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Publication Date

2020

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UNIVERSITY OF CALIFORNIA SAN DIEGO

Impacts of hybrid breakdown on the production of the red pigment astaxanthin in the
intertidal copepod *Tigriopus californicus*

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

in

Marine Biology

by

Lucas D. Martz

Committee in charge:

Professor Ronald S. Burton, Chair
Professor Stuart A. Sandin
Professor Martin Tresguerres

2020

The thesis of Lucas D. Martz is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California San Diego

2020

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Acknowledgements

I would like to thank Ron Burton for giving me support and guidance throughout my time as a master's student. Ron has been an incredible influence on my scientific journey and constantly pushed me to do quality research. I would also like to thank Stuart Sandin and Martin Tresguerres for being supportive committee members. All the current and past Burton Lab members deserve an abundance of appreciation as well: Tim Healy, Reggie Blackwell, Satomi Tsuboko, Andrea Odell, Emma Choi, Sumi Hunjan, Antonia Bock, Rebecca Pak, and Jazmin Nijjar. Working in the Burton lab has been an absolute pleasure and I know I will be hard pressed to find such an enjoyable work environment anywhere else. Thank you as well to Dovi Kacev for the advice and guidance you have given me these last few years, your help has been much appreciated. Thank you to my parents for always supporting me and trusting me to take ownership of my own decisions. Thank you to all my friends for being an incredible line of help and support when I needed it most.

This thesis contains unpublished coauthored material currently being prepared for submission for publication. Martz, Lucas; Powers, Matthew. Mr. Martz was the principal author of this material.

ABSTRACT OF THESIS

Impacts of hybrid breakdown on the production of the red pigment astaxanthin in the
intertidal copepod *Tigriopus californicus*

by

Lucas D. Martz

Master of Science in Marine Biology

University of California San Diego, 2020

Professor Ronald S. Burton, Chair

Natural selection favors the co-evolution of the mitochondrial and nuclear genomes. Hybridization of genetically divergent populations forces an interaction between mitochondrial and nuclear genes that have not co-evolved. When genes segregate in the F2 generation, incompatible combinations can result in reduced mitochondrial performance and a general breakdown of fitness. Previous studies have suggested that carotenoid pigment production is tied to mitochondrial performance; this project aims to assess the impacts of

hybridization (and consequent mitochondrial dysfunction) on the production of the carotenoid pigment astaxanthin in the rock pool copepod *Tigriopus californicus*, a species where breakdown of hybrid fitness and mitochondrial function is well documented. Here, we measured astaxanthin production in hybrid copepods alongside their parental populations and found no conclusive evidence that pigmentation is a condition dependent signal of mitochondrial function.

Introduction

Nearly all eukaryotic life is dependent on the mitochondrial conversion of complex carbons into usable energy that takes the form of adenosine triphosphate (ATP). This process is dependent on proteins that are encoded by both the nuclear and mitochondrial genome, and these proteins combine to form the enzyme complexes responsible for ATP synthesis (Rand, Haney, & Fry, 2004). Therefore, it is critical to mitochondrial efficiency that the proteins encoded by both genomes are compatible with one another. However, when two allopatric populations of a species diverge and undergo genetic drift and natural selection separately, their nuclear and mitochondrial genomes may diverge as well (Burton & Barreto, 2012; Burton, Ellison, & Harrison, 2006). In the laboratory, these genetically divergent populations can be crossed, creating F1 offspring with a nuclear genome consisting of equal contributions from both parents, as well as a mitochondrial genome entirely from the maternal population. The F1 generation typically shows no loss of fitness due to the fact that the maternal mitochondrial genome is still compatible with a full set of maternal nuclear genes. Sometimes, F1 individuals exhibit increased fitness, known as hybrid vigor, which is likely due to the masking of deleterious recessive genes by beneficial dominant alleles. However, recombination of nuclear genes in the F2 population forces the maternal mitochondrial genome to interact with divergent alleles in the paternal nuclear genome. Since the two genomes have diverged over time, they may produce incompatible proteins, leading to the phenomenon known as hybrid breakdown, where the F2 generation shows decreased levels of fitness (Ellison & Burton, 2006). Because these incompatibilities arise from nuclear recombination, F2 hybrids show a wider range of variation in fitness measurements, as some produce more incompatible paternal proteins than others (Ellison & Burton, 2006).

The intertidal rockpool copepod *Tigriopus californicus* is ideal for the study of hybrid breakdown because there is limited connectivity between populations due to the nature of their isolated environment (Burton, 1983, 1990). Remarkably, these populations are so isolated, that copepods found on separate rocky outcrops as little as 500m apart have shown genetic differentiation (Burton, 1983). Alongside their genetic isolation, the copulatory behavior of *T. californicus* is also advantageous to the study of hybrid breakdown. Males clasp immature virgin females for up to a week before copulation, and evidence indicates that after copulation, females do not mate again (Burton, 1985). Therefore, by separating clasped pairs before copulation, one can be certain that the female has not yet mated with any male in her population. Separating pairs and placing females in a dish with males from a genetically divergent population can assure the creation of inter-population hybrids without the risk of accidental intra-population mating.

Previous studies on *T. californicus* have found numerous examples of how hybrid breakdown can lead to decreased fitness. Hybrids are known to have slower development rates and lower rates of ATP synthesis (Burton, 1990; Burton et al., 2006; Ellison & Burton, 2008). Hybrids also suffer from elevated oxidative damage due to imperfections in the oxidative phosphorylation pathway (OXPHOS) which cause a leakage of reactive oxygen species (ROS) (Barreto & Burton, 2013). Out of the five enzyme complexes that make up the OXPHOS, four are encoded by both mitochondrial and nuclear genes, and these four complexes show depressed activity in hybrids, while the one complex that is only encoded by nuclear genes remains unaffected (Ellison & Burton, 2006). When incompatible proteins are used to build these complexes, structural problems may arise, which can lead to decreased ATP output and increased production of ROS (Cocheme & Murphy, 2009). This emphasizes

how the effects of hybrid breakdown are only seen where incompatible mitochondrial and nuclear genes are forced to interact. In fact, when hybrids have a higher proportion of maternal nuclear genes, there is less mito-nuclear incompatibility, and these individuals show higher fitness than those with more paternal nuclear genes (Healy & Burton, 2020).

Despite extensive studies into the effects of hybrid breakdown on *T. californicus*, research has yet to be conducted on the impacts of hybrid breakdown on pigment production. *T. californicus* is among many species of crustacean that accumulate the red ketocarotenoid pigment astaxanthin in their tissues (Goodwin & Srisukh, 1949; Maoka, 2011). Nearly all animal species are unable to synthesize carotenoids *de novo*, so they must rely on converting ingested dietary carotenoids into astaxanthin (Britton & Goodwin, 1982; Parker, 1996). Previous research has shown the ability of *T. californicus* to convert the dietary carotenoids zeaxanthin, canthaxanthin, lutein, and β -carotene into the ketocarotenoid astaxanthin (Weaver, Cobine, & Hill, 2018). In terms of function, it is well understood that carotenoids play a vital role as antioxidants, due to their ability to quench singlet oxygen and protect the cell from oxidative damage (Ahmed et al., 2014; Maoka, 2011; Miki, 1991). Copepods that are deficient in carotenoids accumulate more oxidative damage and are more likely to suffer mortality during an oxidative challenge (Weaver, Wang, Hill, & Cobine, 2018). Not only are carotenoids important antioxidants, but they also provide photoprotection against UV radiation (Davenport, Healy, Casey, & Heffron, 2004; Maoka, 2011). When subjected to UV stress, carotenoid-deficient copepods showed a significantly higher mortality rate than their carotenoid-rich counterparts (Davenport et al., 2004). While carotenoids are clearly vital to surviving oxidative and UV stress, research has yet to be conducted on the relationship between mitochondrial function and carotenoid production.

It has been theorized that red ketocarotenoid coloration may be used as an honest signal of condition and quality in pigmented vertebrate species (Hill, 2014). In the house finch, evidence shows that male pigmentation is linked to an individual's parasite load and infection rate, where males with lower parasite counts and infection rates exhibited brighter plumage (Brawner, Hill, & Sundermann, 2000; Thompson, Hillgarth, Leu, & McClure, 1997). It was previously proposed that carotenoids may be a limited biological resource, and thus individuals with more pigment must be fit enough to maintain cellular functions as well as accumulate ornamental pigment; however, research suggests that this resource-tradeoff hypothesis may not hold up, because pigmentation can still be variable when carotenoids are not limited (Hill, 2011; Weaver, Gonzalez, Santos, & Havird, 2020). An alternative to the resource-tradeoff hypothesis is a proposition that the pathways for carotenoid synthesis may be tied to cellular respiration, indicating that pigmentation could act as a direct proxy for mitochondrial efficiency. In goldfinch species, research has shown that plumage color is positively correlated with resting metabolic rate, suggesting a link between pigment and respiration (Kelly, Murphy, Tarvin, & Burness, 2012). Some evidence additionally indicates that carotenoid oxidation may take place in the inner mitochondrial membrane, which would provide an explanation as to why we might expect carotenoid production to be linked to mitochondrial efficiency (Johnson & Hill, 2013). Although copepods do not use red pigmentation for sexual selection (Powers, Hill, & Weaver, 2020), they do offer the opportunity to study the condition-dependency of astaxanthin production in a model species with manipulatable mitochondrial function.

Here we combined the study of mitochondrial hybrid breakdown in *T. californicus* with the theory that ketocarotenoid production is an honest signal of fitness. If pigmentation is

in fact a visual proxy for mitochondrial efficiency, we predict that copepods suffering from hybrid breakdown will show depressed levels of astaxanthin production. Additionally, if pigmentation is directly linked to mitochondrial fitness, we expect to find rates of pigmentation to be positively related to signs of high fitness, such as high ATP synthesis and fast development time.

A conceptual diagram (Figure 1) has been constructed to explain the methodology behind the experiments conducted for this project and how each one allows us to better understand the relationship between astaxanthin and mitochondrial function. Primarily, we must understand how different diets affect astaxanthin production, and then we can compare rates of astaxanthin production between hybrids and parental populations. Then, each population's astaxanthin production can be compared to its mitochondrial function to determine if a relationship exists. Lastly, we can use a UV radiation assay to determine the importance of astaxanthin in surviving UV stress. The final box in Figure 1, outlined in red, describes an experiment that was not completed before the publication of this thesis. That experiment was designed to determine if differences in the rate astaxanthin production between populations would result in measurable differences in survival.

