UNIVERSITY OF CALIFORNIA SAN DIEGO

RNA-seq analysis of *Tigriopus californicus* under multiple dietary conditions.

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

in

Marine Biology

by

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Professor Martin Tresguerres

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The Thesis of Antonia K. Bock is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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Dedications

I am deeply grateful to Ron Burton for his mentorship as my advisor. He has given me the chance and the support to explore my research interests and learn about doing exciting and rigorous science at every step of the process. Thank you, Ron! I am also grateful to Tim Healy for training me when I was an undergraduate, and for his advice on this project and comments on a draft of this thesis. Thank you to every member, former and current, of the Burton lab with whom I shared time: Reggie Blackwell, Rebecca Pak, Tatum Bernat, Michaela Labare, Emma Choi, Lucas Martz, Satomi Tsuboko-Ishii, Laura Furtado, Elena Duke, Andrea Odell, Sumi Hunjan, Alice Harada, Por Tangwancharoen, Gary Moy, Cody Hargadon, Abby Lindemood, and Natalie Faivre. I'm incredibly lucky to have worked with such a friendly, smart, and overall wonderful group of people. My family and friends have buoyed me up, for which I thank and love them, and learned more than they ever expected to learn about copepods, for which they are welcome. Finally, I'd like to thank my committee members, Dede Lyons and Martin Tresguerres, for valuable and thought-provoking comments and conversations on several iterations of my master's project.

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ABSTRACT OF THE THESIS

RNA-seq analysis of *Tigriopus californicus* under multiple dietary conditions.

by

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Master of Science in Marine Biology

University of California San Diego, 2021

Professor Ronald Burton, Chair

In supralittoral splash pools, the copepod *Tigriopus californicus* is frequently exposed to environmental extremes, and synthesis and accumulation of the red ketocarotenoid astaxanthin from dietary precursors may be an important component of survival. To search for evidence of genes involved in the synthesis pathway and for potential further-reaching effects of the pigment, we evaluated the transcriptomic response to astaxanthin synthesis. By comparing RNA sequencing of copepods fed diets with varying amounts of astaxanthin precursors (*Tetraselmis* algae, spirulina, nutritional

yeast) across two timescales (lifelong and raised on yeast but exposed for 16 hours to *Tetraselmis*), we found limited or mixed responses to the shift in carotenoids and other dietary antioxidants. Although we did not observe evidence in favor of any candidate carotenoid ketolases, the lack of differential expression in response to diet is not evidence against these candidates. A carotenoid oxygenase homologous to vertebrate BCO2 did shift in expression over a short-term exposure to carotenoids, potentially to prevent oxidative stress induced by carotenoid accumulation. Other changes in gene expression may relate to nutrient sensing and assimilation pathways. Both the upregulation of myosins in spirulina-fed copepods and shifts in neural signaling transcript levels in copepods exposed briefly to *Tetraselmis* may be related to the highly conserved ways that organisms sense, assimilate, and respond to nutrient changes. These include the TOR and IIS pathways. Protease and protease inhibitor levels could be influenced by multiple factors, including shifts in both antioxidant and nutrient assimilation systems.

Introduction

The importance of diet to crustacean health has long been a topic of intense interest in aquaculture, and its recognized ecological relevance has expanded drastically, as the accumulation of a variety of dietary components permits some crustaceans to withstand challenging environments. Some copepod species are able to survive and reproduce in salinities in excess of 300 parts salt per thousand parts water (ppt), ten times the salinity of seawater, using exoosmolytes they accumulate from algae (Anufriieva, 2015), for example. Additionally, the evolution of some crustacean immune systems may have occurred in the context of carotenoid (yellow to red pigments able to scavenge free radicals) accumulation stimulating the immune system and preventing self-harm (Cornet et al., 2007), and both carotenoids and MAAs may protect against the effects of UV radiation, with MAAs functioning as a "sunscreen" that absorbs damaging rays (Davenport et al., 2004; Caramujo et al., 2012; Helbling et al., 2002). However, not all of the genes involved in the accumulation and conversion of relevant compounds are known, and while there is evidence of interaction between multiple dietary components and the regulation of endogenous enzymes, the typical manipulation of or focus on one dietary variable can obscure the full context in which each nutrient or enzyme acts. Analyzing the whole transcriptome in response to a change in diet, from one associated with elevated oxidative stress (Weaver et al., 2018; Li et al., 2019) to a diet more similar to that found in the natural habitat, provides an opportunity to uncover the timescale of changes related to dietary compounds, as well as to test hypotheses related to diet and search for unexpected dietary effects.

Tigriopus californicus is an intertidal harpacticoid copepod found in supralittoral splash pools, where it is frequently exposed to environmental extremes in salinity, temperature, UV radiation, and dissolved oxygen (e.g. Powlik, 1999; Schoville et al., 2012; Russell & Phillips, 2009; Davenport et al., 2004; Truchot & Duhamel-Jouve, 1980). The red-orange coloration of *T. californicus* copepods, which is due to their synthesis and accumulation of the red ketocarotenoid astaxanthin from dietary precursors (Weaver et al., 2018), may be an important adaptation to environmental extremes. The variety of fitness benefits potentially provided by astaxanthin include protection against UV radiation (e.g. Davenport et al., 2004; Caramujo et al., 2012) and pollutants (e.g. Caramujo et al., 2012), increased growth and reproduction (e.g. Schneider et al., 2016; Wang et al., 2020), longer survival in low dissolved oxygen conditions (Chien & Shiau, 2005), and antioxidant activity (Weaver et al., 2018; Li et al.. 2019). The variety of benefits apparently offered by astaxanthin and other carotenoids may help to explain why they are accumulated when mycosporine-like amino acids (MAAs), also derived by crustaceans from an algal diet, act as a "sunscreen" without the predation-related tradeoff of bright coloration (e.g. Helbling et al., 2002). Despite all of these proposed benefits, the pathway by which *T. californicus* synthesizes astaxanthin is not known. In the lab, *T. californicus* can be reared on yeast, which lacks any carotenoids to serve as precursors to astaxanthin and therefore produces colorless copepods with higher markers of oxidative stress and lower tolerance of UV radiation (Weaver et al., 2018; Li et al., 2019; Davenport et al., 2004). Because the genome of *T. californicus* is published (Barreto et al., 2018), *T. californicus* is an excellent candidate for studies involving RNA-sequencing, and therefore for

searching for transcriptome changes associated with astaxanthin synthesis. The transcriptomic response to astaxanthin synthesis could provide evidence for genes involved in the synthesis pathway and other effects of the pigment relating to the hypothesized antioxidant and overall survival benefits.

Previous dietary manipulations in *T. californicus* (Weaver et al., 2018a,b; Li et al., 2019) and the related species *T. brevicornis* (Davenport et al., 2004) have consisted of feeding some cultures baker's or nutritional yeast (*Saccharomyces cerevisiae*), and others cultured algae. We followed this approach, using RNA-seq to compare transcriptomes of adult males from the San Diego population fed with yeast, *Tetraselmis chuii* algae, powdered spirulina (cyanobacteria *Arthrospira* sp.), and copepods originally fed yeast but exposed to *Tetraselmis* for 16 hours. Our approach was intended to provide some idea of timescale of transcriptomic response to dietary change, which likely happens before nutrients are accumulated to wild-type levels. For example, Weaver et al. (2018) found that yeast-fed copepods supplemented with zeaxanthin can synthesize astaxanthin to biologically relevant levels (significantly affecting oxidative status) within 48 hours, although red coloration appears before that point, around the 16 h mark. In contrast, Hapette & Poule (1990) found in calanoid copepods that the rate of vitamin C uptake from algae after starvation was 1.54% per hour, which, while five times higher than the rate of decrease, was not fast enough to restore the initial pool of vitamin C in over 66 hours of observation. In addition to the timescale of changes following 16 hours of exposure to *Tetraselmis*, there are several key differences between a yeast diet, a *Tetraselmis* diet, and a spirulina diet, meaning that we did not manipulate a single nutritional variable. Rather, we evaluated the transcriptomic

response to a broad dietary change, which has a number of potential results. For example, key antioxidants (including vitamin C/ascorbic acid, vitamin E/tocopherol, and non-astaxanthin carotenoids) are present in *Tetraselmis* and spirulina and absent in yeast (see Tables S1, S2, S3, S4). Despite this, copepods fed *Tetraselmis* produce and accumulate high quantities of astaxanthin, while copepods fed spirulina do not, potentially due to the lower levels of carotenoids present in spirulina. Additionally, spirulina and yeast differ in amino acid composition, and both differ from *Tetraselmis* in protein:carbohydrate ratio. Because RNA-seq records the whole transcriptome, it is not constrained by our predictions. Therefore, while we found limited responses to the shift in carotenoids and antioxidants, we also saw unexpected shifts in gene expression that may relate to nutrient sensing and assimilation pathways.

