent because populations of copepods distributed in different geographic locations have evolved varying gene pools and phenotypes, well-adapted to the local thermal tolerance (Willett, 2010; Kelly et al., 2012). This can be a contributing factor to the subsequent coadaptation of mitochondrial and nuclear genomes (Sloan et al., 2017; Barreto et al. 2018).

Phenotypic linkages

My experiment sought to identify to what extent mitonuclear interactions affect the developmental times and longevity of *Tigriopus californicus* and their linkages to each other. The first tested phenotypic trait was developmental rate, which was measured in days from when the first developmental stage copepods (nauplii) hatched from the egg sac to when they metamorphosed into juvenile copepods (copepodids). Developmental rates were split into 3 categories: fast developers, medium developers, and slow developers. The subsets of high fitness (fast developers) and low fitness (slow developers) copepods were then each tested for longevity to determine potential linkages amongst phenotypic traits. Longevity is a life-history trait that has not yet been tracked in *T. californicus* hybrids and I wanted to link it to developmental rates, a trait that has been well-studied in *T. californicus* hybrids. In this experiment, we predict that

1) there will be a negative correlation between lifespan and developmental time, where fast developers will have longer lifespans compared to slow developers because F_2 fast developers have been shown to retain favorable mitonuclear interactions through keeping certain maternal nuclear genes, and these favorable mitonuclear interactions would be expected to increase lifespan, and 2) the F_2 hybrids will experience an increased range of developmental rates (Ellison and Burton, 2006) and longevity due to potential mitonuclear incompatibilities not found in the parental populations. We predict that not all F_2 hybrids will experience hybrid breakdown as we would still expect to produce fast-developing F_2 hybrids; rather, that many that do will exhibit increased developmental times and longevity, which would increase the overall average of these traits in F_2 hybrids compared to the parental populations. We also predict that there will be a difference in longevity of F_2 hybrids as the ATP synthesis rates through CI was shown to be significantly different between fast and slow developers of different hybrid crosses (Healy and Burton, 2020).

RESULTS

The developmental times of both parental populations, SD and SCN, and F_2 hybrids, SD ♀ x SCN ♂ and SCN ♀ x SD ♂, were compared (Figure 1). SCN ♀ x SD ♂ and SD ♀ x SCN ♂ displayed lower mean developmental times compared to that of the SCN parental population, while SCN ♀ x SD ♂ exhibited a similar mean developmental time to the SD parental population's developmental time, and SD ♀ x SCN ♂ showed a mean developmental time in between that of the SD and SCN parental populations. The means and standard deviations were 7.3 days (+/- 2.2 days) for the SD parental population, 11.5 days (+/- 5.1 days) for the SCN parental population, 9.0 days (+/- 2.7 days) for the SD ♀ x SCN ♂ F_2 hybrid cross, and 7.3 days (+/- 1.7 days) for the SCN ♀ x SD ♂ F_2 hybrid cross. After conducting F tests for variances between pairs of samples, t-tests assuming unequal variances were run. All groups were found to be statistically significant ($P < 0.05$) from each other except for SD and SCN ♀ x SD ♂ (two-tail $P = 0.75$).