Methods

Culturing of Copepod Populations

Specimens of *Tigriopus californicus* were collected from upper intertidal rock pools from multiple locations along the western coast of North America. Stock populations from these collections have been maintained in the laboratory in 400mL beakers with filtered seawater. Since copepods are unable to synthesize astaxanthin *de novo* (Britton & Goodwin, 1982), controlling their diet allows for standardized measurements of astaxanthin production. To create clear-colored, carotenoid restricted copepods, egg sacs from stock populations were transferred into fresh 400mL beakers with at least 200mL of filtered seawater and stored at 20°C and entirely in the dark to prevent algal growth. All of these cultures were fed a carotenoid-free (Table 2) nutritional yeast (Bragg) diet that produced adult copepods lacking red pigmentation. Yeast-fed populations were established from the following stock populations: Abalone Cove (AB, 33°74'N, 118°37'W), Bird Rock (BR, 32°81'N, 117°27'W), Bufadora (BUF, 31°72'N, 116°72'W), Catalina (CAT, 33°27'N, 118°29'W), Pescadero (PES, 37°26'N, 122°41'W), Santa Cruz (SCN, 36°95'N, 122°05'W), and San Diego (SD, 32°74'N, 117°26'W).

Recombinant inbred lines (RILs) developed for other projects in the lab were also transferred into yeast-fed beakers. RILs were established by creating two replicate groups in which females from one population were crossed with males from another. When these offspring matured and formed clasped pairs, males from one group were crossed with females of the other to make the F2 generation, and this was repeated again until F3, maintaining discrete non-overlapping generations each time. Gravid females from the F3 generation were isolated in individual wells of a 6-well plate and removed after the hatching of their first egg

sac. The first clasped sibling pair to form in each well was moved to a new well and this process was repeated until F7. At that point, each RIL was moved into a 400mL beaker with filtered seawater and dried *Spirulina* and allowed to grow in numbers with overlapping generations. RIL nomenclature is defined as follows: “female population” x “male population” followed by the ID number of the isolated gravid female. Yeast-fed populations were established for the following RILs: ABxCAT11, BRxSD45, BRxSD50, BRxSD56, BUFxSD19, BUFxSD24, BUFxSD4, CATxAB27, PESxAB20.

Some egg sacs from each yeast-fed beaker were then used to create pigmented versions of all the yeast-fed populations. These fresh 400mL beakers were filled with at least 200mL of filtered seawater and were fed with live *Tetraselmis chuii* algae. These *Tetraselmis*-fed populations were also stored in the dark at 20°C to avoid the growth of any foreign algae species that might introduce different carotenoids into the diet.

New hybrid crosses were established to further test our hypothesis on early-generation hybrids, where variation in performance is highest. Forty clasped pairs were isolated from SD and SCN stock cultures. Pairs were separated, and males from SD were added to a petri dish with SCN females, and vice versa, creating reciprocal SDxSCN and SCNxSD crosses. After all females became gravid, males were removed from the dishes. When F1 offspring formed clasped pairs, they were transferred to a new petri dish to seed the F2 generation. The process was repeated once more to create an F3 generation.

1-Week Tetraselmis supplementation

Preliminary experiments were designed to supplement yeast-fed copepods with zeaxanthin powder for one week, as was done in Weaver et al., 2018. However, in our tests, one week of zeaxanthin supplementation did not produce a strong astaxanthin signal, and

mortality during experimentation was high. Instead, we used the green algae *Tetraselmis chuii*, which is known to contain the dietary precursors necessary to lead to astaxanthin production in copepods (Davenport et al., 2004). In order to compare astaxanthin production rates among populations, small subsets of each yeast-fed population were transferred into a petri dish and supplemented with *Tetraselmis* ad libitum. The copepods were left to feed on this algae for 7 days at 20°C on a 12 hour light/dark cycle. After 7 days, copepods were transferred into fresh filtered seawater for two hours to allow clearance of gut contents to ensure no carotenoids remained undigested in the gut. During gut clearance, individual pieces of aluminum foil were weighed on a microbalance (Cahn C-31). After gut clearance, the copepod samples were transferred onto their respective pieces of aluminum foil and left to desiccate in a fume hood for 30 minutes. After the desiccation, the pieces of foil were weighed again, dry weight was recorded (± 0.001 mg), and the copepods were momentarily rehydrated with DI water to create a static-free transfer into a 0.5mL screwcap microtube. Microtubes were stored at -80°C.

Additionally, samples were prepared to analyze the pigment content of the different diets used in this study. To assess the natural diet of the copepods, filamentous algae was taken from a San Diego rock pool that was abundant with copepods. The copepods were seen attached to the algae in the rock pools and were seen feeding on it in the lab. These filamentous algae were later identified as two different species of cyanobacteria of the genus *Oscillatoria*. Dried *Oscillatoria*, *Tetraselmis*, *Spirulina*, and yeast were weighed and added to 0.5mL screwcap tubes and stored at -80°C.

Pigment Extraction for HPLC

Microtubes containing copepods or dry diet were removed from the freezer, and approximately 280mg of .5mm zirconia-silicate ruptor beads and 500uL of HPLC grade acetone was added to the tubes. Tubes were placed in a Mini-Beadbeater machine (Biospec Products) and homogenized at top speed for 30 seconds to break apart the copepod tissue and suspend the pigments in acetone. The acetone was then transferred into a 1.7mL microcentrifuge tube and spun down at 5000g for 5 minutes. The supernatant was then passed through a disposable 13mm .45um polypropylene filter (Whatman) with an additional 500uL of HPLC grade acetone into a fresh 1.7mL microcentrifuge tube. Tubes were evaporated to dryness in a centrivap concentrator (Labconco). The concentrated contents were resuspended in 50uL HPLC grade acetone, capped with nitrogen gas, and stored at -80°C until ready for HPLC pigment analysis.

HPLC pigment analysis

The concentrated extractions were removed from the -80°C freezer. 40uL of HPLC grade water was added to each sample, and samples were vortexed and then spun down. The entire sample (90uL) was loaded into a 200uL flat bottom glass insert within 2mL autosampler vial (Agilent). Pigments were separated using an Agilent HPLC 1260 Infinity II LC system from a 75ul injection into a Waters Symmetry C8 column. We used mobile phases A) 50:25:25 Methanol: Acetonitrile: 0.25M Aqueous Pyridine and B) 20:60:20 Methanol: Acetonitrile: Acetone in a linear gradient as follows: starting at 100% A: 0% B to 60% A: 40% B over 18 min, then to 0% A: 100% B over 4 min, hold at 0% A: 100% B for 16 minutes, then back to 100% A: 0% B over 2 min and held for 8 minutes (Zapata, Rodríguez, & Garrido, 2000). Absorbance was measured at 450nm. Carotenoid peaks were identified by comparison to authentic standards (DHI) run through the same method. A final measurement

of astaxanthin concentration was normalized to dry weight, resulting in a final measurement of micrograms of astaxanthin per milligram of copepod tissue.

Development Time Assays

Gravid females were removed from yeast-fed populations and placed into individual wells of a 6-well plate with filtered seawater. These wells were supplemented with powdered *Spirulina* instead of yeast because mortality of gravid females in 6 well plates was high when fed yeast. Plates were monitored daily, and whenever an egg sac hatched, the date was recorded, and the female and any unhatched eggs were removed. Daily monitoring of plates continued, and the number of animals that metamorphosed from the naupliar form to the copepodid form was recorded for each well on each day.

For the F2 and F3 generations of the reciprocal SD and SCN crosses, development time was assayed differently, to get a more accurate grouping of copepods by development time. Roughly 50 gravid females were placed in a petri dish with filtered seawater and *Spirulina*. The following day, all females and unhatched egg sacs were transferred to a fresh petri dish, leaving behind any nauplii that hatched by that date. This was repeated for 5 days, and the hatch date of each dish was recorded. Dishes were monitored daily, and each day animals that metamorphosed from the naupliar stage to the first copepodid stage were counted and separated into fresh dishes according to their development rate. Each dish consisted entirely of copepods that developed on the same day in relation to their hatch date, thus grouping the copepods by their development rate.

ATP Synthesis Assays

In order to determine the rate of ATP synthesis in each population, an ATP synthesis assay derived from Ellison & Burton, 2006 was conducted. Ten males and ten females were

homogenized with Dounce homogenizers. Their mitochondria were isolated by placing homogenized samples in a 4°C centrifuge at 1000g for 5 minutes. The supernatant was transferred into a new tube and placed in the centrifuge at 11000g for 10 minutes. The supernatant was discarded, and the pellet was resuspended in assay buffer (560mM sucrose, 100mM KCL, 10mM KH₂PO₄, and 70mM HEPES). Suspended mitochondria (25uL) from each sample were then added to 5uL of either Complex I (CI) substrate (100mM ADP, 800mM malate, 2M glutamate, and assay buffer) or Complex II (CII) substrate (100mM ADP, 1M succinate, 15uM rotenone, and assay buffer). Samples incubated for 10 minutes at 20°C on a thermo cycler. During incubation, ATP standards of 10,000nM, 5,000nM, 1,000nM, 500nM, 100nM, 100nM, 10nM, 5nM, and 0nM concentrations were added into an opaque white 96-well assay plate preloaded with 25uL of CellTitre-Glo (Promega). A time=0 control group was created, where 25uL of each mitochondrial resuspension was quickly added to 5uL of CI substrate, mixed briefly, and then immediately added into the assay plate preloaded with CellTitre-Glo. After incubation, 25uL of each sample was loaded into the plate and run immediately on a Fluoroskan Ascent FL plate reader (Thermo Labystems). Using the generated standard curve and subtracting the control group, a final measurement of nanomoles of ATP produced per minute was reported.

The remainder (30uL) of each sample used in the ATP synthesis assay was saved for normalization with a citrate synthase assay (Spinazzi, Casarin, Pertegato, Salviati, & Angelini, 2012). Five microliters of each sample were added to 50uL of 200mM Tris buffer with 0.2% Triton-X (Sigma), 24uL DI water, 10uL 1mM DTNB (5, 5'-dithiobis(2-nitrobenzoic acid)), and 6uL acetyl coenzyme A. Absorbance was measured at 412nm for 5 minutes to check for background activity. Immediately after the background run, 5uL of

10mM oxaloacetic acid was added to each sample and read again at 412nm. The slope measured over 5 minutes was used to calculate the concentration of mitochondria in the original sample. ATP synthesis results were divided by this concentration to normalize the data.