MATERIALS AND METHODS

T. californicus copepods were collected from the San Diego population at Ocean Beach, San Diego County (32° 45′ N, 117° 15′ W) (Harada et al., 2019) and maintained in the lab under control conditions (12 hour light cycle and combination of ground spirulina and natural algal growth for food) for at least one generation before dietary treatments. In order to prevent uncontrolled algal growth contaminating the diet, all cultures used in this experiment were kept in the dark. Temperature remained at 20℃. Experimental cultures were established, each with thirty to forty egg sacs from multiple control SD cultures, in 250 mL of filtered sea water (FSW) in 400 mL beakers, and from hatching were fed either nutritional yeast (Bragg brand), live *Tetraselmis chuii*, or dried spirulina (*Arthrospira* sp., Jade brand, Salt Creek Inc.). Copepods were fed two to three times per week with enough food for them to just clear the water before the new feeding. Forty adult males per sample for three pooled RNA samples were then selected from each experimental line. An additional three samples were taken from the yeast-fed line for exposure to *Tetraselmis* for 16 hours, just long enough for red-orange to appear in their bodies under a microscope, in 200 mL of clean FSW in unused beakers. This was the 16 hour exposure treatment. For consistency, all samples were placed in 200 mL clean FSW with food overnight before freezing. Samples were then moved to petri dishes with clean FSW and no food to allow for one hour of gut clearance before snap freezing in 1.5 mL tubes in liquid nitrogen. All samples were taken at the same time of day. Frozen samples were stored at -80℃ until RNA isolation.

In summary, there were three replicate samples for four treatments: 1) copepods reared on yeast, 2) copepods reared on spirulina, 3) copepods reared on live *Tetraselmis,* and 4) copepods reared on yeast and transferred to *Tetraselmis* for 16 hours.

RNA isolation followed the manufacturer's protocol for TRIzol Reagent (Invitrogen). We placed 1000 µL TRIzol in each 1.5 mL sample tube and added approximately 0.250 g of 0.1 mm silica beads to each tube. Samples were then homogenized at full speed in a beadbeater for 1 minute. Samples stood at room temperature for five minutes before the addition of 200 µL chloroform. Sample tubes were shaken manually for thirty seconds until thoroughly combined, then stood at room temperature for fifteen minutes. We then spun samples in a cooled 4℃ centrifuge at 112,500 x g for fifteen minutes in order to fully separate phases. The clear, top, aqueous phase containing the RNA, approximately 500 µL, was carefully removed to new 1.5 mL tubes, avoiding the red phenol-chloroform interface. To precipitate the RNA, we added 500 µL ice-cold isopropanol to each tube, tilted to mix, and let sit at room temperature for ten minutes before spinning at 12,500 x g at 4℃ for another ten minutes. Liquid was decanted from the tubes and pelleted RNA was resuspended with 1000 µL chilled 75% ethanol made with DEPC-treated water. Tubes were again tilted to mix and spun at 12,500 x g at 4℃ for five minutes. Liquid was decanted and the remaining ethanol was carefully removed with a P10 pipette before allowing ethanol to evaporate from the pellets for 10 minutes at room temperature. The pelleted RNA was then resuspended in 12 µL DEPC-treated water and treated to remove any contaminating DNA using a TURBO DNase Kit (Thermo Fisher). RNA samples were transferred to 200 µL PCR tubes and 1.2 µL 10X DNase buffer was added to each tube. After the addition of 1 µL TURBO DNase

enzyme, each sample was mixed gently before incubating at 37℃ for 30 minutes. Samples were transferred to 0.5 mL tubes and 2 µL TURBO DNase Inactivation Reagent were added. Tubes were flicked to mix frequently at room temperature for five minutes. Tubes were then spun down at 10,000 x g for 3 minutes before the supernatant containing the purified RNA was transferred to new tubes and stored at -80℃ until sequencing.

RNA was quantified using a Qubit RNA HS Assay Kit (Thermo Fisher). Quality was evaluated at the Institute for Genomic Medicine (IGM) Genomics Center at UC San Diego with a TapeStation (Agilent). Library preparation used a TruSeq Stranded mRNA Kit (Illumina) and sequencing occurred using 100 bp paired end reads on a NovaSeq 6000 (Illumina) DNA sequencer. Resulting sequence data were trimmed of adapters and sequences mapped to the San Diego reference genome (Barreto et al., 2018) using CLC Genomics Workbench (Qiagen). We used DESeq2 (Love et al., 2014) to analyze read counts for differentially expressed genes, with the default pre-filtering (see Table 1) and the default Benjamini-Hochberg correction of p-values for multiple testing. We considered genes with Benjamini-Hochberg corrected p-values, referred to as adjusted p-values, $p < 0.01$ in pairwise comparisons between treatments to be differentially expressed genes. The stringent cutoff of $p < 0.01$ was selected in order to account for multiple comparisons and reduce false positives. Fold change was not used as a criterion to account for the potentially subtle differences in expression of our genes of interest.

RESULTS

Before trimming or alignment, raw read counts ranged from around 15.6 million to 28 million pairs (Table 1) per sample. After mapping, library sizes range from 1.7 to 5.7 million pairs for non-Tetraselmis samples, and from 0.56 to 0.86 million pairs for Tetraselmis samples which, based on Tapestation results, appeared to have more degraded RNA (Table 1). However, average mapped pair counts are comparable between non-Tetraselmis groups. In pairwise comparisons involving Tetraselmis samples, 1.2-2% (180-286) of genes are upregulated in Tetraselmis, with corrected p-values < 0.01, while 1.7-2.2% (245-315) are downregulated in Tetraselmis (Table 2). The fewest genes were differentially expressed in either direction in the Tetraselmis-16 hour comparison, while for the Tetraselmis-yeast and Tetraselmis-spirulina comparisons more genes tend to be downregulated in Tetraselmis-fed copepods than upregulated. Relatively small numbers of genes were differentially expressed in comparisons between non-Tetraselmis groups, ranging from 0.082-0.2% (12-30), and the fewest genes differentially expressed in either direction (although not significantly in the case of upregulation) in the yeast-16 hour comparison (Table 2). Of these differentially expressed genes, the most (24) were shared between the two comparisons against spirulina (Figure 4).

Principal component analysis (PCA) including all samples and treatments showed separation of Tetraselmis samples from all others along PC1, explaining 50% of variance (Figure 1). Two of three spirulina-fed samples were separable from the yeast-fed and 16 hour exposure samples along PC2, explaining 13% of variance in this model. However, we also found that all of the Tetraselmis-fed samples had final read counts below one million mapped pairs (Table 5), and previous extrapolation of

sequencing depth guidelines for the human transcriptome from the ENCODE Project to T. californicus transcriptome sizes suggests that approximately 2 million mapped reads per sample are required for adequate depth, on average (Li et al., 2019). Despite the fact that the PCA including all samples uses normalized counts, meaning that library size has been accounted for, it is possible that the Tetraselmis samples cluster together partially due to differences in coverage and not only due to the substantial nutritional differences between Tetraselmis and the other diets.

PCA run on a model excluding the Tetraselmis samples revealed that two of three spirulina-fed samples differ from the yeast-fed and 16-hour samples along PC1, explaining 29% of variance, and the third spirulina sample separates out along PC2, accounting for 19% of variance (Figure 2). Despite the clustering of one spirulina sample seemingly away from all other samples, PCA using only differentially expressed genes showed strong clustering within all three treatments examined (Figure 3).

Cytochrome P450 (CYP450) genes were potentially important in the search for a transcriptomic response to astaxanthin synthesis, as carotenoid ketolases that produce astaxanthin in birds, spider mites, and potentially shrimp (Mundy et al., 2016; Lopes et al., 2016; Wybouw et al., 2019; Weaver et al., 2020) all belong to this superfamily. In pairwise comparisons, however, no CYP450 genes were differentially expressed between yeast-fed and 16 hour exposure groups, and of the 46 putative CYP450s in the T. californicus genome, only two were differentially expressed between the Tetraselmis treatments and all three other groups in comparisons (Figure 5). Of the T. californicus CYP450s named as candidate carotenoid ketolase enzymes in the bioinformatic analysis of Weaver et al. (2020), none were differentially expressed in any comparisons.

Two groups of genes potentially related to carotenoid processing, scavenger receptor class B (SCARB) and lipoproteins, did not show differential expression between yeast and 16 hour exposure groups. However, one beta,beta-carotene 9',10'-oxygenase (homolog of BCO2 in vertebrates) showed strong upregulation in copepods exposed for 16 hours to *Tetraselmis* relative to all other groups (Figure 6).