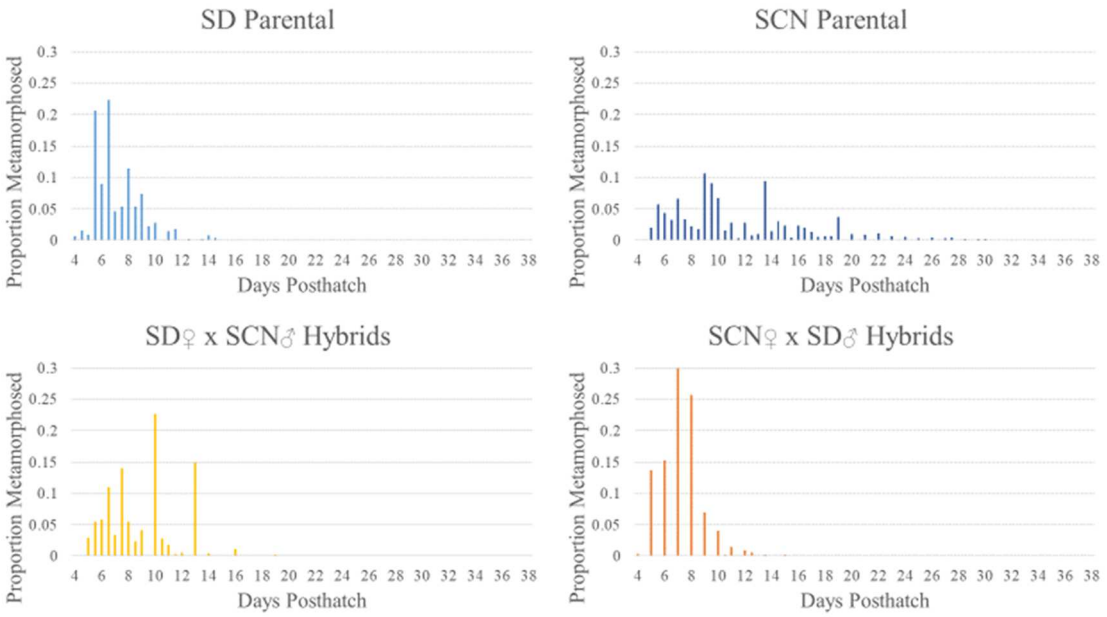


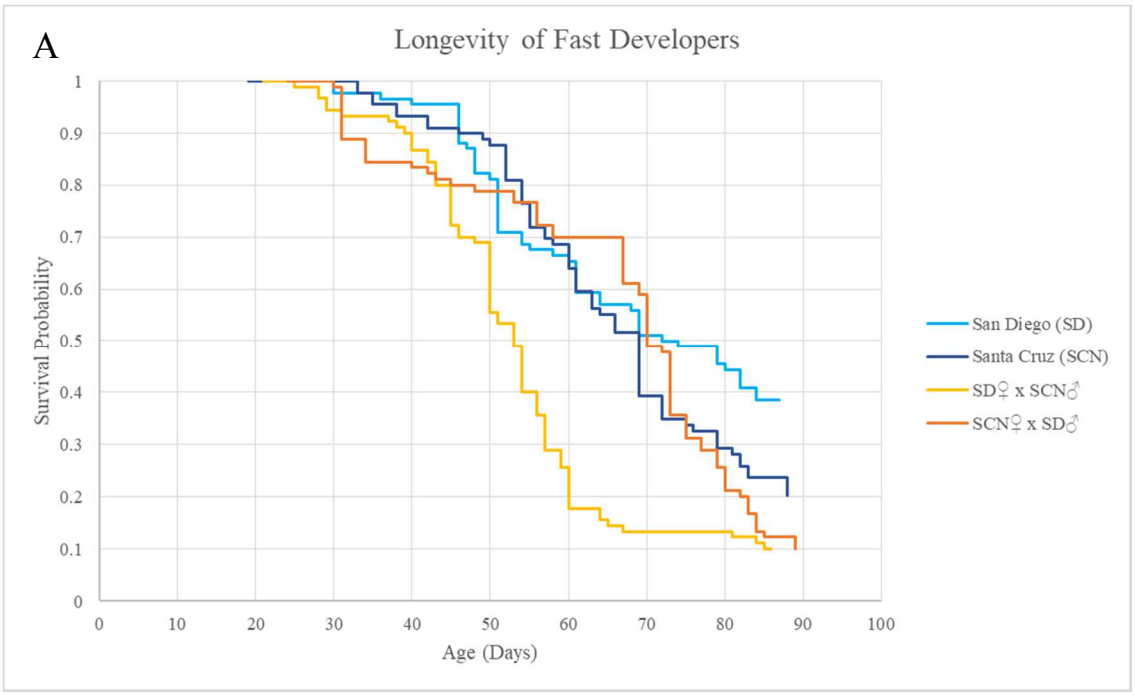
Figure 1. Developmental times of San Diego (SD) and Santa Cruz (SCN) parental populations and F_2 hybrids display greater variance in parental populations than hybrids. Developmental times were measured in proportion metamorphosed over days posthatch from hatching to juveniles (copepodids). Warm color bars (yellow, orange) on the left represent the parental population and hybrids (respectively) with SD mitochondria, and cool colors (blue, light blue) on the right represent the parental population and hybrids (respectively) with SCN mitochondria. Hybrid crosses were named with the maternal population preceding the paternal population.

The developmental times ranged from 4 to 33 days for the SD parental population, 5 to 38 days for the SCN parental population, 5 to 22 days for the SD ♀ x SCN ♂ F_2 hybrid cross, and 4 to 25 days for the SCN ♀ x SD ♂ F_2 hybrid cross (Figure 1). A larger range was displayed in the parental populations, SD and SCN, for days posthatch. Because the hybrid crosses did not show as much or more variation than the parental populations, they did not exhibit hybrid breakdown, or the deterioration and increased variation of a phenotypic trait due to the introduction of new alleles from different populations. The total number of copepods that underwent metamorphosis into copepodids was 1,668 from 92 egg sacs for the SD parental population, 3,451 copepodids from 200 egg sacs for the SCN parental population, 1,506 copepodids from

319 egg sacs for the SD ♀ x SCN ♂ F_2 hybrids, and 3,622 copepodids from 190 egg sacs for the SCN ♀ x SD ♂ F_2 hybrids. Because the number of egg sacs varied per population or hybrid, the proportion metamorphosed was calculated to adjust for the varying number of animals per cross. The variance in developmental times could potentially be due to variances in the condition of the wells of the six-well plates. Some wells grew more algae than others. However, there were numerous egg sacs and plates used to measure developmental times, so these are less likely to be factors contributing to the differences in developmental times amongst groups.

Depending on the developmental times, fast and slow developers were determined by calculating the fastest 25% and slowest 25% of developing individuals for each population or F_2 hybrid cross, although most individuals used for the longevity assay were within the fastest 15% and slowest 15% of their population or cross. To measure the longevity of the SD parental population's fast developers, adult copepods with a developmental time less than or equal to 5.5 days (fastest 23.7% of SD individuals) were used; the longevity of SCN parental population fast developers consisted of adult copepods with developmental times less than or equal to 6 days (fastest 12.1% of SCN individuals) were used; SD ♀ x SCN ♂ F_2 hybrids consisted of adult copepods with developmental times less than or equal to 6 days (fastest 14.1% of SD ♀ x SCN ♂ F_2 hybrid individuals) were measured; and SCN ♀ x SD ♂ F_2 hybrids consisted of adult copepods with developmental times less than or equal to 5 days (fastest 14.0% of SCN ♀ x SD ♂ F_2 hybrid individuals) were measured. Measuring longevity of slow-developing individuals from each group included SD copepods with developmental times greater than or equal to 10 days (slowest 8.7% of SD individuals),

SCN copepods with developmental times greater than or equal to 20 days (slowest 6.9% of SCN individuals), SD ♀ x SCN ♂ F_2 copepods with developmental times greater than or equal to 14 days (slowest 2.3% of SD ♀ x SCN ♂ F_2 individuals), and SCN ♀ x SD ♂ F_2 copepods with developmental times greater than or equal to 10 days (slowest 8.2% of SCN ♀ x SD ♂ F_2 individuals).