UV Survivorship Assays

In order to mimic the natural environment, ultraviolet radiation (UVR) measurements were taken over the course of 10 days in June 2020 in San Diego, CA (Table 3). A digital ultraviolet radiometer (Solarmeter) was used to measure total UV intensity, and then a 320nm longpass glass filter (Schott) was placed over the radiometer to measure isolated UVA intensity.

In the laboratory, two lamps were available to test UVA and UVB tolerance separately. A 366nm model UVL-21 Blak-Ray UV lamp (UVP) was used for UVA experiments, and a 311nm narrow band phototherapy lamp (Philips) was used for UVB experiments. Copepods were placed in a single well of a 6 well plate filled with 8mL of filtered seawater. The UVA plate was positioned 1.9cm below the lamp with the 320nm longpass filter placed on top of the well to filter out any UVB or UVC radiation. The UVB plate was positioned 9.6cm below the UVB lamp with no filter necessary due to the narrow spectrum. The UVR intensity of the local natural sunlight as well as both experimental setups is listed in Table 3.

Copepods were checked hourly, dead animals were removed, and mortality was recorded. All experiments continued until 100% mortality or at least 10 hours had passed, while some were monitored for longer to ascertain a difference between treatments that lasted longer than 10 hours. Final data are reported as the proportion of the starting population

surviving after each hour. Controls were conducted to check for mortality on copepods not subjected to UV radiation for 10 hours, and no mortality was observed.

Statistics

All statistics were completed in R studio. Comparisons between multiple populations' astaxanthin production or ATP synthesis were analyzed with an ANOVA. When the *p* value of the ANOVA was greater than 0.05, a Tukey-Kramer *post-hoc* test was conducted to find which pairwise comparisons held statistical significance. For comparisons between two populations' means, a Student's *t*-test was conducted, and *p*-values were reported. When analyzing the relationship between two variables, linear models were constructed, and we report the model results as the effect size estimate (β) and *p*-value.

Results

Hybrids vs Parentals

Throughout the 12 months of research on this project, the yeast diet caused consistent problems with culturing and experimentation that is not observed with other food sources. Populations often crashed, mortality was high, and gravid females rarely produced successful egg sacs in small volumes of water. This indicated that the yeast diet was stressful and nutrient poor compared to algal diets.

When comparing astaxanthin production between RILs and their respective parental populations, we found that female data showed no statistically significant differences ($p>0.05$) in astaxanthin production (Figure 2). The female data had little variation, and astaxanthin values were lower than that of their male counterparts. After this data was collected, it was decided that no additional female measurements would be made on astaxanthin production, and all of the following astaxanthin data are from adult males. BUFxSD19, BUFxSD24, BUFxSD4, and BRxSD56 all produced less astaxanthin than both parental populations, while BRxSD45 and BRxSD50 produced less astaxanthin than just the SD parental population ($p<0.05$) (Figure 2). CATxAB27 and ABxCAT11 seemed to produce more astaxanthin than both parental populations, though the difference was not statistically significant ($p>0.05$). PESxAB20 had astaxanthin production levels in between its two parental populations, though not significantly different than either ($p>0.05$) (Figure 3). Hybrids from the SD and SCN crosses did not show any decrease in astaxanthin production compared to SD and SCN until the F3 generation, where one F3 hybrid population (SCNxSD F3) made significantly less astaxanthin than the SD parental population ($p = 0.048$) (Figure 4).

Development Time vs. Astaxanthin

In the F1 generation of the SD and SCN crosses, there was a statistically significant relationship between development time and astaxanthin production ($\beta = 0.04$, $p = 0.025$), where longer development is associated with higher pigmentation (Figure 5A). The F2 generation of hybrids showed no statistically significant relationship ($\beta = -0.005$, $p = 0.909$) between development time and pigment production (Figure 5B). The F3 generation of hybrids also showed no statistically significant relationship ($\beta = -0.002$, $p = 0.346$) (Figure 5C). When we compare the average development time of each hybrid generation against each generation's average astaxanthin production, there is a statistically significant relationship ($\beta = -0.03$, $p = 0.017$) between slower development and lower pigment production (Figure 6).

ATP

ATP synthesis measurements were taken for 7 RILs and 7 parental populations (Figure 7), all of which were yeast-fed. There were no statistically significant differences between any RIL and their respective parental populations ($p > 0.05$). There is a nearly statistically significant relationship ($\beta = -0.0003$, $p = 0.068$) where high ATP synthesis is associated with low astaxanthin production reported in Figure 8.

CI ATP synthesis rates SD, SCN, and F3 hybrids are reported in Figure 9A, all of which were *Tetraselmis*-fed. There were no statistically significant differences in ATP production for any pairwise comparisons ($p > 0.05$). A relationship between ATP and astaxanthin production for these populations was drawn in Figure 9B, with a nearly statistically significant positive trend ($\beta = 1036.8$, $p = 0.058$).

BUF vs. BUFxSD19

After analyzing the astaxanthin production rates between the SD, BR, and BUF populations and their RILs (Figure 2), it was evident that the largest disparity in astaxanthin

production between a RIL and parental population was that of BUF and BUFxSD19. To properly test our hypothesis that differences in astaxanthin production are driven by mitochondrial efficiency, this pair was subjected to two fitness assays: ATP synthesis and development rate. For both development rate and CI ATP synthesis rate (within diet), there was no statistically significant difference between the two ($p>0.05$) (Figure 10).

UV Experiments

The previously described finding that *Tetraselmis*-fed copepods are protected from UV radiation (Davenport et al., 2004) was upheld in this research (Figure 11A&B). Additionally, a third diet group (*Spirulina*) was added to test if the mortality of yeast-fed copepods was a factor of poor diet rather than lack of pigmentation. *Spirulina*-fed copepods survived longer than yeast-fed copepods, but only by a few hours, and still experienced mortality before *Tetraselmis*-fed copepods (Figure 12). Exposing newly hatched nauplii from mothers that had been feeding on *Tetraselmis* vs. yeast demonstrated that those from *Tetraselmis*-fed mothers were more tolerant of UV radiation (Figure 11C&D). When exposed to UVA radiation, the nauplii from the *Tetraselmis*-fed mothers were able to survive ten hours with no mortality, while the nauplii from the yeast-fed mothers began to show mortality after four hours (Figure 11C). For UVB radiation, the nauplii from the *Tetraselmis*-fed mothers were able to survive for five hours before showing signs of mortality, and were unable to survive past eight hours, while the nauplii from the yeast-fed mothers began to show mortality after just two hours and were unable to survive past five hours (Figure 11D). Egg sacs from populations feeding on *Tetraselmis*, *Spirulina*, and yeast were analyzed for pigments in the HPLC, and the eggs from the *Tetraselmis*-fed population contained significantly higher levels of astaxanthin than both *Spirulina* ($p = 0.0004$) and yeast ($p = 0.0003$) (Figure 13).

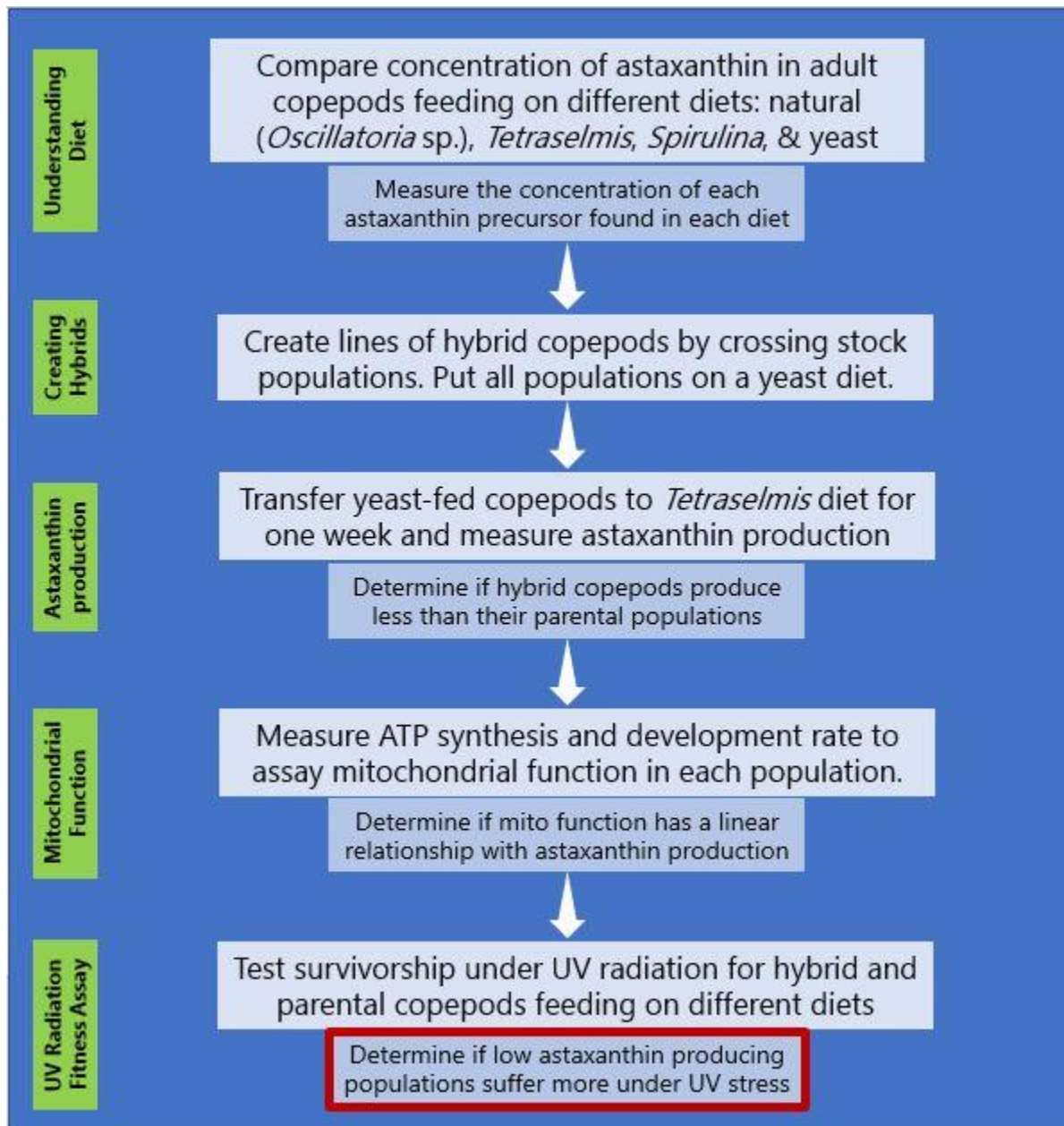


Figure 1: A conceptual diagram of this project. The method outlined in red was not completed in time to be included in this thesis.