Genes from thirteen antioxidant families were analyzed for differential expression in all comparisons, as well as proteases. Glutathione s-transferases, thioredoxins, ascorbate peroxidases, and oxidoreductases all had at least one differentially expressed gene in comparisons with lifetime *Tetraselmis*-fed samples. One ascorbate peroxidase was downregulated in spirulina relative to all other samples samples (Figure 7).

Nine potential protease genes and five potential protease inhibitor genes were differentially expressed, with a trend towards spirulina-fed animals appearing at the extremes: eight of nine proteases and four of five protease inhibitors were most highly up- or down-regulated in spirulina-fed animals (Figures 8-9).

Finally, seven myosins were upregulated in the spirulina-fed group relative to the yeast-fed group, the 16 hour exposure group, or both (Figure 10) and nine neuropeptides were differentially expressed between the groups (Figure 11).

DISCUSSION

Both the clustering of samples in PCA plots (Figure 1) and the percentages of differentially expressed genes (Table 2) suggest that the *Tetraselmis* samples cluster far from the others, with more genes differentially expressed between *Tetraselmis*-fed animals and any other dietary conditions. It is possible that this is due to the substantial differences in protein:carbohydrate:lipid ratios between *Tetraselmis*, spirulina, and nutritional yeast and the differences in vitamin and carotenoid content between *Tetraselmis* and yeast (Tables S1-S4). However, all results involving *Tetraselmis*-fed samples should be interpreted with caution, given the relatively high degradation and low sequencing depth of those particular samples. When the potentially problematic *Tetraselmis* samples are excluded from the model, spirulina samples tend to cluster together (with the exception of one) and yeast and 16-hour exposure samples form another cluster (Figure 2). The final spirulina sample does cluster with the other two of its kind when only differentially expressed genes are used in the PCA (Figure 3), potentially supporting the idea that patterns in the most significant genes are mainly consistent within the spirulina-fed group.This is borne out in the percentages of differentially expressed genes, which are the smallest in the yeast-16 hour comparison (Table 2), suggesting that while some significant regulatory changes may occur in the first 16 hours of exposure to a new and substantially different food source, many mRNA levels are slower to shift towards those typical of the new diet.

Despite the lack of a widespread pattern of changes in genes relating to carotenoid transport and metabolism, one gene, TCALIF_13295, annotated as a likely beta,beta-carotene 9',10'-oxygenase and homologue of BCO2, was significantly

upregulated in the 16 hour exposure to *Tetraselmis* group relative to all other groups. This suggests that the BCO2-like enzyme may be involved in the immediate reaction to an influx of dietary carotenoids, including beta-carotene and lutein, which are found in *Tetraselmis* at concentrations about seventeen and sixty times those of spirulina, respectively (Martz, 2020). On the one hand, polymorphisms in BCO2-like genes are associated with changes in carotenoid accumulation and pigmentation (Toews et al., 2017) in salmon (Lehnert et al., 2019), sheep adipose tissue (Våge & Boman, 2010), cow milk (Berry et al., 2009), and chicken skin (dela Seña et al., 2016), while the related carotenoid oxygenase BCO1(-like) is involved in the orange pigmentation of some scallops (Li et al., 2019). On the other hand, the contribution of BCO2-like polymorphisms to pigmentation in these situations is by its *downregulation* or mutations leading to its relative ineffectiveness at cleaving carotenoids. Additionally, BCO2-like enzymes appear to act on a wide variety of carotenoid substrates, both carotenes (which do not contain oxygen) and xanthophylls (such zeaxanthin and lutein, which do contain oxygen) (e.g. Mein et al., 2011) by cleaving at both the 9,10 and 9′,10′ carbon–carbon double bond (Mein et al., 2011) which removes one of the rings characteristic of astaxanthin and its known precursors, and therefore does not play an obvious role in any proposed astaxanthin synthesis pathway (Rhodes, 2006; Martín et al., 2008). Instead, while carotenoids as a large category are often considered by default to be antioxidants, in excess or in unprocessed states they can in fact interfere with mitochondrial function and increase oxidative stress (Amengual et al., 2011). BCO2 appears to be essential in preventing oxidative stress induced by carotenoid accumulation (Amengual et al., 2011), and so our results emphasize that carotenoids can play multiple opposing roles *in*

vivo and that pathways both to accumulate them and to remove them must be considered.

The potential response of cytochrome P450 (CYP450) genes to the dietary shift was originally of particular interest in this project, both for previous work connecting CYP450 gene expression with carotenoids (albeit in varying directions, e.g. Rühl et al., 2004; Paolini et al., 2001; Satomi et al., 2013; Wang & Leung, 2010; Zheng et al., 2013), and for their potential role in astaxanthin synthesis. In contrast to some of these previous works, we did not observe much effect of dietary shift on CYP450s, including candidate carotenoid ketolases. CYP450s are an ancient superfamily of enzymes that oxygenate organic compounds, and are involved in metabolism of both exogenous drugs/toxins and endogenous compounds (Lewis et al., 1998). In an example of convergent evolution, certain CYP450s are also believed to function as carotenoid ketolases in birds, spider mites, and potentially shrimp (Mundy et al., 2016; Lopes et al., 2016; Wybouw et al., 2019; Weaver et al., 2020). In *Tigriopus japonicus*, 52 CYPs belonging to five families (CYP20 is the addition) have been tentatively identified (Han et al., 2017); searching the annotated *T. californicus* genome revealed 46 potential CYPs. Bioinformatic work has pointed to several candidate ketolase enzymes responsible for the production of astaxanthin in *T. californicus* (Weaver et al., 2020), as well as a candidate hydroxylase in a calanoid copepod (Mojib et al., 2014). However, while some previous transcriptomic studies have successfully identified genes related to carotenoid metabolism (e.g. Ahi et al., 2020; Liu et al., 2015), these studies have generally relied on either population or tissue differences in coloration, perhaps suggesting constitutive rather than inducible differences in expression, unlike in our

study system. It is also possible that the candidate genes in question perform multiple functions (i.e. promiscuous enzymes) and so did not display a significant upregulation in response to dietary stimulus. For example, the gene identified as a carotenoid ketolase in spider mites and believed to be highly conserved across the Trombidiformes order of mites, CYP384A1 (Wybouw et al., 2019) had been previously identified as a key component of resistance to the pesticide fenpropathrin in a spider mite species of the same genus as the one in which CYP384A1 was identified as a ketolase (Shi et al., 2016). Furthermore, bioinformatic analysis by Weaver et al. (2020) suggests that *T. californicus* CYP2J3 and *T. japonicus* CYP3029A1 are CYP2-like hits. In addition to known variation by sex and lifestage in CYP3029A1, CYP3029A1 is significantly upregulated in response to the pollutant benzo[α]pyrene (Han et al., 2017). Overall, then, while we did not observe evidence in favor of any candidate carotenoid ketolases, the lack of differential expression in response to diet is not evidence against these candidates.

The variety of antioxidant compounds (carotenoids, vitamin E/tocopherols, and vitamin C/ascorbic acid) found in *Tetraselmis* and spirulina, but not in nutritional yeast, provides an opportunity to evaluate the interactions of exogenous dietary antioxidants and endogenous antioxidant enzymes in *T. californicus*. However, there was no discernible pattern of up- or downregulation: instead, *Tetraselmis*, which has the highest concentrations of all antioxidants measured, tended to appear at the extreme ends of the comparisons. Additionally, only one gene, an ascorbate peroxidase, was differentially expressed in non-*Tetraselmis* comparisons, although it was downregulated in the spirulina group, which is in keeping with previous results. There is substantial precedent

for decreases in the activity of antioxidant enzymes such as superoxide dismutase, glutathione peroxidase, and catalase with dietary supplementation of vitamin C (e.g. Selman et al., 2006), vitamin E (e.g. Li et al., 2018), and carotenoids (e.g. Palozza et al., 2000; Babin et al., 2015) although transcription of the same enzymes has been observed to increase with astaxanthin supplementation in shrimp (Wang et al., 2020). Previous work in *T. californicus* has also suggested that some antioxidant-related genes (heat shock protein, 2 oxidoreductases and 2 glutathione S-transferases) are upregulated in yeast-fed adult males relative to spirulina and TetraMin-fed (Li et al., 2019). The pattern of decreasing antioxidant enzyme activity or expression with increasing dietary antioxidants is explained by the fact that it is only necessary to achieve balance between antioxidant capacity and reactive oxygen species (ROS) to prevent oxidative stress (Monaghan et al., 2009). Production of antioxidant enzymes with sufficient dietary antioxidants may be an unnecessary expenditure of energy. HSPs and proteases were also evaluated due to their known upregulation during oxidative stress in *T. californicus* (Li et al., 2020; Kim et al., 2014; Foley et al., 2020) due to their roles in refolding or recycling damaged proteins, and showed greater numbers of differentially expressed genes in both the *Tetraselmis* and non-*Tetraselmis* comparisons. Proteases were not exclusively up- or downregulated in any group, but *Tetraselmis* and spirulina-fed animals did tend to appear at opposite extremes, suggesting shifts that could be influenced by multiple factors (see below).