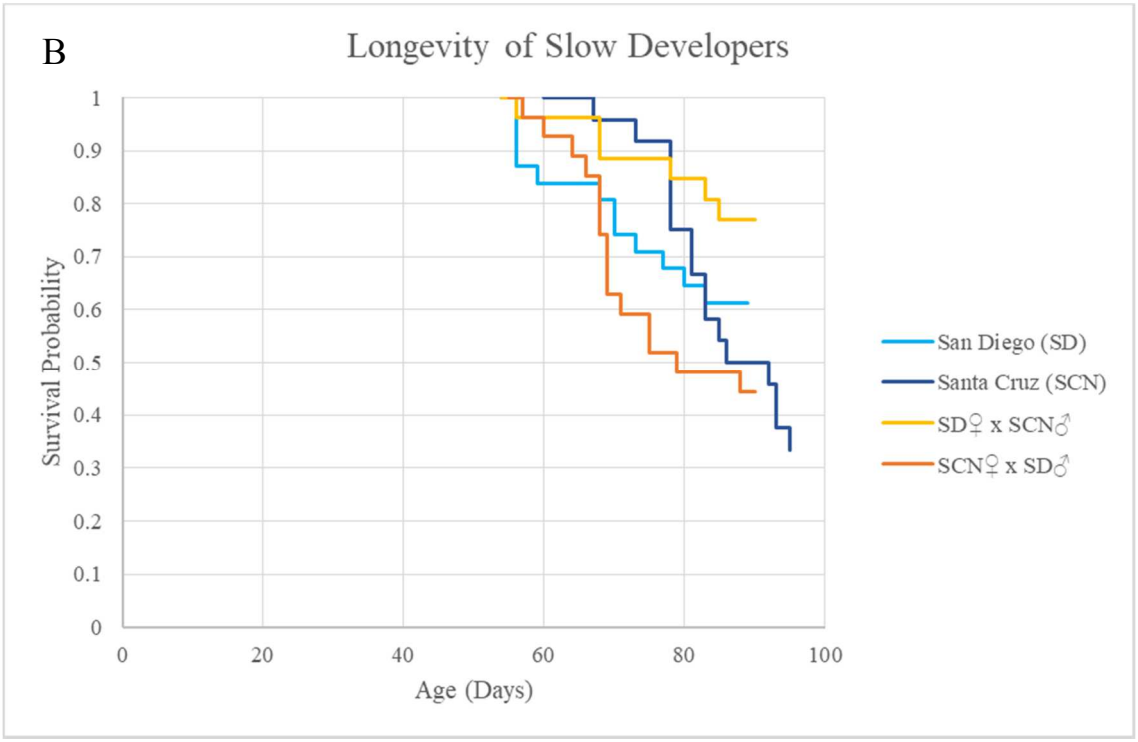


Figure 2. Survival curves of SD and SCN parental populations and SD ♀ x SCN ♂ and SCN ♀ x SD ♂ F_2 hybrid crosses show increased lifespans for slow developers. The proportion of adult copepods that survived was recorded for each population and each F_2 hybrid cross for (A) fast- and (B) slow-developing copepods. Proportion of surviving copepods were measured with the number of days survived.

The survival of copepods from the San Diego (SD) and Santa Cruz (SCN) populations and the F_2 hybrid crosses, SD ♀ x SCN ♂ and SCN ♀ x SD ♂, were recorded every two to four days and the proportion of copepods that survived were calculated and plotted onto a survival curve. Using the log-rank test on pairwise comparisons of the Kaplan-Meier survival curves, SD copepods exhibited a significantly higher survival probably than the hybrid crosses for fast developers, while SCN only had a significantly higher survival probability than SCN x SD (Table 1). The hybrid crosses were also calculated to be significantly different from each other in the longevity of fast developing copepods. This finding was not consistent in the longevity of slow developers, however, where the only groups statistically different from each other were SCN and SD x SCN as well as the hybrid crosses, SD x SCN and SCN x SD (Table 2). Patterns between the longevity of fast developers and slow developers were not found.

For the longevity of fast developers, SD ♀ x SCN ♂ F_2 hybrids survived comparatively worse from 50-80 days posthatch, which could potentially be attributed to hybrid breakdown. However, SD ♀ x SCN ♂ F_2 hybrids maintained the highest proportion of surviving copepods at 90 days posthatch for the longevity of slow developers. SCN ♀ x SD ♂ F_2 hybrids along with SD ♀ x SCN ♂ F_2 hybrids had the lowest proportion of surviving copepods by 86 days old for the longevity of fast developers while SD and SCN parental populations showed the highest proportion of surviving copepods at 87 days old for the longevity of fast developers. The parental populations did not maintain higher survival probabilities compared to the hybrid

groups for the longevity of slow developers. Therefore, it is possible that hybrid breakdown plays a role in the low proportion of surviving hybrids compared to parental populations for fast developing copepods, but there are other factors that must play a role in the patterns between developmental time and longevity that need to be studied to find potential correlations.