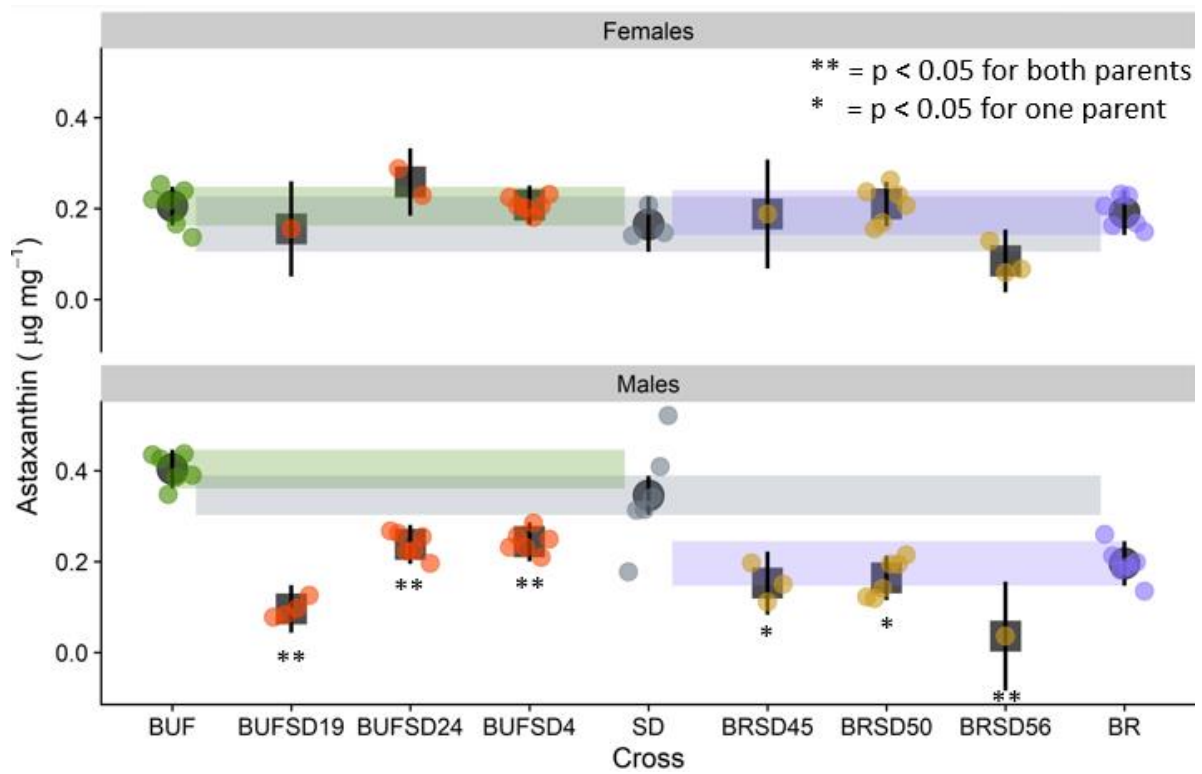


Figure 2: Astaxanthin concentration (micrograms of astaxanthin per milligram of dry copepod tissue) of parental and RIL populations after one week of feeding on *Tetraselmis*. Black bars represent 95% confidence intervals (CI), and shaded bars are extensions of each parental population's CI. Results are separated by sex. ** = astaxanthin production is significantly lower than both parental populations ($p < 0.05$). * = astaxanthin production is significantly lower than one parental population ($p < 0.05$).

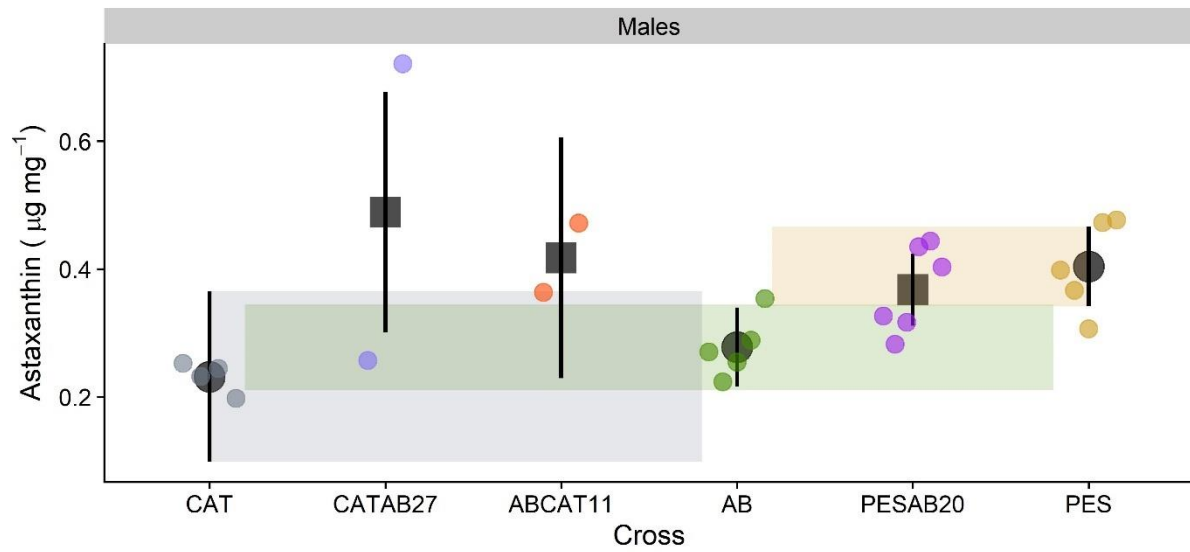


Figure 3: Astaxanthin concentration (micrograms of astaxanthin per milligram of dry copepod tissue) of parental and RIL populations after one week of feeding on *Tetraselmis*. Black bars represent 95% confidence intervals (CI), and shaded bars are extensions of each parental CI.

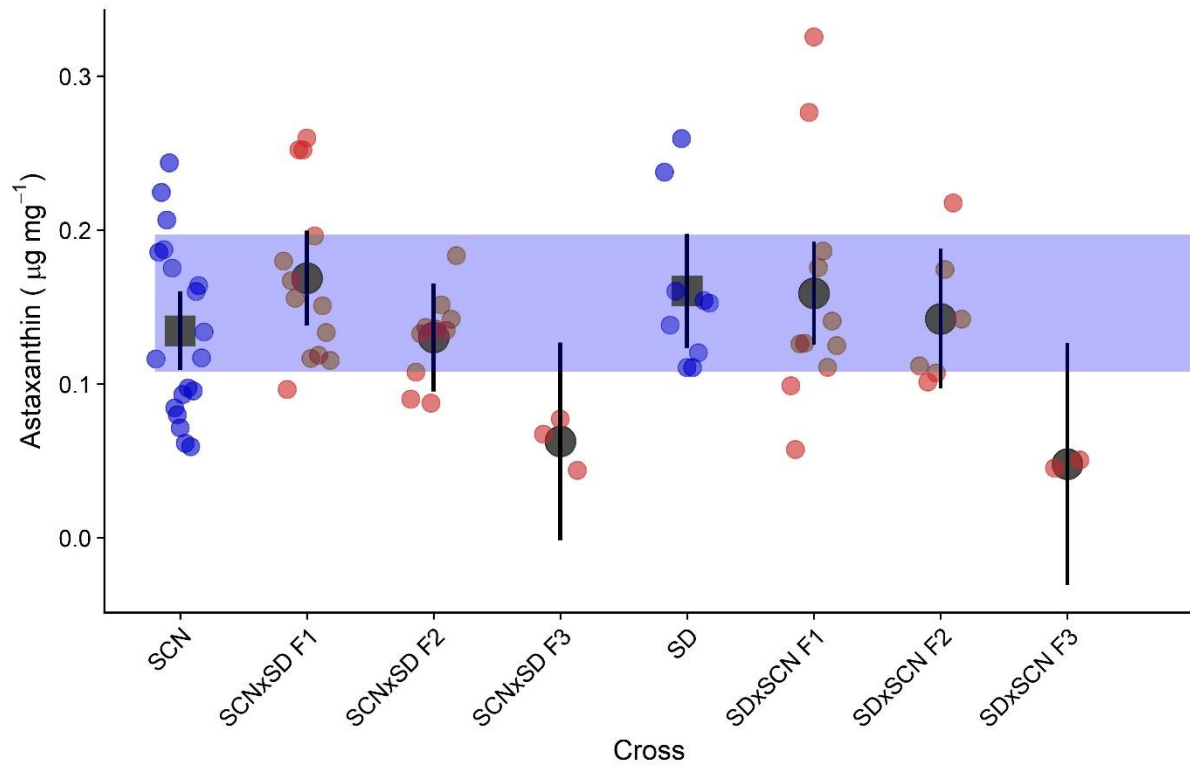


Figure 4: Astaxanthin concentrations (micrograms of astaxanthin per milligram of dry copepod tissue) of adult males in SD, SCN, and their reciprocal hybrid crosses after one week of feeding on *Tetraselmis*. The blue shaded bar represents the combined 95% CI of SD and SCN. The only statistically significant difference in means is between SD and SCNxSD F3 ($p = 0.048$). The difference between SD and SDxSCN F3 is near significance ($p = 0.054$)