The consistent upregulation of myosins in spirulina-fed copepods was an unexpected but potentially intriguing result. Several previous studies have shown spirulina in the diet to increase protein synthesis in farmed red sea bream (Mustafa et

al., 1993), carp (Nandeesha et al., 1998), and rats (Volaterelli & de Mello, 2008), as well as to increase myosin content (Volaterelli & de Mello, 2008) and decrease intestinal protease activity, which was attributed to improved protein digestibility (Nandeesha et al., 1998). More specifically, despite the roughly equivalent protein:carbohydrate ratios in nutritional yeast and spirulina, the differences in percentages of amino acids in the two diets could trigger the insulin/Igf-like signalling (IIS) and the Target of Rapamycin (TOR) pathway (Voltarelli & de Mello, 2008), the lengthy set of genes that are involved in cascades in response to nutrient availability (Camus et al., 2019). This could in turn upregulate muscle protein synthesis/myosin transcription (Voltarelli & de Mello, 2008). Protein content or composition may also contribute to the differential expression seen in proteases and protease inhibitors, as spirulina has substantially higher protein content than *Tetraselmis.*

Additionally, nine neuropeptides and neural signaling genes were differentially expressed. The largest number of genes in this category tended to be differentially expressed between the spirulina and yeast groups, with the 16 hour exposure group matching either spirulina or yeast expression levels. Transcript levels of genes relating to neurological processes are known to correspond to diet and nutrient availability, as they are also required to regulate the response (Camus et al., 2019). Overall, both the upregulation of myosins in spirulina-fed copepods and the shifts in neuropeptides may be related to the highly conserved ways that organisms sense, assimilate, and respond to nutrient changes.

In addition to the typical limitation of RNA-seq, which is that mRNA transcript levels do not always correspond to protein levels for a variety of reasons (some of

which are discussed above), there are several limitations of our particular dataset relating to RNA quality. Mapping percentages were relatively low for pooled RNA-seq in *T. californicus*, ranging from 34.6 to 49.16% reads not mapping to the reference genome in non-*Tetraselmis* samples. The *Tetraselmis* samples, which were the most degraded, had 50.69 to 55.36% reads not mapping. The relatively high percentages of reads not mapping are at least partially attributable to contamination caused by the short one hour gut clearance; however, this was expected, as we hoped to ensure that we captured the response to the diet as it happened rather than a hunger response. Both the mapping percentages and potential contamination are not unknown in transcriptomic studies of *T. californicus* (Li et al., 2019).

However, previous extrapolation of sequencing depth guidelines for the human transcriptome from the ENCODE Project to *T. californicus* transcriptome sizes suggests that approximately 2 million mapped reads per sample are required for adequate depth, on average (Li et al., 2019). All sample groups except *Tetraselmis* exceed this requirement.

APPENDIX

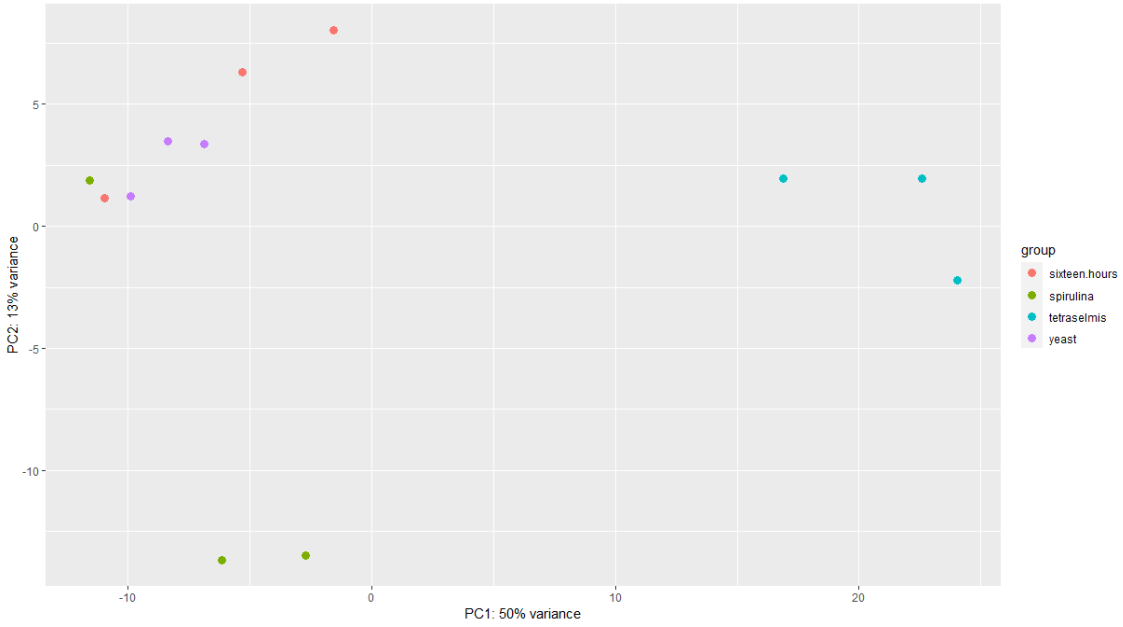

Figure 1: Principal component analysis (PCA) including all samples and treatments. PC1 explains 50% of variance, and separates the *Tetraselmis*-fed samples from all other treatments, while PC2 explains 13% of variance and partially separates the spirulina samples from the yeast-fed and 16 hour exposure samples.

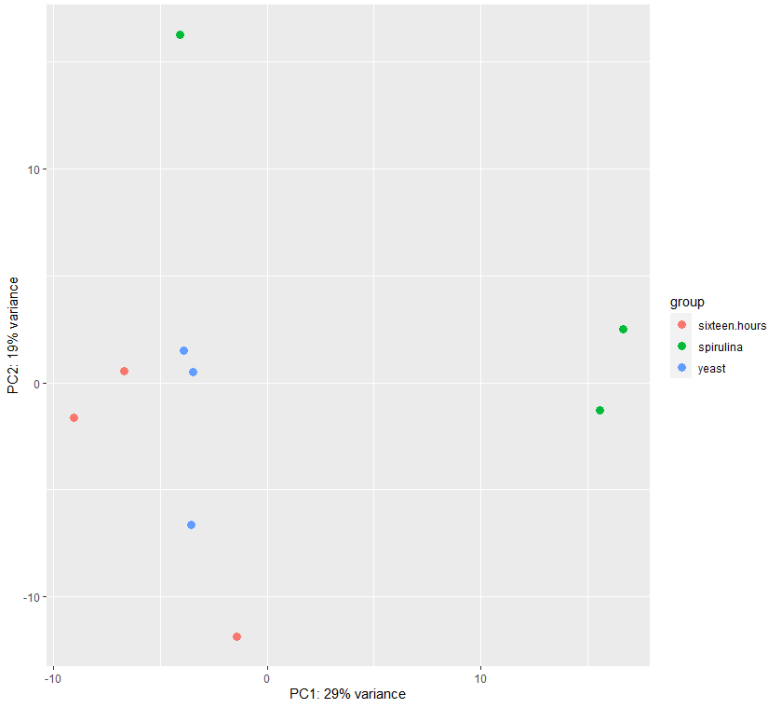

Figure 2: PCA excluding the *Tetraselmis* samples. PC1 explains 29% of variance and separates two of three spirulina samples from the yeast and 16 hour exposure samples, while PC2 explains 19% of variance and allows the last spirulina sample to be separated from the other samples.