Table 1. Comparing longevity amongst fast-developing groups.

Fast Developing Groups	Log Rank Test $\chi^2 = \Sigma \frac{(\Sigma O_{jt} - \Sigma E_{jt})^2}{\Sigma E_{jt}}$ (Chi-Square)	Significant Difference (P) (Critical Value ≥ 3.841 → P-value < 0.05)
SD vs. SD x SCN	34.016	<0.00001
SCN vs. SCN x SD	0.32056	0.57131
SD vs. SCN x SD	10.854	0.00099
SCN vs. SD x SCN	24.862	<0.00001
SD vs. SCN	3.5414	0.05986
SD x SCN vs. SCN x SD	14.000	0.00018

Table 2. Comparing longevity amongst slow-developing groups.

Slow Developing Groups	Log Rank Test $\chi^2 = \Sigma \frac{(\Sigma O_{jt} - \Sigma E_{jt})^2}{\Sigma E_{jt}}$ (Chi-Square)	Significant Difference (P) (Critical Value ≥ 3.841 → P-value < 0.05)
SD vs. SD x SCN	1.9518	0.16239
SCN vs. SCN x SD	0.45693	0.49908
SD vs. SCN x SD	1.4577	0.22730
SCN vs. SD x SCN	5.0095	0.02521
SD vs. SCN	2.2589	0.13285
SD x SCN vs. SCN x SD	6.5622	0.01042

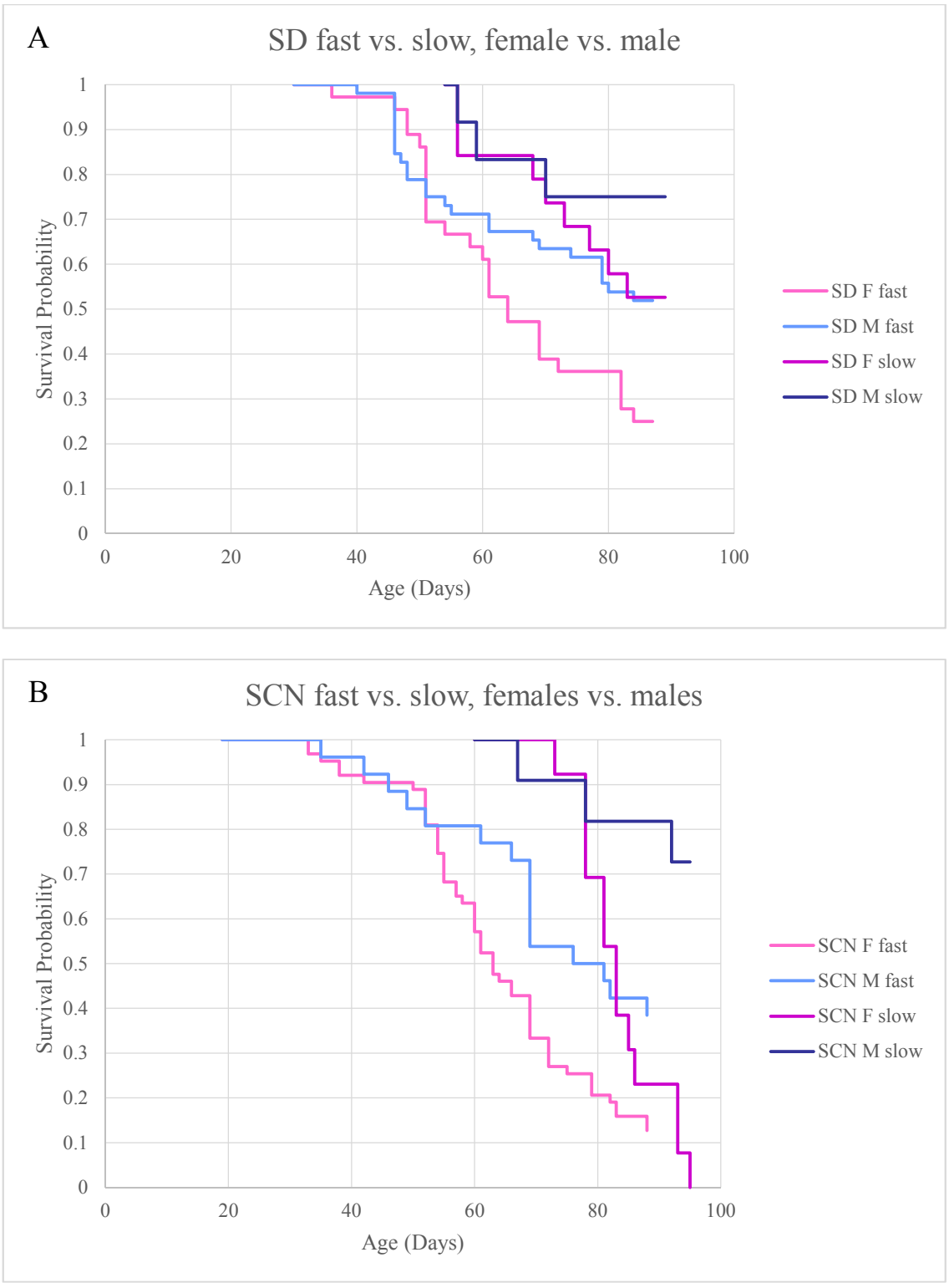
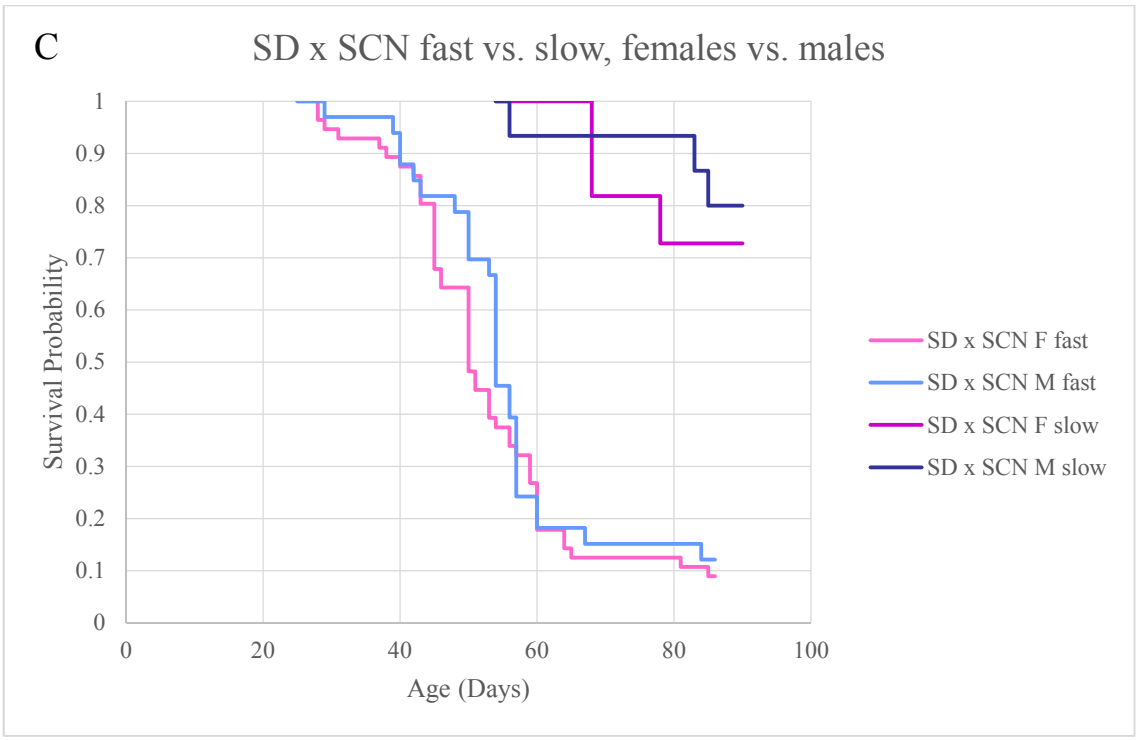