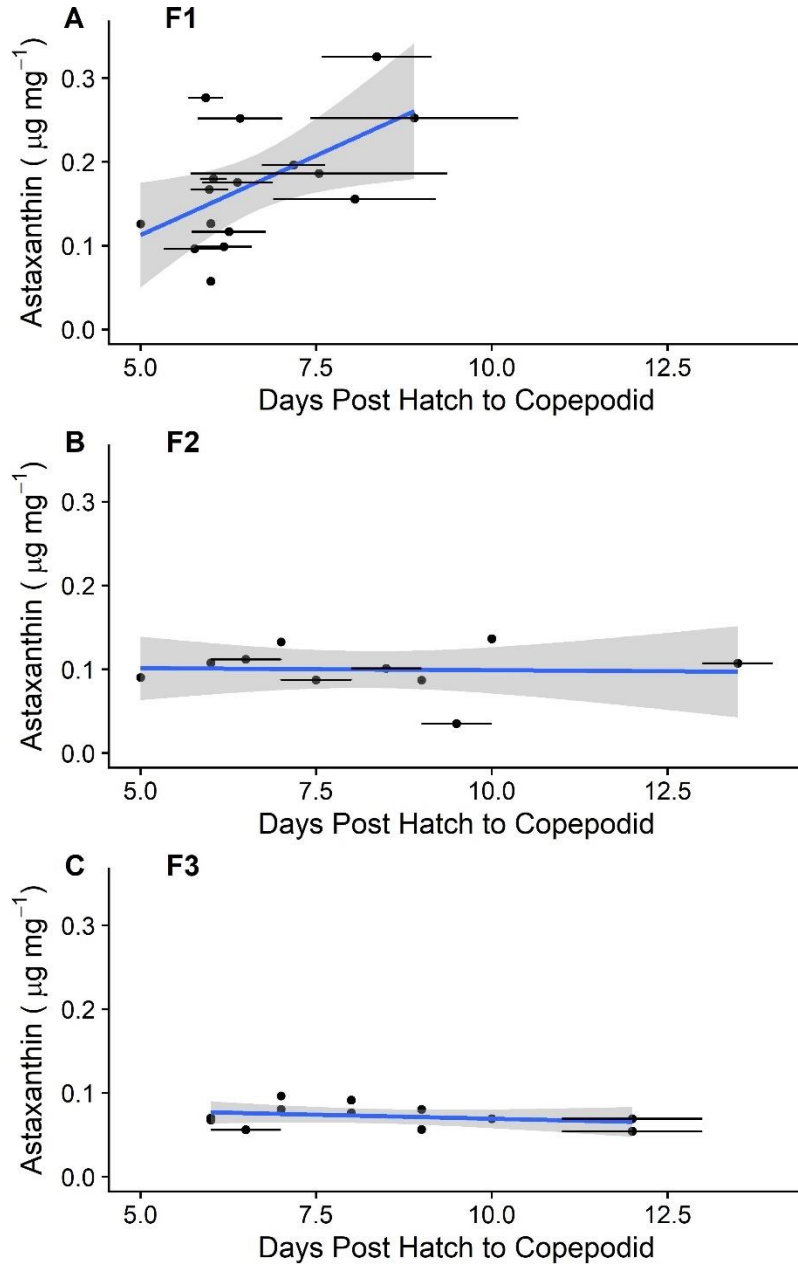


Figure 5: Astaxanthin concentrations (micrograms of astaxanthin per milligram of dry copepod tissue) F1, F2, and F3 hybrids after one week of feeding on *Tetraselmis* compared to development rate. A. Each point represents the astaxanthin production of pooled F1 adult males from a single egg sac where all nauplii were scored for development rate. Horizontal bars show the variation in development rate within each egg sac. ($\beta = 0.04$, $p = 0.025$) B. Each point represents the astaxanthin production of F2 adult males from groups separated by development rate. ($\beta = -0.0005$, $p = 0.909$). C. Astaxanthin production of adult males from groups separated by development rate. ($\beta = -0.002$, $p = 0.346$) Horizontal bars for 4B and 4C represent instances where sample size was low enough that two neighboring development rate groups were combined.

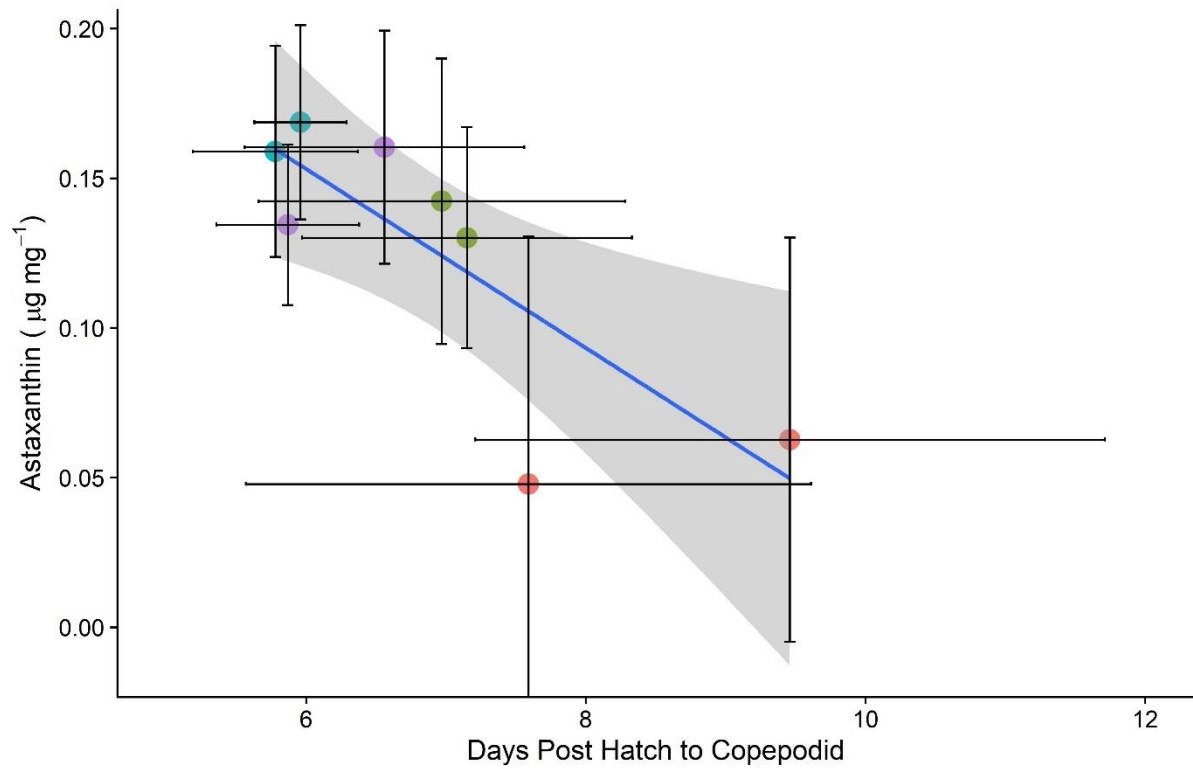


Figure 6: Astaxanthin concentrations (micrograms of astaxanthin per milligram of dry copepod tissue) of adult males in SD&SCN parental populations (purple), F1 hybrids (blue), F2 hybrids (green), and F3 hybrids (red) after one week of feeding on *Tetraselmis* compared to average development rate for each population. Vertical bars represent 95% CI, and horizontal bars represent one standard deviation around the mean development time. ($\beta = -0.03$, $p = 0.017$)

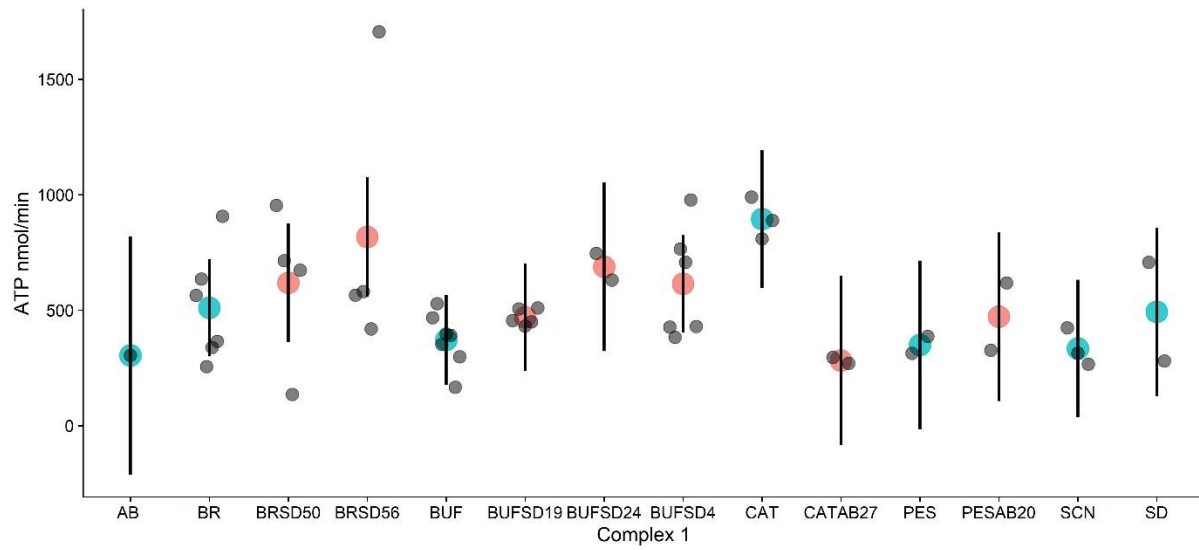


Figure 7: CI ATP synthesis of all RILs (orange) and parental populations (blue). Black bars represent 95% CI.