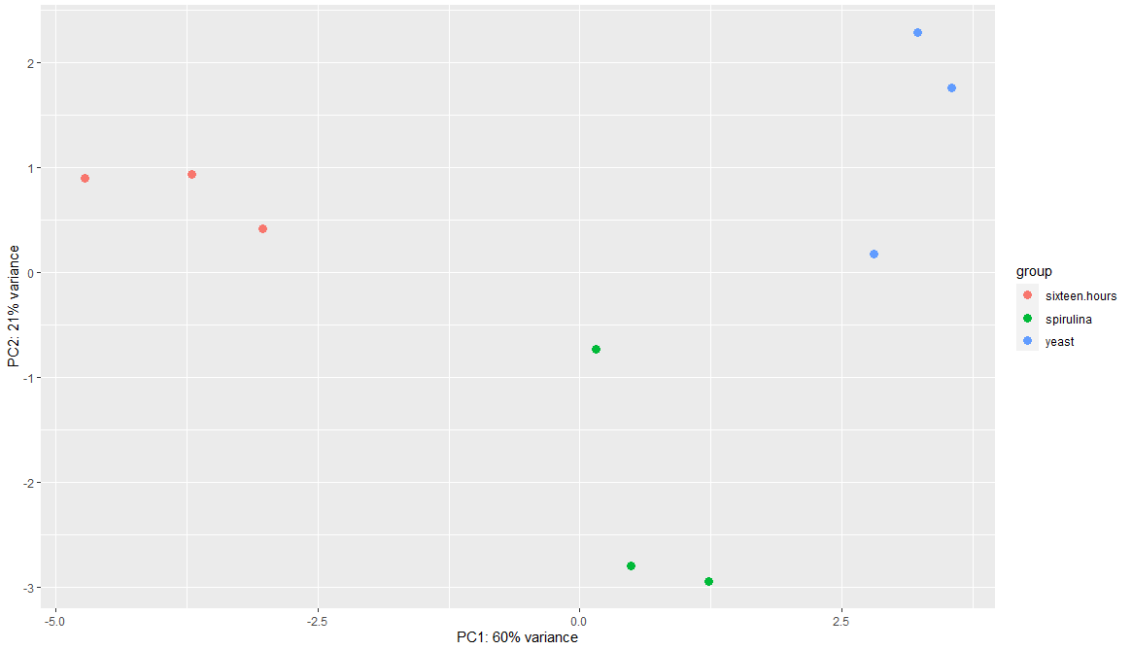

Figure 3: PCA including only differentially expressed genes in the model of all three non-*Tetraselmis* samples (n=40). The dietary treatments clearly cluster together; however, sample size is small here.

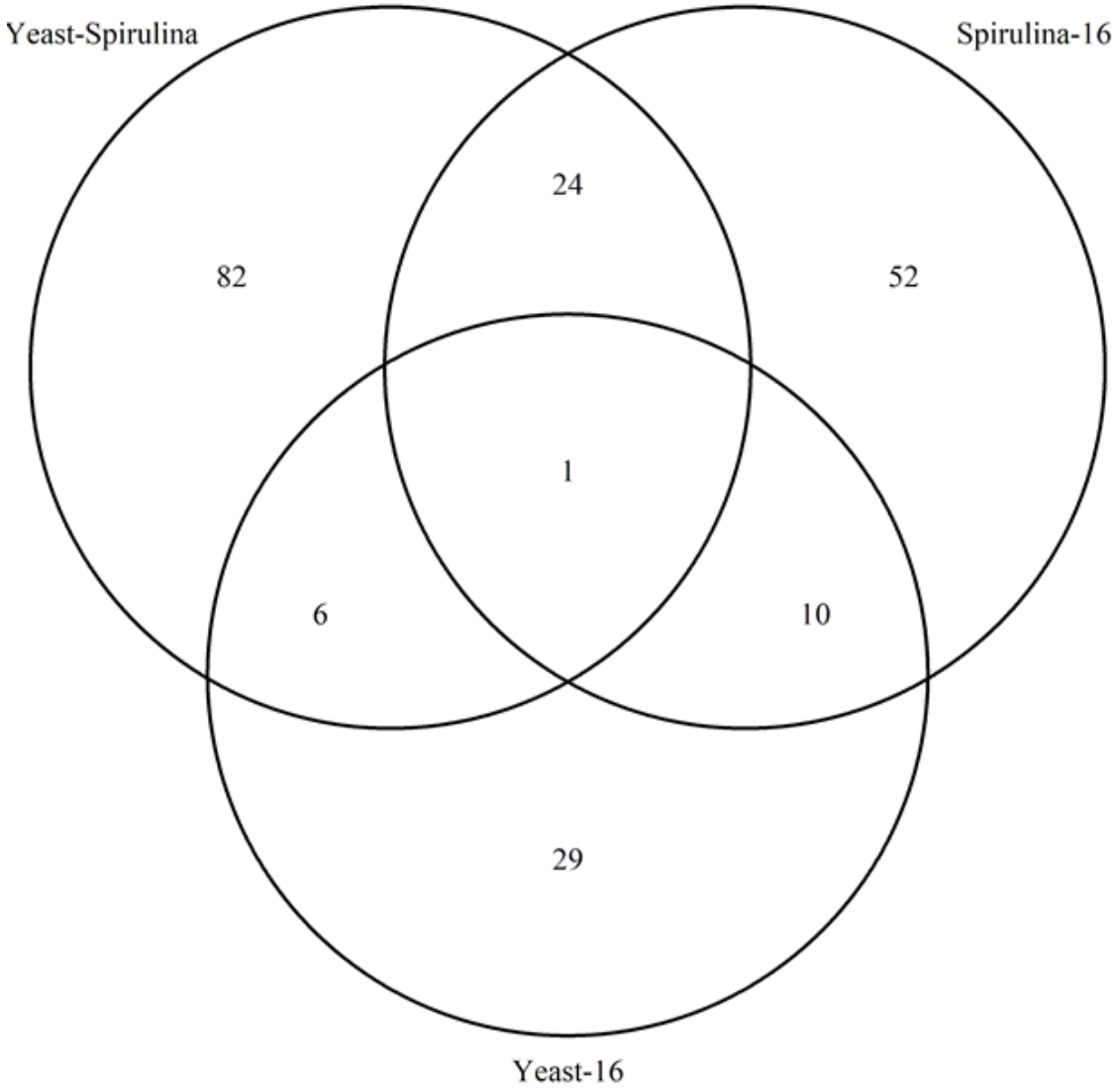

Figure 4: Comparison of differentially expressed gene IDs between three pairwise comparisons. The only gene that showed significant differences in each comparison was a putative thyroid peroxidase (TCALIF_10485), which was most highly expressed in spirulina-fed samples, followed by yeast, followed by 16 hour exposure samples. (*Tetraselmis* samples were indistinguishable from 16 hour exposure samples).

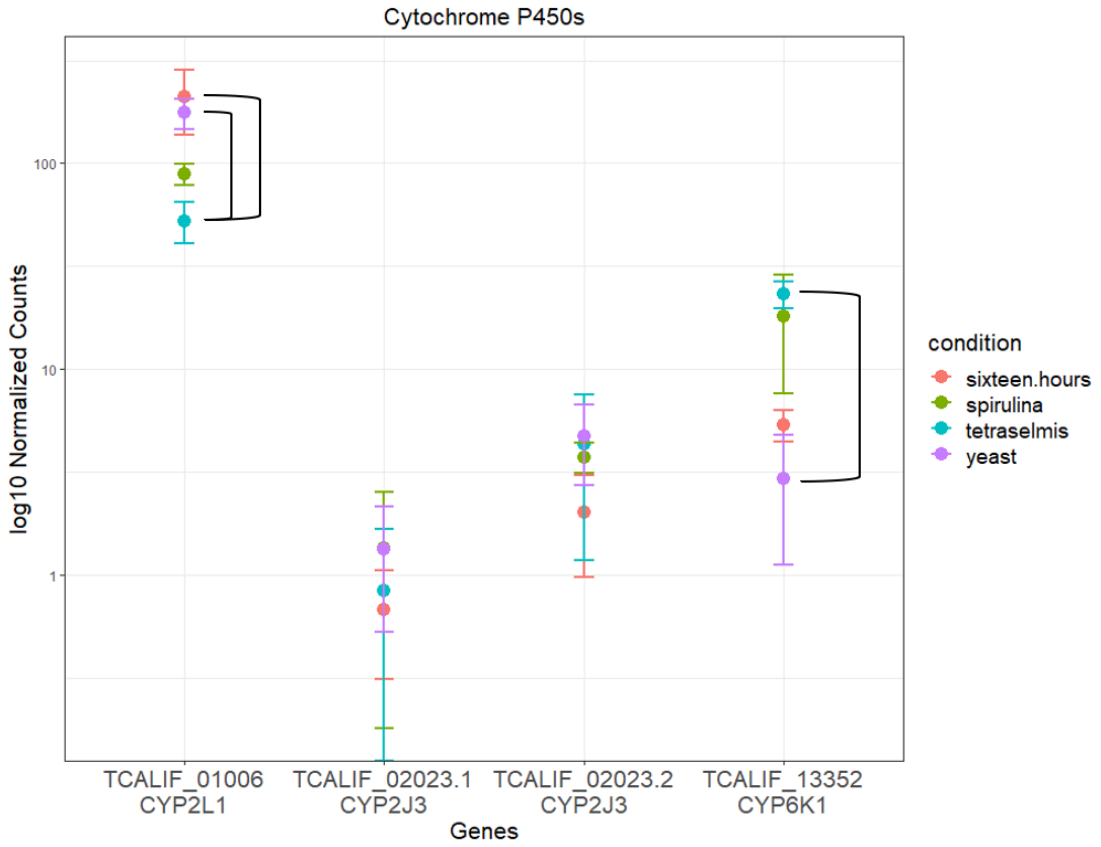

Figure 5: Expression levels of selected cytochrome P450s of interest. Searching all 46 likely cytochrome P450 (CYP450) enzymes in the *T. californicus* genome revealed differential expression in only two, CYP2L1 Cytochrome P450 2L1 (TCALIF_01006) and CYP6K1 Cytochrome P450 6k1 (TCALIF_13352), which only showed differential expression in pairwise comparisons with the problematic *Tetraselmis* samples. The potential carotenoid ketolase candidate from Weaver et al. (2020) is CYP2J3, and did not show differential expression in our analysis. Points represent the mean of a set of samples and error bars are standard error. Significant comparisons (adjusted $p < 0.01$) between a pair of conditions are indicated by brackets.