Figure 3. Longevity curves comparing the proportion survival of females and males for the (A) SD and (B) SCN parental populations. The proportion of alive copepods of each sex were recorded on the number of days survived.



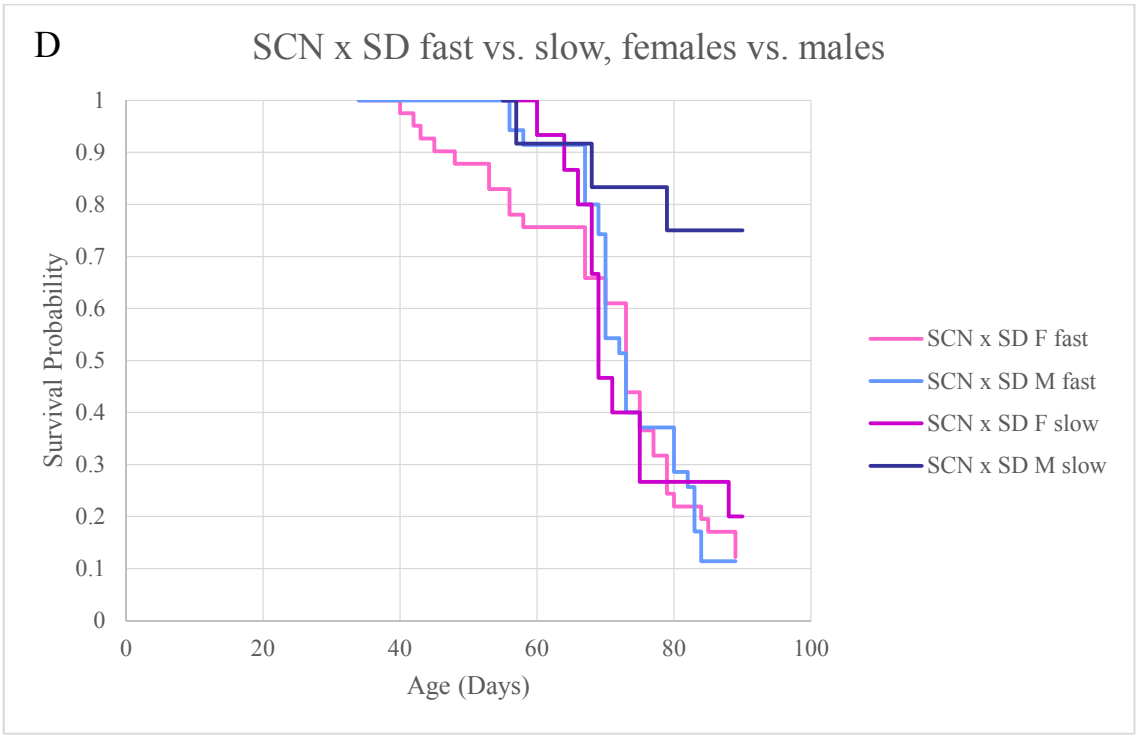


Figure 3. Longevity curves comparing the proportion survival of females and males for the (C) SD♀ x SCN♂ and (D) SCN♀ x SD♂ hybrid crosses. The proportion of alive copepods of each sex were recorded on the number of days survived.

The longevity of fast and slow developers was further divided into females and males for each parental population and hybrid. The proportions of surviving female and male copepods were calculated and graphed in Figure 3. The log-rank test was performed in pairwise comparisons of the survival curves of females vs. males (Table 3) and fast-developers vs. slow-developers by sex (Table 4). For most of the groups (SD and SCN parental populations for fast developers and SD, SCN, SD♀ x SCN♂, and SCN♀ x SD♂ for slow developers), males maintained a higher proportion of surviving copepods than reproducing females did when approaching 90 days. SD♀ x SCN♂ and SCN♀ x SD♂ hybrid crosses of fast developers did not exhibit a significant difference between the proportion of females and males that survived during the recorded time, while the parental populations did possess a significant difference between fast developing females and males. Slow-developing females and males exhibited significant differences in the SCN and SCN x SD groups.

Log-rank tests were also conducted on females and males of the same population or hybrid using the pairwise comparison of fast-developers and slow-developers for each of these groups. SD x SCN showed significant survival curve differences between fast- and slow-developing individuals for both females and males. SCN x SD males also showed a significant difference between fast- and slow-developing male individuals.

Table 3. Comparing longevity between females and males of fast-developing and slow-developing groups.

Female vs. Male Comparisons	Log Rank Test $\chi^2 = \Sigma \frac{(\Sigma O_{jt} - \Sigma E_{jt})^2}{\Sigma E_{jt}}$ (Chi-Square)	Significant Difference (P) (Critical Value ≥ 3.841 → P-value < 0.05)
SD fast	5.2856005	0.02150
SCN fast	7.4155741	0.00647
SD x SCN fast	0.661133663	0.41617
SCN x SD fast	0.003489335	0.95350
SD slow	1.317916	0.25097
SCN slow	14.26043	0.00016
SD x SCN slow	0.279390367	0.59716
SCN x SD slow	8.412508196	0.00373

Table 4. Comparing longevity between females of fast-developing and slow-developing groups and between males of fast-developing and slow-developing groups.

Fast-developing and Slow-developing Group Comparisons	Log Rank Test $\chi^2 = \Sigma \frac{(\Sigma O_{jt} - \Sigma E_{jt})^2}{\Sigma E_{jt}}$ (Chi-Square)	Significant Difference (P) (Critical Value ≥ 3.841 → P-value < 0.05)
SD Females	1.0005009	0.31719
SCN Females	1.29963715	0.25429
SD x SCN Females	9.2138	0.00240