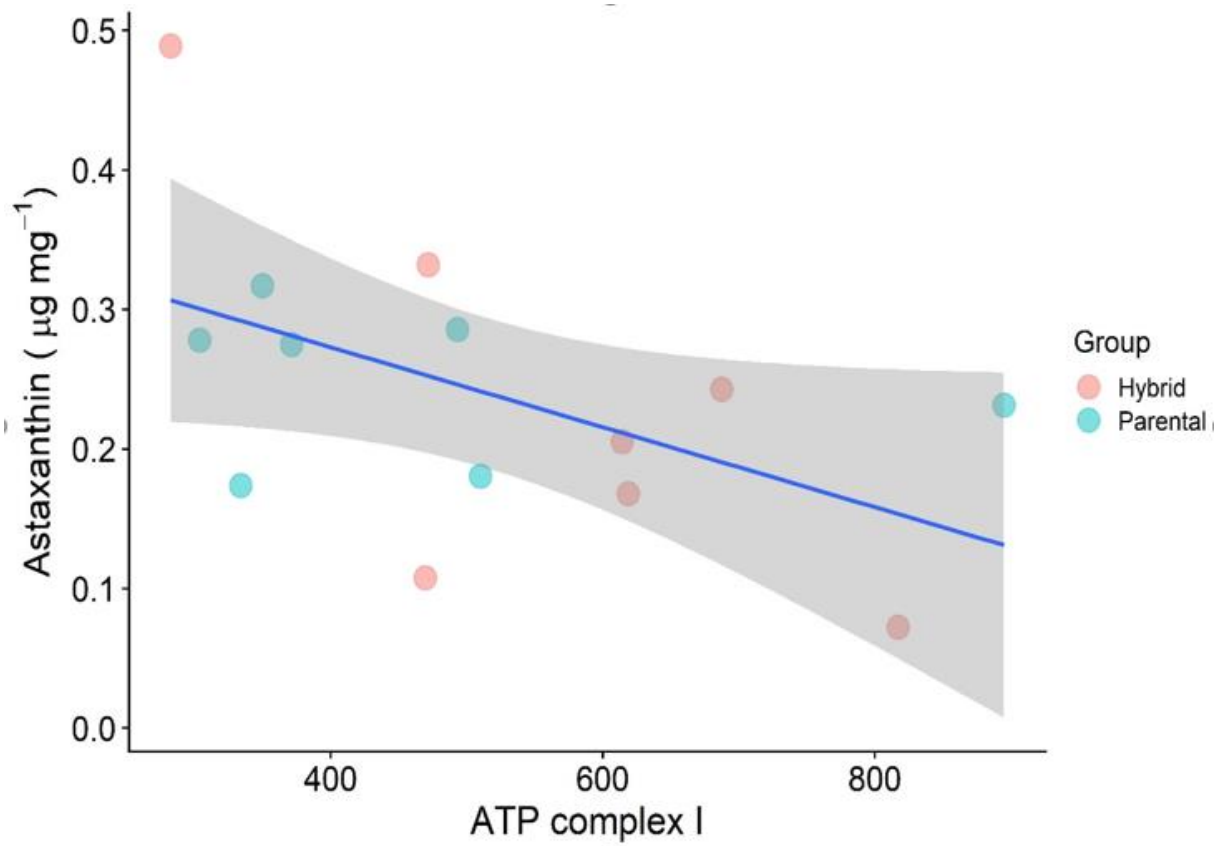


Figure 8: Astaxanthin concentration of adult males (micrograms of astaxanthin per milligram of dry copepod tissue) in RILs (red) and parental populations (blue) after feeding on *Tetraselmis* for one week compared to averaged CI ATP synthesis rates from yeast-fed copepods. ($\beta = -0.0003$, $p = 0.068$)

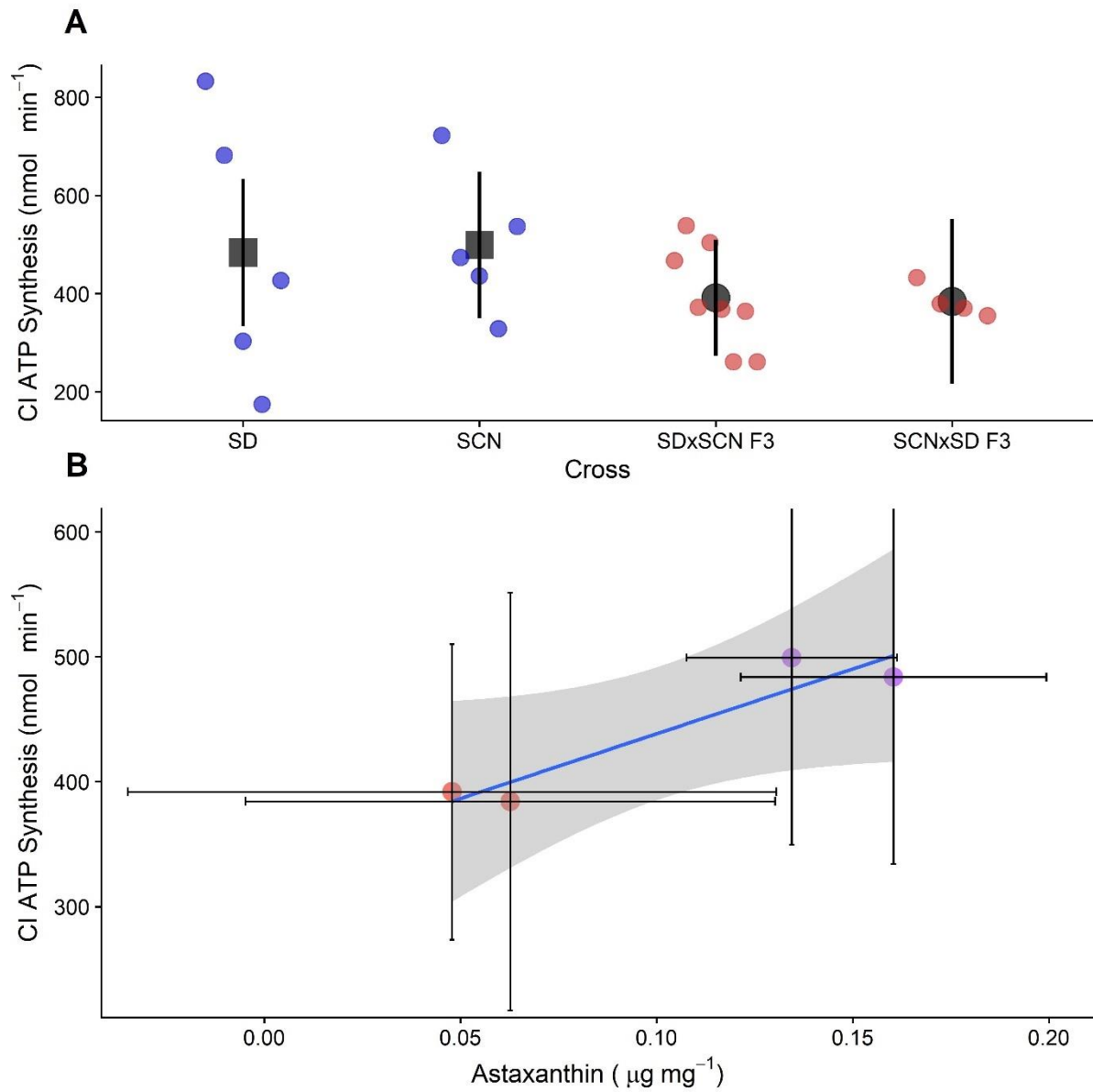


Figure 9: A. CI ATP synthesis rate of SD and SCN populations compared to their F3 hybrids. Black bars represent 95% CI. B. CI ATP synthesis of *Tetraselmis*-fed parental populations (purple) and F3 hybrids (red) compared to astaxanthin concentration of adult males (micrograms of astaxanthin per milligram of dry copepod tissue). ($\beta = 1036.8$, $p = 0.058$)

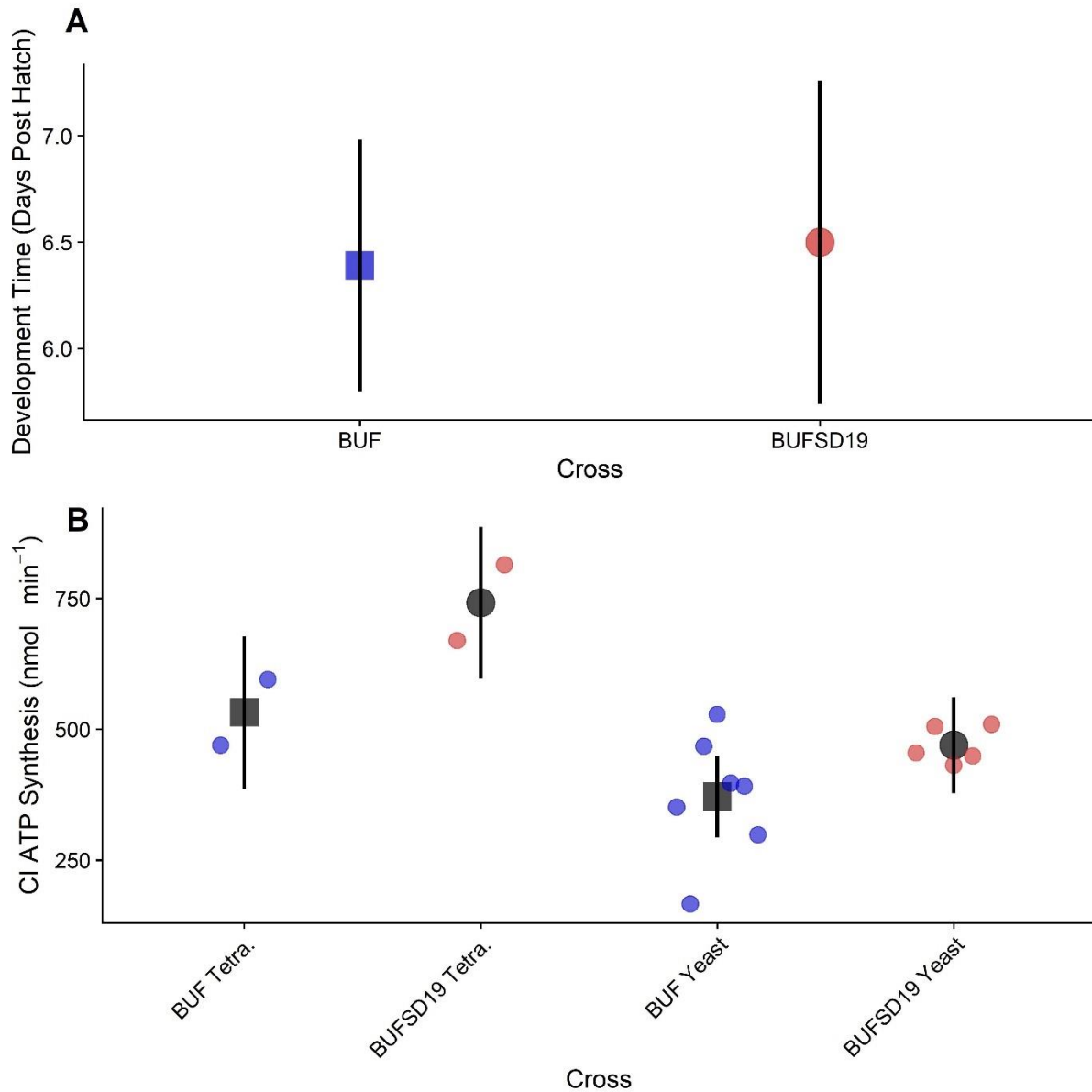


Figure 10: A. Average development time of BUF and BUFxSD19. Black bars show 95% CI. The difference in development time was not statistically significant ($p>0.05$) B. CI ATP synthesis rates from BUF and BUFxSD19 copepods that had been transferred from a yeast diet to a *Tetraselmis* diet for one week before experimentation. For comparisons within diet, the difference in ATP production was not statistically significant ($p>0.05$).