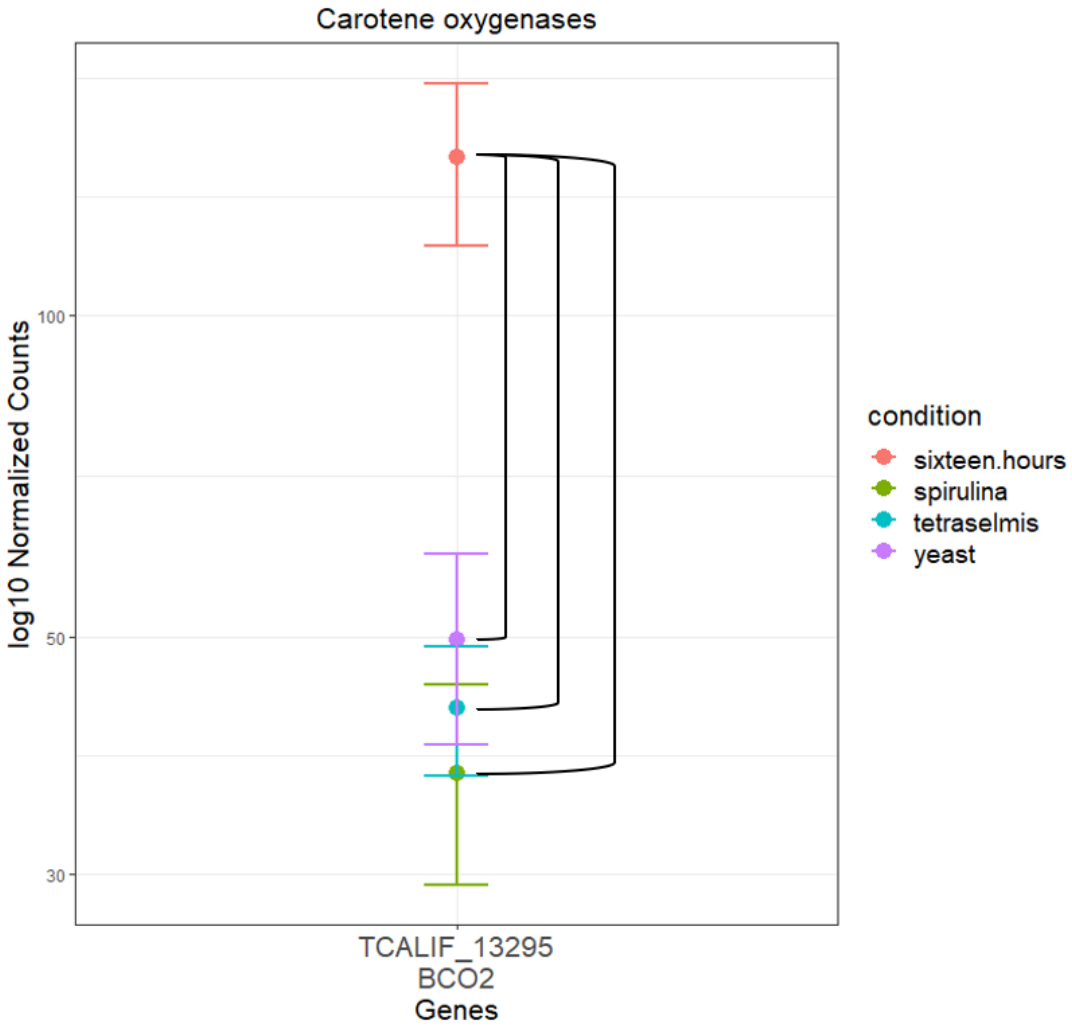

Figure 6: Expression levels of one beta-carotene oxygenase. BCO2, which is a beta,beta-carotene 9',10'-oxygenase (TCALIF_13295) shows strong differential expression in pairwise comparisons between the 16 hour exposure group and all other groups. The other three genes annotated as beta-carotene oxygenases were all BCO1 homologs, beta,beta-carotene 15,15'-dioxygenases, and did not show differential expression. Points represent the mean of a set of samples and error bars are standard error. Significant comparisons (adjusted $p < 0.01$) between a pair of conditions are indicated by brackets.

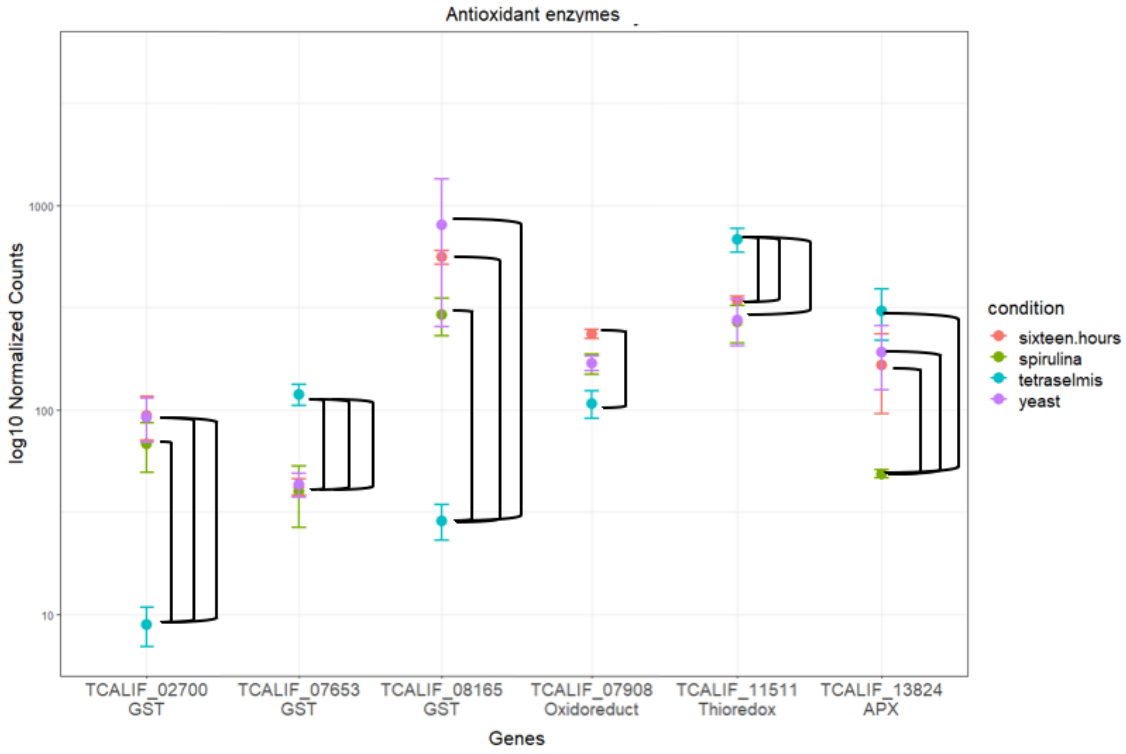

Figure 7: Expression levels of putative antioxidant enzymes. Three glutathione S-transferases, an oxidoreductase, a thioredoxin, and an ascorbate peroxidase were differentially expressed. Lifetime *Tetraselmis*-fed animals consistently appear at the extremes, with either the most upregulated or the most downregulated expression of antioxidant enzymes. Points represent the mean of a set of samples and error bars are standard error. Significant comparisons (adjusted $p < 0.01$) between a pair of conditions are indicated by brackets.