SCN x SD Females	0.328335955	0.56666
SD Males	0.0210507	0.88478
SCN Males	3.5565566	0.05931
SD x SCN Males	11.90959592	0.00056
SCN x SD Males	13.26288328	0.00027

DISCUSSION

This experiment was conducted under ideal lab conditions with a fixed temperature, a fixed light/dark cycle, and a constant food source, which would differ from what we would see out in the field. In the case that hybrids survive in the wild, we would expect hybrid breakdown to exert stronger effects as the copepods are locally adapted and have coevolved mitochondrial and nuclear genes to optimize their metabolism for their local habitat (Blier et al., 2001; Barreto et al., 2018).

Contrary to other published data, which showed hybrid breakdown in the developmental times of hybrid crosses (Ellison and Burton, 2006; Healy and Burton, 2020), our data do not reveal hybrid breakdown with developmental times. Our results revealed that the Santa Cruz (SCN) parental population displayed both increased variation (25.9 days variance compared to SD's 4.7 days, SD♀ x SCN♂'s 7.1 days and SCN♀ x SD♂'s 3.0 days) and a longer developmental time average compared to those of the San Diego (SD) parental population and the F_2 hybrid crosses, SD♀ x SCN♂ and SCN♀ x SD♂. Additionally, the F_2 hybrids showcased a smaller range of days posthatch compared to the parental populations, which also indicates a lack of hybrid breakdown. The increased variation in the SCN and SD parental populations could be a result of natural variation in the random subset of parental copepods used for this experiment, although this is unlikely as the sample sizes were large. The developmental time mean for SCN was longer, but the developmental time mean for SD was the shortest along with the SCN ♀ x SD ♂ F_2 hybrid cross. This is possible if the hybrid cross individuals that survived to the life stage of copepodid (juvenile) had genetically more compatible mitonuclear interactions, making them more fit, and the unfavorable

mitonuclear mismatches were lethal. Because hybrids receive a random mix of nuclear genes from both the paternal and maternal lines, it is possible that the copepods that metamorphosed to copepodids inherited nuclear genes that encoded subunits compatible with the mitochondrial encoded subunits. Possessing compatible mitonuclear interactions could potentially result in phenotypic fitness similar to that of the parental populations. Moreover, the hybrid cross SD♀ x SCN♂ had a developmental time mean that was 2 days higher than that of the SD parental population, which contains the same mitochondrial background. When comparing the SD parental population to the hybrid cross SD♀ x SCN♂ with SD mitochondria, SD♀ x SCN♂ does reveal some extent of hybrid breakdown in developmental time and this difference is significant ($P < 0.05$). Overall, however, hybrid breakdown in developmental time was not observed since the SCN parental population had the greatest mean and variation compared to the other tested groups.