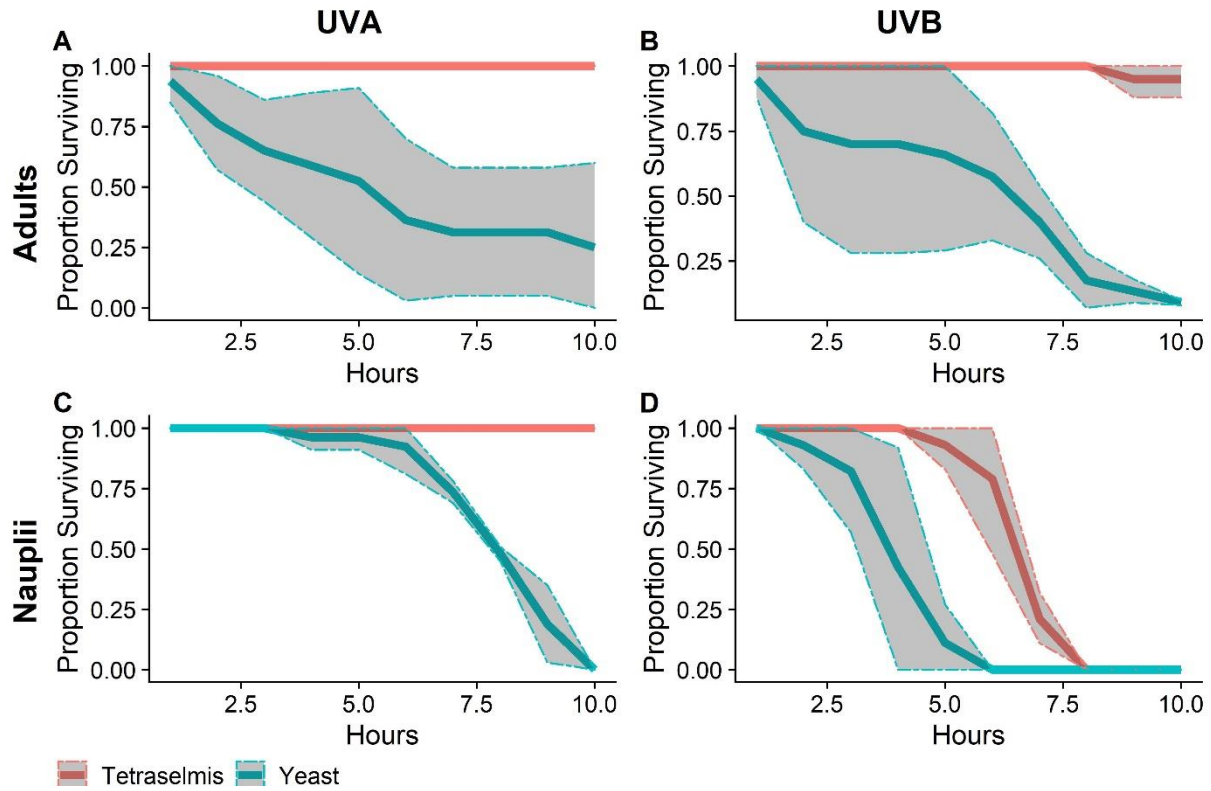


Figure 11: Survivorship of copepods under UV stress. Shaded regions show one standard deviation above and below the mean. A. *Tetraselmis*-fed (red) vs. yeast-fed (blue) adults under UVA radiation. B. *Tetraselmis*-fed vs. yeast-fed adults under UVB radiation. C. Freshly hatched nauplii from mothers sourced from *Tetraselmis*-fed vs. yeast-fed populations under UVA radiation. D. Freshly hatched nauplii from mothers sourced from *Tetraselmis*-fed vs. yeast-fed populations under UVB radiation.

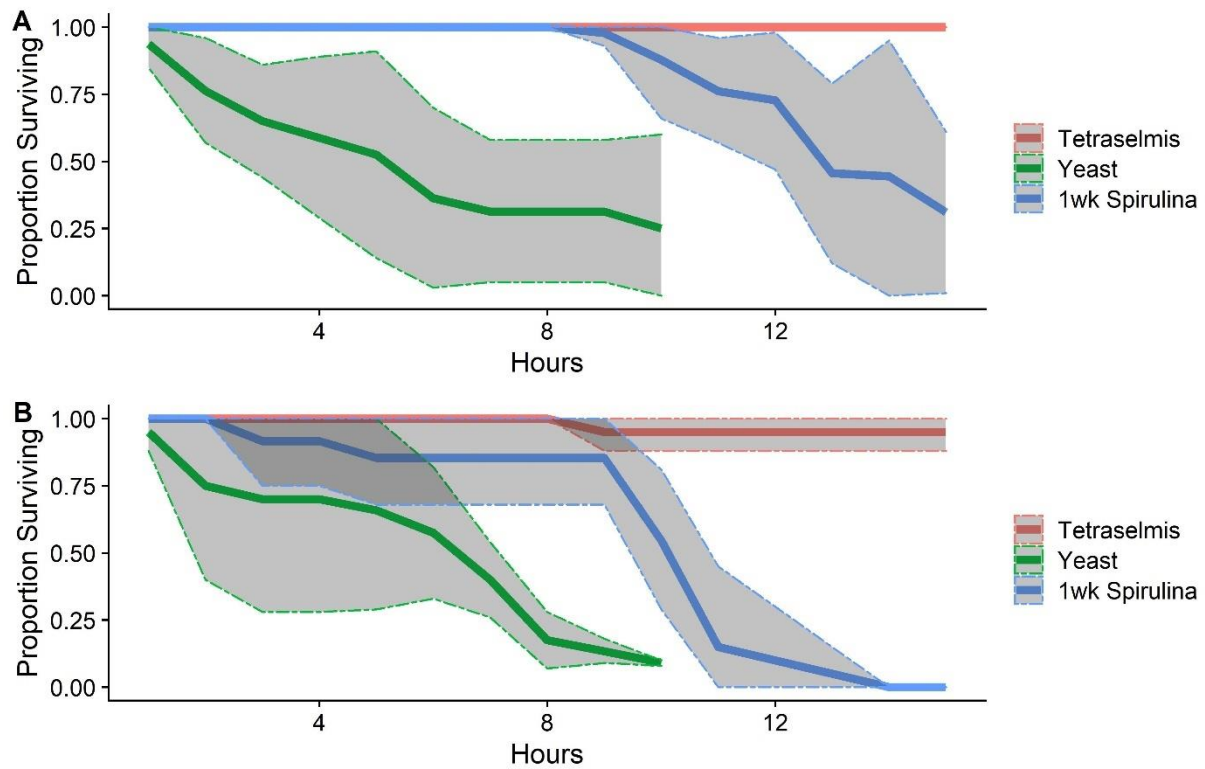


Figure 12: Survivorship of copepods under UV stress. Shaded regions show one standard deviation above and below the mean. A. *Tetraselmis*-fed (red) vs. *Spirulina*-fed (blue) vs. yeast-fed adults under UVA radiation. B. *Tetraselmis*-fed vs. *Spirulina*-fed vs. yeast-fed adults under UVB radiation.

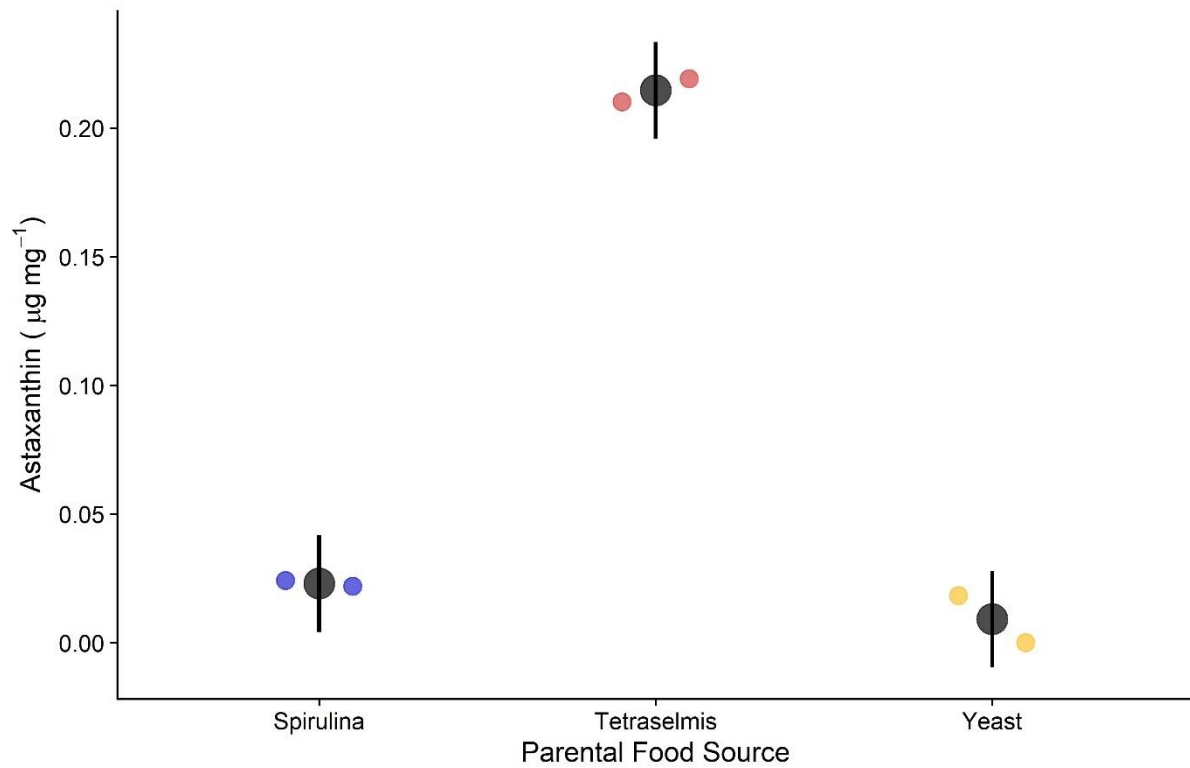


Figure 13: Astaxanthin concentration (micrograms of astaxanthin per milligram of dry egg sac) in egg sacs from mothers feeding on *Spirulina*, *Tetraselmis*, and yeast. Eggs from *Tetraselmis*-fed mothers show significantly higher astaxanthin content than both *Spirulina* ($p = 0.0004$) and yeast ($p = 0.0003$). Black bars represent 95% CI.