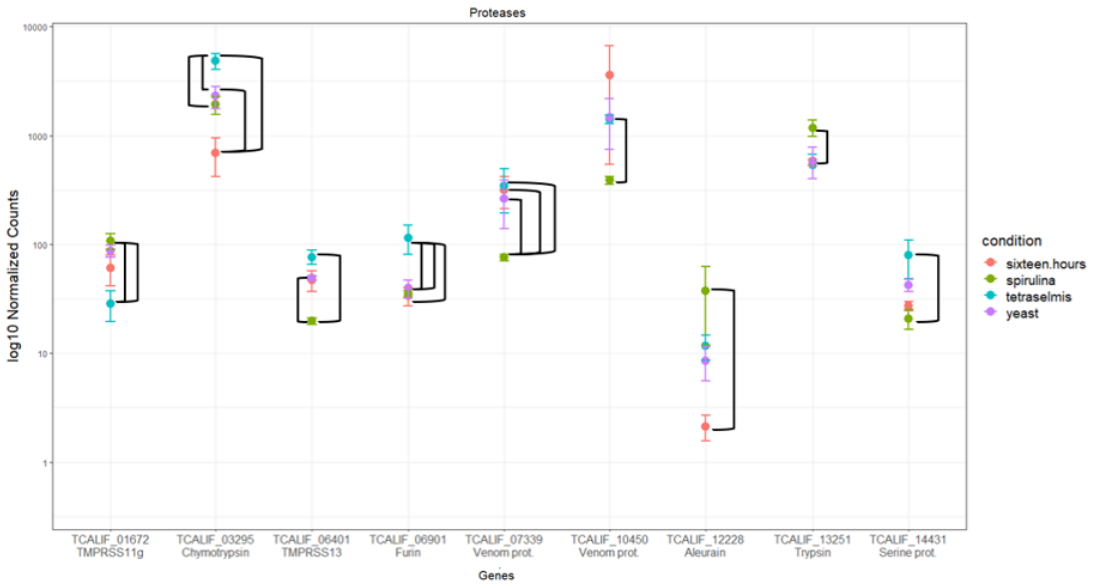

Figure 8: Expression levels of proteases. Nine proteases were differentially expressed in at least one pairwise comparison. Points represent the mean of a set of samples and error bars are standard error. Significant comparisons (adjusted $p < 0.01$) between a pair of conditions are indicated by brackets.

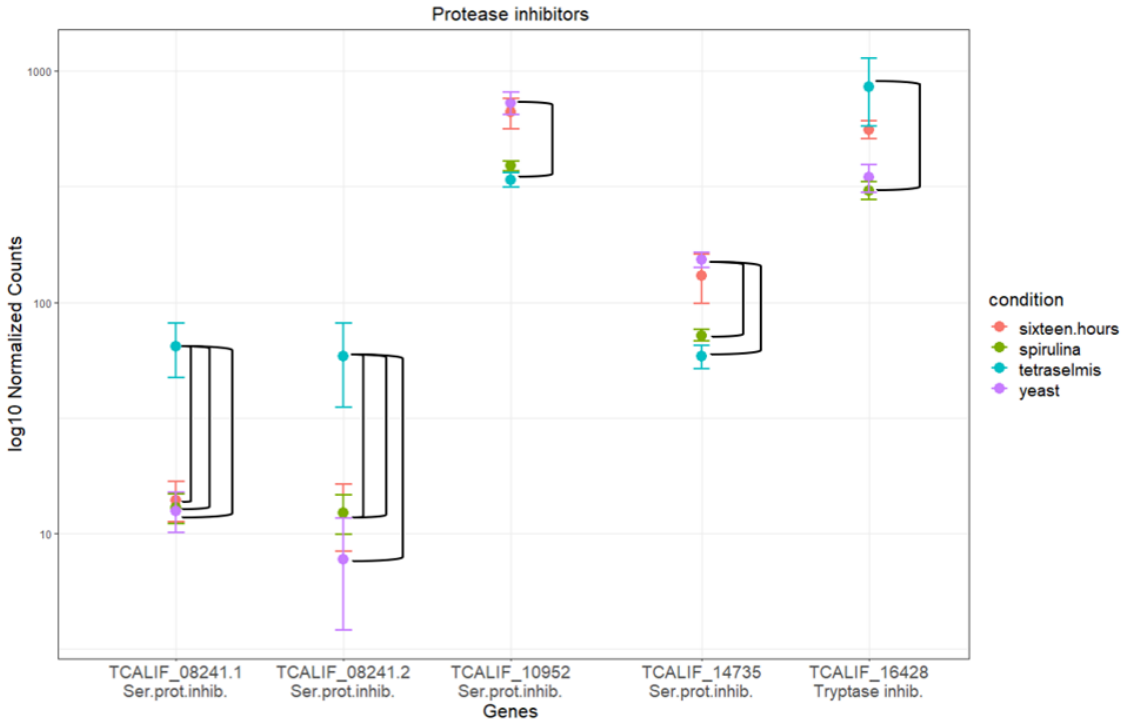

Figure 9: Expression levels of protease inhibitors. Five protease inhibitors were differentially expressed in at least one pairwise comparison. Points represent the mean of a set of samples and error bars are standard error. Significant comparisons (adjusted $p < 0.01$) between a pair of conditions are indicated by brackets.

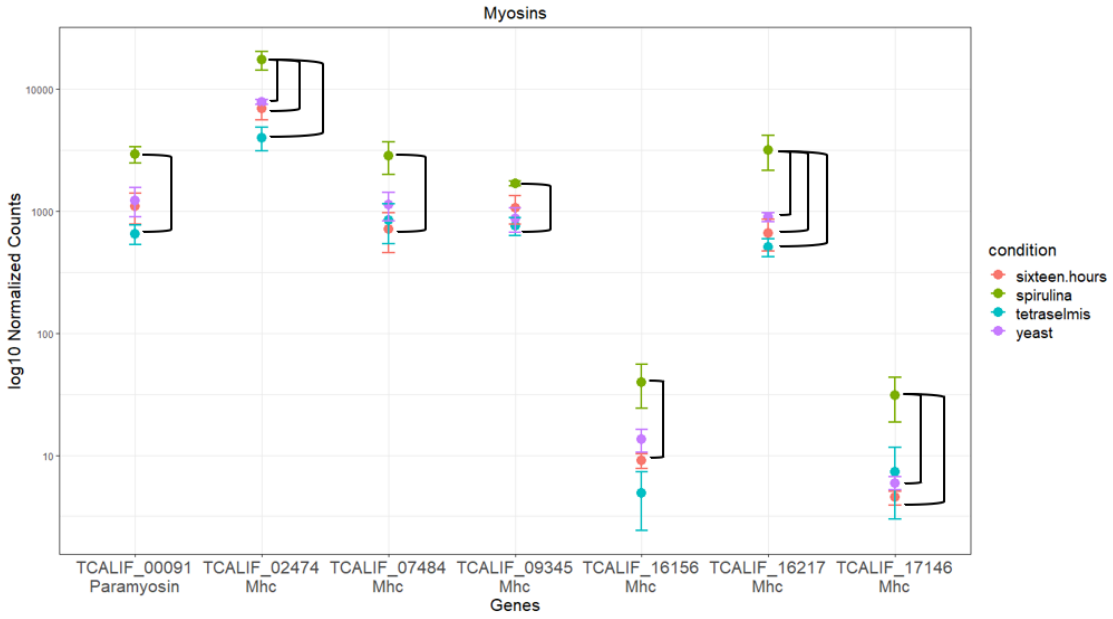

Figure 10: Expression levels of thick filament proteins: myosins and paramyosin. Six myosin heavy chain (Mhc) genes and one paramyosin were upregulated in spirulina-fed groups. Points represent the mean of a set of samples and error bars are standard error. Significant comparisons (adjusted $p < 0.01$) between a pair of conditions are indicated by brackets.