The developmental time means of each population and hybrid cross did not seem to affect the longevity of fast and slow developers. While SD and SCN ♀ x SD ♂ F_2 hybrids had the fastest mean developmental times and earliest days posthatch copepods used for the fast development longevity assay, they contrasted drastically in the proportion of copepods survived at 86 days for the longevity of fast developers. Similarly, the slow development cutoff for SD and SCN ♀ x SD ♂ F_2 hybrids was greater than or equal to 10 days, which was 4 days and 10 days earlier than the SD♀ x SCN♂ F_2 hybrid and SCN population, respectively. However, their survival proportion differed by 0.17, indicating that specific developmental times were not a good predictor for the outcome of longevity.

There was not an overarching relationship between parental populations and F_2 hybrid crosses in the life history trait of longevity. However, in the longevity of fast developers, the SD and SCN parental populations had the highest proportion of surviving copepods at day 86, whereas both F_2 hybrid crosses had a 0.1 decreased proportion of surviving copepods in comparison. This difference was statistically different ($P < 0.05$) for the pairwise comparisons of SD and SD♀ x SCN♂, SD and SCN♀ x SD♂, SCN and SD♀ x SCN♂, and between SD♀ x SCN♂ and SCN♀ x SD♂. Based on the statistical differences found from conducting log rank tests, there was some extent of hybrid breakdown in the longevity of fast developers. This pattern did not continue into the longevity of slow-developing individuals because SD♀ x SCN♂ F_2 hybrids had greater than 0.15 increased proportion of surviving copepods at day 89. Additionally, SCN and SD♀ x SCN♂, and SD♀ x SCN♂ and SCN♂ x SD♀ are significantly different in the longevity of slow developers, but in the opposite direction of that of fast developers. A previous study showed that there is a correlation between the fast and slow developing groups in ATP production rates through CI, which would be expected to affect other life history traits (Healy and Burton, 2020). However, there does not appear to be a definitive correlation between fast- and slow-developing individuals of each group in my experiment. Although the fast-developing SD♀ x SCN♂ and SCN ♀ x SD ♂ F_2 hybrid crosses had a proportion survival at around 0.1 on day 86, their slow-developing counterparts had survival proportions of 0.77 and 0.48, respectively, on day 86, which reflects the best and worst longevity of the four groups.

Comparing the longevity of females and males of each group to each other revealed statistically significant differences in fast-developing SD and SCN parental

population females and males and slow-developing SCN and F_2 SCN x SD females and males, differing from the findings of Foley et al. (2019). In almost all of the females vs. males comparisons, males had a higher survivability proportion compared to their egg-producing female counterparts. This trend was consistent across parental population and hybrid groups of fast and slow developers. It is possible that additional energy expenditure on producing egg sacs for females led to a lower survivability proportion, but further studies should be conducted to learn about the ATP production rates of reproducing females and its potential relationship with longevity.

It should be noted that when calculating significance between groups, only copepods that already died were accounted for when calculating age. Copepods that survived after the assay stopped were not counted in the average age, which consequently decreased the sample size for the calculations and decreased the mean age of these groups. The survival assay was not completed until the death of all copepods due to the onset of the pandemic, and therefore, limited the available data to one replicate. The surviving copepods were not assigned a minimum age because the groups were at various stages of their survival curves. This pitfall and limitation make it important for the experiment to be conducted again to completion.

In relation to development and longevity, the proportion of copepods that survived was higher for the slow-developing copepods compared to the fast-developing copepods for every population and hybrid cross. This could suggest a potential tradeoff between efficient early life stage development and longevity, contrary to the 'uncoupling to survive' hypothesis. This could be due to the shortcoming of the longevity assay starting to track adult copepods after 19 to 24 days post-hatch for fast

developers and 54 to 60 days for slow developers, where deaths of copepods prior to these days post-hatch were not accounted for. Therefore, the remaining copepods could be the more fit or long-living copepods, especially for those selected for slow development. On the other hand, we have yet to study the correlation between ATP production rates in early-life stage development and adulthood in *T. californicus*. Perhaps early-life stage development metabolism rates do not determine the metabolism that *T. californicus* exhibits throughout adulthood, in which case, a tradeoff between efficient early-life development and longevity could be explained.

Because longevity is potentially compromised for efficient early-stage development, future studies can include measuring different fitness traits in relation to longevity and development rate. It would be interesting to study the effects of earlier life history characteristics, such as egg clutch size, age of the mother when she laid the egg sac, or how long the mother lived and these effects on longevity. Additionally, measuring post-developmental characteristics and their effects on longevity would be insightful to determine if longevity can be greatly affected by variances later in life. For example, determining the effects of pairing for females and males on longevity and measuring the difference between females that lay fertile egg sacs throughout their lives versus virgin females could be independent variables to measure longevity. Another trait that could be measured is lifetime egg production of females and how that is correlated with longevity. ATP production alongside ROS production could also be measured in future studies and correlated to longevity, although the linkages are not expected to be easy to predict due to the potential nature of asymmetrical hybrid breakdown. Different hybrid crosses may also be tested with populations possessing

equally divergent mitochondria as San Diego and Santa Cruz (20% sequence divergence). Numerous characteristics can be measured with longevity to determine linkages between life history traits.