Table 1: Astaxanthin concentration (micrograms of astaxanthin per milligram of dry copepod tissue) for copepods feeding on different diets: Rock Pool (sampled directly from SD), *Tetraselmis*, *Spirulina*, and nutritional yeast. Astaxanthin determined by HPLC (retention time was 23.65 minutes).

	Rock Pool	<i>Tetraselmis c.</i>	<i>Spirulina</i>	Yeast
Astaxanthin (ug/g tissue) 23.65 min	586 ± 127	500 ± 91	57 ± 21	6 ± 3

Table 2: Concentrations (micrograms of carotenoid per milligram of dry food source) of the four astaxanthin precursors found in each food source. *Oscillatoria* species were discovered in rock pools abundant with *T. californicus*. nd = no detection. HPLC retention times are listed below each precursor.

<u>Food Source</u>	Zeaxanthin (ug/g) 28.16 min	Lutein (ug/g) 28.28 min	Canthaxanthin (ug/g) 29.62 min	Beta Carotene (ug/g) 38.09 min
<i>Oscillatoria</i> <i>sp. A</i>	51.5	nd	10.1	239.1
<i>Oscillatoria</i> <i>sp. B</i>	37.2	nd	6.7	92.9
<i>Tetraselmis c.</i>	2.0	172.7	1.5	169.0
<i>Spirulina</i>	4.0	3.6	1.0	10.4
Yeast	nd	nd	nd	nd

Table 3: UVR measurements taken over a 10 day period in June 2020 in San Diego, California. Field measurements were taken outside in direct sunlight (Sunny Afternoon) or under moderate cloud cover (Partially Cloudy Afternoon). Laboratory measurements were taken underneath the experimental UVA and UVB lamps at the height of the water's surface.

	UVAR (mW cm⁻²)	UVBR (mW cm⁻²)
Sunny Afternoon	4.1 ± 0.4	0.8 ± 0.1
Partially Cloudy Afternoon	2.5 ± 1.0	0.5 ± 0.2
Experimental Lab Setup	2.7 ± 0.2	1.0 ± 0.1

Discussion

Field Data

The concentrations of astaxanthin in copepods feeding on different diets exemplifies the impact of diet on pigment production. Copepods taken directly from San Diego rock pools had similar astaxanthin concentrations to those raised on *Tetraselmis*, indicating that *Tetraselmis* provides adequate carotenoid precursors for astaxanthin production (Table 1). Copepods feeding on the *Spirulina* and yeast diets produced much less astaxanthin than the natural or *Tetraselmis* diet (Table 1). The relative levels of the four known astaxanthin precursors were measured in each diet, in an attempt to discern which precursors are being used by the copepods. For the purpose of this discussion, we will assume that the rock pool copepods were feeding on *Oscillatoria* species A&B. Since the highest astaxanthin content was found in copepods feeding in the rock pools and on *Tetraselmis*, looking at what precursors they have in common may help identify the preferred precursor for astaxanthin production. Beta-carotene was the common precursor found in high concentration in each of these diets, suggesting it may be responsible for most of the astaxanthin production in these copepods (Table 2). That being said, it is entirely possible that other precursors are used as well, as the *Oscillatoria* species had elevated zeaxanthin levels, and *Tetraselmis* had elevated lutein levels. However, the *Oscillatoria* species had no detectable lutein and *Tetraselmis* had an extremely low zeaxanthin content, suggesting that neither is essential for the level of pigment found in rock pool and *Tetraselmis*-fed copepods (Table 1).

UV

Compared to the natural environment, our experimental setup had similar UVA radiation and more extreme UVB radiation (Table 3). However, both UVA and UVB

radiation levels were close to natural conditions, suggesting that our results are ecologically relevant.

When adult copepods were subjected to UV stress, we found that copepods feeding on *Tetraselmis* (and thus producing astaxanthin) were protected against both UVA and UVB radiation (Figure 11A&B), confirming results previously described in Davenport et al., 2004. *Spirulina*-fed copepods, which are shown to have low levels of astaxanthin but a less problematic diet, survived longer than the yeast-fed copepods, but still displayed mortality a few hours past the yeast-fed copepods (Figure 12). This suggests that astaxanthin is providing UV protection for the *Tetraselmis*-fed copepods.

Additional tests on newly hatched nauplii show that those hatched from *Tetraselmis*-fed females are more UV tolerant than those from yeast-fed females, suggesting that these nauplii reap the protective benefits from the astaxanthin that their mothers provide for them in the egg sacs (Figure 13). This maternal endowment is likely vital for the survival of nauplii in their natural environment, as they often hatch in exposed rock pools. If hatching occurs during daylight hours, having astaxanthin at birth gives these nauplii the ability to survive hours of direct sunlight before finding a source of food that contains the necessary precursors to start producing astaxanthin on their own.

Hybrid vs. Parental Astaxanthin Production

Preliminary experiments showed that female copepods did not produce as much astaxanthin as males (Figure 2). The constant reproductive cycle of egg sac production and release is likely the cause of this disparity, especially since we know gravid females deposit astaxanthin into their egg sacs (Figure 13) before releasing them. Therefore, we decided to continue experimentation explicitly on adult male copepods.

Of the nine RILs tested, six showed significantly lower pigment production than at least one of their parental populations (Figure 2), initially supporting the concept that hybridization can lead to depressed astaxanthin synthesis rates. This was not the case for all RILs though, as we found three that showed no decrease in astaxanthin production (Figure 3), introducing uncertainty into the trend of hybrids making less pigment. This result, however, is not entirely surprising when we consider how RILs are created. Since each RIL is formed from an isolated F3 female, and there is a wide variation in hybrid fitness, some RILs may be created from higher fitness females that do not suffer the effects of hybrid breakdown to a measurable degree. That, combined with the fact that the lowest fitness lines die off before they can even be experimented on, leaves us with a selection of higher fitness RILs.

The hybrid crosses made using the SD and SCN populations also present some complex findings. Compared to the SD population, one line of F3 hybrids shows a statistically significant decrease in astaxanthin production, and the other F3 hybrid line is near statistical significance (Figure 4); however, it is unclear why we did not see this trend in F2 hybrids, where breakdown is typically observed (Burton et al., 2006). Interestingly, astaxanthin production gradually decreases (while variation increases) as the hybrid generations progress from F1 to F2 to F3 (Figure 6). This relationship between low pigment production and slow development is the only statistically significant evidence reported in this paper that suggests a positive relationship between astaxanthin and fitness.

We report contradictory relationships between ATP synthesis and astaxanthin production. The results from our ATP synthesis assays on RILs and parental populations present a nearly statistically significant negative relationship between ATP synthesis and astaxanthin production (Figure 8); whereas, the results from SD/SCN F3 hybrids present a

nearly statistically significant positive relationship between ATP synthesis and astaxanthin production (Figure 9B). The main difference between these two datasets was the diet of the copepods used in the ATP assays. RIL ATP data was collected on yeast-fed copepods and SD/SCN F3 ATP data was collected on *Tetraselmis*-fed copepods, indicating how large of an impact diet has on assays of mitochondrial function. Adding more contradictions to the analysis, the BUF and BUFxSD19 development time and ATP data (Figure 10) suggest that there is no difference in mitochondrial function between the two, despite the large disparity in astaxanthin production (Figure 2). Altogether, the results of these assays provide contradictory relationships between mitochondrial function and pigment production, but certainly do not suggest a clear and definitive relationship between pigmentation and mitochondrial function.

Focusing more directly on the relationship between development time and astaxanthin within hybrid populations, the separate trends for F1, F2, and F3 hybrids tell quite an interesting story, as the slope of the trendline is positive for the F1 generation, but flat for the F2 and F3 (Figure 5). In the F1 generation, we saw a hint of hybrid vigor where F1s had a slight but non-significant increase of fitness (Figure 6) and a relationship between longer development and increased pigment production (Figure 5A). Since all F1 copepods developed within the normal parental range, there were likely no low-fitness hybrids that would show the expected trends of hybrid breakdown. However, it is unknown why there would be a relationship between longer development and increased pigment production. In the F2 and F3 generations, there is no trend and far less variation in astaxanthin production compared to the F1 (Figure 5B&C), which is unusual, since variation typically increases past the F2 generation (Ellison & Burton, 2006). The lack of consistency in these trends makes it evident there is no

clear relationship between development time and astaxanthin production, especially since the trends in Figure 5A and Figure 6 have statistically significant slopes with opposite signs.

Although some hybrid populations had decreased levels of astaxanthin production, we were unable to show that these differences were a result of decreased mitochondrial function. The number of contradicting slopes and trends presented in this research suggests the possibility that there is some other driver behind differences in astaxanthin production. Since astaxanthin is an important antioxidant, it is possible that oxidative stress could have had a greater impact on the concentrations of astaxanthin than previously thought. It is known that hybrids have elevated oxidative stress (Barreto & Burton, 2013), and if that causes an upregulation of the production of antioxidants like astaxanthin, it could have heavily influenced our results. Additionally, our measurement of astaxanthin concentration may not have been an accurate prediction of the rate of astaxanthin production over a week if a large portion of that astaxanthin was simultaneously being consumed as an antioxidant. Future research could focus on measuring oxidative stress in each population to determine if that may have obscured a clearer relationship between astaxanthin production and mitochondrial function; however, with the current results, we cannot conclusively define a clear relationship between astaxanthin production and mitochondrial function.

Acknowledgments

This thesis contains unpublished coauthored material currently being prepared for submission for publication. Martz, Lucas; Powers, Matthew. Mr. Martz was the principal author of this material.

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