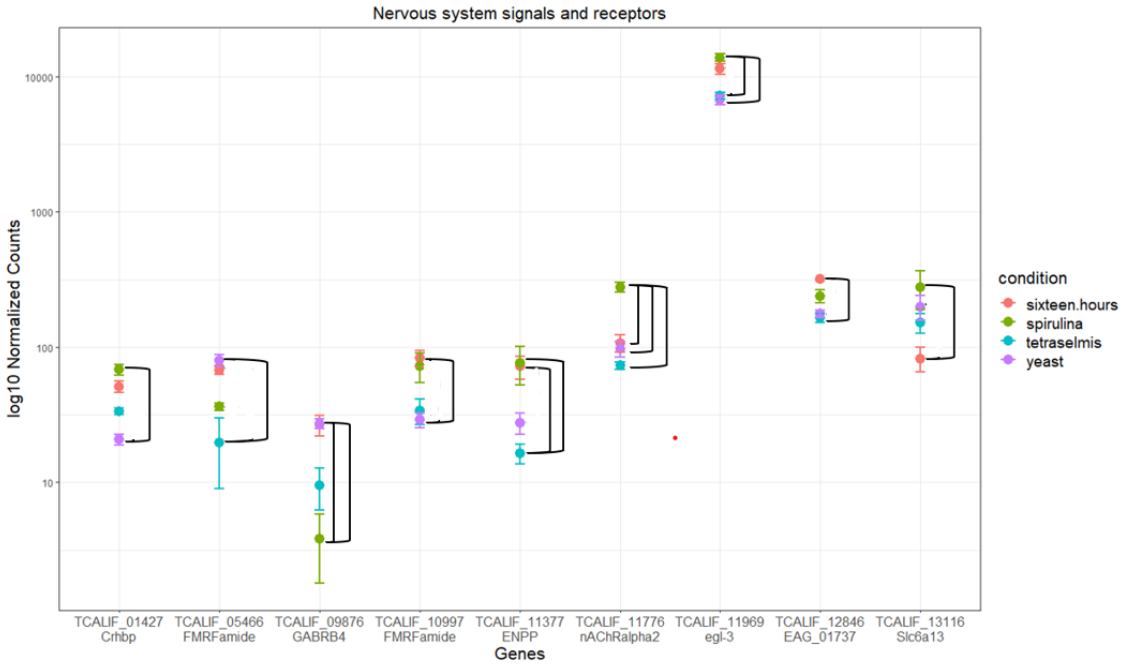

Figure 11: Expression levels of potential neural signaling-related genes. Nine neuropeptide or neural signaling genes were differentially expressed in the comparison groups. Abbreviated annotations stand for similarity to corticotropin-releasing factor-binding protein, FMRFamide receptor, gamma-aminobutyric acid receptor subunit beta-4, FMRFamide-related peptides type HF-4, enterin neuropeptides, acetylcholine receptor subunit alpha-like 2, egl-3 neuroendocrine convertase 2, EAG_01737 IDLSRF-like peptide, and Sodium- and chloride-dependent GABA transporter 2, respectively. Points represent the mean of a set of samples and error bars are standard error. Significant comparisons (adjusted $p < 0.01$) between a pair of conditions are indicated by brackets.

Table 1: RNA library sizes.

Sample name	**Pre-mapping reads**	**Mapped reads**
161	28008018	4580488
162	21087311	3025777
163	15600383	1817134
Spir1	17104237	1724554
Spir2	19649365	5265042
Spir3	19463611	5736028
Tetra1	17465681	612108
Tetra2	16465299	561184
Tetra3	19469615	864165
Yeast1	18572673	2398563
Yeast2	21871348	3895550
Yeast3	20362482	3220491

Table 2: Summary of differential expression in pairwise comparisons. For ease of comparison across groups, for all comparisons involving Tetraselmis, the reference level is the other condition, so that upregulation or downregulation is of Tetraselmis. For the yeast-16 hour and spirulina-16 hour comparisons, the reference levels are yeast and spirulina, so that downregulation or upregulation are of the 16 hour group relative to the others. Finally, in the yeast-spirulina comparison, spirulina is the reference level, so that downregulation or upregulation are of yeast relative to spirulina. Nonzero read count is the total number of genes tested in a comparison, as the read count must be greater than zero. Log fold change (LFC) equal to zero was the null hypothesis in our analysis, and so LFC > 0 and LFC < 0 are the numbers and percentages of genes up- or downregulated significantly (adjusted p-value < 0.01) in our analysis. Outliers are determined in DESeq2 by Cook's distance and are excluded from analysis. DESeq2 by default performs pre-filtering on genes by optimizing the number of genes with significant adjusted p-values and excluding genes with mean normalized counts below a threshold based on that number. The number and percentage of low count genes and the mean count cutoff are included in the table.

	Tetra-yeast	Tetra-spir	Tetra-16	Yeast-spir	Yeast-16	Spir-16
Nonzero read count	14574	14598	14511	14742	14690	14700
LFC > 0 (up)	286, 2%	185, 1.3%	180, 1.2%	26, 0.18%	12, 0.082%	30, 0.2%
LFC < 0 (down)	246, 1.7%	315, 2.2%	245, 1.7%	15, 0.1%	9, 0.061%	17, 0.12%
Outliers	7, 0.048%	15, 0.1%	4, 0.028%	17, 0.12%	10, 0.068%	15, 0.1%
Low count	4222, 29%	4789, 33%	3923, 27%	2564, 17%	567, 3.9%	3408, 23%
Mean count <	8	11	6	5	0	10

Table S1: Contents of Bragg Nutritional Yeast (Bragg, Santa Barbara, CA, US). Calculated using the nutrition facts label. “Not a significant source of vitamin A, vitamin C, or calcium”, or vitamin E/tocopherols.

“Serving size”	**5g (1 tablespoon)**	**Per 100g**
Kilocalories	20	400
Total fat (g)	0	0 (in theory)
Sodium (mg)	0	0
Total carbohydrate (g)	2	40
Dietary fiber (included in total carbohydrate) (g)	1	20
Protein (g)	3	60
Iron (mg)	0.36	7.2
Riboflavin (B2) (mg)	2.72	54.4
Pyridoxine (B6) ()	2.8	56
Vitamin B12 ()	2.4	48
Zinc (mg)	0.9	18
Thiamine (B1) (mg)	2.7	54
Niacin (B3) (mg)	14	280
Folic Acid ()	160	3200
Pantothenic Acid (mg)	3	60
Selenium ()	7	140

Table S2: Contents of Tetraselmis species. The species used in this analysis was Tetraselmis chuii; three additional Tetraselmis strains are included in this table and two additional strains are included in Table S3 below to provide potential estimates for unrecorded values. However, the high variability among strains should be noted.

	Tetraselmis sp. CTP4	**Tetraselmis chuii**	**Tetraselmis suecica**	**Tetraselmis sp. CS-362**
Serving size	100 g	100 g	100 g	
Kilocalories	297 ± 12	n.r.	n.r.	
Total fat Lipids (g)	7.04 ± 0.42	5–8	8.0	
Sodium (g)	1.18			
Total carbohydrate (here from digestible + fiber) (g)	~42.68	~32-35	~25.8	
Digestible carbohydrates (g)	18.08 ± 4.18	30–32	22.4	
Dietary fiber (included in total carbohydrate) (g)	24.60 ± 3.85	2–3	3.4	
Protein (g)	31.20 ± 0.48	35–40	48.7	
Iron (mg)	32.3	173.4		
Riboflavin (B2) (mg)	0.53			2.6
Pyridoxine (B6) (mg)	6.9			0.6
Vitamin B12 ()	7.8			195
Zinc (mg)	2.9	6.4		

Table S2: Contents of Tetraselmis species, continued.

	Tetraselmis sp. CTP4	**Tetraselmis chuii**	**Tetraselmis suecica**	**Tetraselmis sp. CS-362**
Thiamine (B1) (mg)	0.18			10.9
Niacin (B3) (mg)	7.98			n.r.
Folic Acid ()	0.02			2000
Pantothenic Acid (mg)	0.65			n.r.
Selenium	“Although low amounts of selenium and iodine were detected, it should be noted that both elements were not included in the industrial culture medium used for growth.”			
A—Retinol (µg/100 g)	<4			220
B7—Biotin (mg/100 g)	n.d.			0.13
C—Ascorbic acid (mg/100 g)	79.2			300
E—Tocopherol (mg/100 g)	20.28			7

Table S3: Pigment contents of Tetraselmis species. Information on Tetraselmis combined from Pereira et al. (2019). (n.r.: not recorded, n.d.: not detected).

Pigments (mg/100 g)	**Tetraselmis sp. CTP4**	**Tetraselmis chuii**	**Tetraselmis sp. M8**
Chlorophyll a and b	3531.2 ± 152.1	n.r.	n.r.
Violaxanthin	130.8 ± 5.7	54.6	22.9
Antheraxanthin	n.d.	20.1	12.6
Neoxanthin	236.4 ± 11.9	n.d.	n.d.
Zeaxanthin	10.8 ± 1.3	n.d.	n.d.
Lutein	225.6 ± 8.5	62.4	66.5
α-carotene	n.d.	17.4	3.0
β-carotene	8.4 ± 0.7	94.1	105.7

Table S4: Spirulina nutritional breakdown. Total carotenoids 300 mg/100 g. Information on spirulina obtained from the Jade-brand spirulina container used in feeding.

Protein	55% min.
Fat	4% min.
Fiber	7% max
Ash	7% max
Moisture	7% max

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