MATERIALS AND METHODS

Intertidal copepods were collected from intertidal areas in Ocean Beach in San Diego, CA and in Santa Cruz, CA. The copepods were transported from these tidepools to laboratory incubators which were kept at 20℃ with 12-hour light and dark cycles. They were maintained in 400 mL glass beakers filled to 250 mL with seawater and fed spirulina weekly for a month before being used for experiments. Copepod culture maintenance included seawater changes of one-third of the seawater volume in the beaker monthly to maintain a salinity of 35 ppt. Cultures were fed ground spirulina weekly.

Hybrid crosses were made by pairing females from one parental population with males from another parental population. Copepod pairs consist of an adult male clasped onto a virgin female. Virgin females were necessary in creating hybrids to ensure that the offspring inherited paternal genes of the desired population. Initially, 100 San Diego (SD) pairs and 100 Santa Cruz (SCN) pairs were split and males and females of each population were separated into petri dishes filled half-way with filtered seawater and spirulina for 2 days. 50 SD virgin females were paired with 50 SCN adult males and 50 SCN virgin females were paired with 50 SD adult males. The remaining 50 females and males in each population were paired with the opposite sex of the same population to create the parental control populations. Each cross or parental population was kept in their own petri dish with filtered seawater and additions of spirulina every other day.

The offspring that hatched from the egg sacs of gravid females were the F_1 generation. As the F_1 generation matured into copepodids (juvenile copepods), the

parental adult copepods were removed from the petri dishes. Regular feeding of spirulina every other day was performed as the F_1 generation matured to adults and started to pair with other F_1 individuals. Once F_1 females produced their first egg sacs, the F_1 gravid females were moved to a separate petri dish with filtered seawater and spirulina. When the first egg sac of the F_1 females was mostly bright orange in color and eye spots on each egg could be detected through microscopic observation, it was dissected off the females with a needle and placed into a well of a six-well plate with filtered seawater and spirulina. One to three egg sacs were placed per well and the next day was recorded as day 0 for when they hatched.

F_2 nauplii were monitored every one to four days to determine the number of days it took for nauplii (larvae) to metamorphose into copepodids (juveniles). These data were recorded for each copepod as "days posthatch" and comprised the developmental times for the F_2 offspring and were then calculated into proportion metamorphosed after all the nauplii became copepodids (Figure 1). Due to developmental times being recorded every one to four days, the average of the days posthatch for metamorphosed copepodids was calculated if at least a day was missed. For example, if copepodids were counted on 11 and 14 days posthatch, then the number of metamorphosed copepodids counted on day 14 posthatch would be graphed in the figure as 12.5 days posthatch. All groups were checked at least twice a week on the same dates with the exception of four staggered time points where the hybrid groups were checked one or two days later than the parental groups. Once metamorphosed, copepodids were separated from the nauplii and kept in separate petri dishes with filtered seawater and spirulina. These copepodids were categorized by developmental

times whereby the fastest 25% developing individuals from each population and cross were pooled to the group “fast developers” and the slowest 25% developing individuals from each population and cross were pooled to the group “slow developers”. Means, standard deviations, and significance (ANOVA) amongst means of developmental times of the parental populations and hybrid crosses were calculated.

After these copepodids matured into adults, they were moved to six-well plates with filtered seawater and spirulina to track longevity for fast-developing and slow-developing individuals. Longevity began to be tracked at 19 to 24 days post-hatch for fast developers and 54 to 60 days for slow developers, although some death of copepods occurred before then and, therefore, was not measured nor recorded in Figure 2. For fast developers, 90 SD copepods were randomly selected out of 395 fast developers, 89 SCN copepods out of 418 fast developers, 90 SD♀ x SCN♂ copepods out of 212 fast developers, and 90 SCN♀ x SD♂ copepods out of 507 fast developers were used to measure longevity. Although the adults were selected randomly for this experiment, future studies should select equal numbers of adult females and adult males if possible. For slow developers, 31 SD copepods out of 145 slow developers, 24 SCN copepods out of 238 slow developers, 26 SD♀ x SCN♂ copepods out of 35 slow developers, and 27 SCN♀ x SD♂ copepods out of 297 slow developers were used to measure longevity. Although there were fewer slow developers to randomly select from, the sample size also decreased due to some mortality prior to the longevity assay being conducted. Every two to three days the number of surviving copepods were recorded. Dead copepods were determined by lack of motion when water was moved in the well and an opaque light orange color of the body was visible. The dead copepods were promptly

removed from the wells. The proportion of living copepods was then calculated. Additionally, the number of surviving copepods of each sex from both fast and slow developers for each group (SD, SCN, SD♀ x SCN♂, and SCN♀ x SD♂) were recorded with the longevity data. The data were plotted as Kaplan-Meier survival curves and further analyzed through pairwise comparisons via the log-rank test. P-values to determine significant differences between survival curves were calculated from the chi-square value obtained from the log-rank test.

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