Chemical manipulation of mitochondrial function affects metabolism of red carotenoids in a marine copepod *(Tigriopus californicus)*

Powers, Matthew J.[1,*], Baty, Alex[1], Dinga, Alexis M.[1], Mao, James H.[1], Hill, Geoffrey E.[1]

[1]Auburn University, Department of Biological Sciences, Auburn, AL, USA

*Corresponding author: Email: [1]mjp0044@auburn.edu

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Summary Statement

Copepods treated with the mitochondria-targeted chemical 2-4-dinitrophenol show an increase in respiration rate and ketolation of astaxanthin. This suggests a potential connection between mitochondrial function and carotenoid metabolism.

Abstract

The Shared-Pathway Hypothesis offers a cellular explanation for the connection between ketocarotenoid pigmentation and individual quality. Under this hypothesis, ketocarotenoid metabolism shares cellular pathways with mitochondrial oxidative phosphorylation such that red carotenoid-based coloration is inextricably linked mitochondrial function. To test this hypothesis, we exposed *Tigriopus californicus* copepods to a mitochondrially-targeted protonophore, 2-4-dinitrophenol (DNP), to induce proton leak in the inner mitochondrial membranes. We then measured whole-animal metabolic rate and ketocarotenoid accumulation. As observed in prior studies of vertebrates, we observed that DNP treatment of copepods significantly increased

respiration and that DNP-treated copepods accumulated more ketocarotenoid than control animals. Moreover, we observed a relationship between ketocarotenoid concentration and metabolic rate, and this association was strongest in DNP-treated copepods. These data support the hypothesis that ketocarotenoid and mitochondrial metabolism are biochemically intertwined. Moreover, these results corroborate observations in vertebrates, perhaps suggesting a fundamental connection between ketocarotenoid pigmentation and mitochondrial function that should be explored further.

Introduction

Ornamental traits can provide reliable information about the condition or quality of an organism (Bonduriansky, 2007; Hill, 2011; Rowe and Houle, 1996). The mechanism by which such honest signaling is achieved remains a focus of debate (Higham, 2014; Searcy and Nowicki, 2010; Smith and Harper, 2003). The Shared-Pathway Hypothesis proposes that some ornaments act as condition-dependent signals of quality because their production is intimately tied to the same vital cellular processes that give rise to individual performance, regardless of the influence of sexual selection (Hill, 2011; Hill, 2014a; Hill, 2014b; Powers and Hill, 2021; Weaver et al., 2017). The hypothesized consequence of such a link is that condition-dependent traits serve as indices of individual quality, such that expression of the ornamental trait reflects the function of internal processes required for the maintenance of life (Biernaskie et al., 2014; Hill, 2011). Specifically, the dynamics of the mitochondrial organelle, a critical, cellular hub of organismal function and condition (Havird et al., 2019; Heine and Hood, 2020; Picard and Sandi, 2020), has been identified as a candidate regulator in the expression of condition-dependent traits (Hill, 2014a; Hill, 2014b; Koch et al., 2017).

A primary and well-studied example of a condition-dependent ornamental trait is bright red coloration that arises from the accumulation of carotenoid pigments in animal tissues (Blount and McGraw, 2008; Hill, 2002; Svensson and Wong, 2011). With few exceptions, animals cannot synthesize carotenoid pigments *de novo*; they must acquire carotenoids through their diet in a trophic manner (Svensson and Wong, 2011). Further, once ingested, many animals metabolize dietary carotenoids into other carotenoid forms in order to produce pigments with new functional roles or new colors (Maoka, 2011; Von Lintig, 2010). This process can occur

through a series of oxidation reactions, such as hydroxylations and/or ketolations, that may cause a color change from the yellow hues of dietary carotenoids to red hues of metabolized carotenoids (Meléndez-Martínez et al., 2007). For example, many crustaceans convert yellow dietary xanthophylls found in algae to red ketolated carotenoids (ketocarotenoids) like astaxanthin. Many animals display astaxanthin-based coloration including birds (Hill and McGraw, 2006), insects (Kayser, 1982), reptiles (Czeczuga, 1980), amphibians (Baruah et al., 2012), fish (Seabra and Pedrosa, 2010), and crustaceans (Maoka, 2011). Astaxanthin is also often recognized as a potent antioxidant and potential immune system regulator (Dose et al., 2016; Naguib, 2000; Weaver et al., 2018b).

The Shared-Pathway Hypothesis, as it applies to ketocarotenoid-based coloration, posits that ketocarotenoid accumulation is intrinsically linked to individual condition through metabolic pathways shared with oxidative phosphorylation (OXPHOS) in the mitochondria (Hill, 2014a; Hill, 2014b; Koch et al., 2017; Powers and Hill, 2021). Under this hypothesis, the bioconversion of red ketocarotenoid pigments is hypothesized to share a biochemical environment with the enzyme complexes in the electron transport system (Hill et al., 2019; Powers and Hill, 2021). These complexes are positioned within the inner mitochondria membrane (IMM) and produce a proton gradient across the membrane needed for the synthesis of ATP. This activity requires a series of reduction-oxidation (redox) reactions driven by coupled electron donors and acceptors, such as NADH/NAD+. The enzymes responsible for the production of ketocarotenoids also metabolize dietary carotenoids using coupled redox reactions, likely utilizing similar electron donor/acceptor systems (Hill and Johnson, 2012; Lopes et al., 2016; Munro et al., 2007; Twyman et al., 2018). Indeed, ketocarotenoids have been observed at the inner mitochondrial membrane in red birds and molecular modeling of the avian carotenoid bioconversion enzyme, CYP2J19, suggests it may localize either in the IMM or mitochondria-associated membranes (MAM) (Ge et al., 2015; Hill et al., 2019). Moreover, positive relationships between resting metabolic rate, mitochondrial damage, lower rates of mitochondrial replacement, and ketocarotenoid-based coloration have been observed in red birds, suggesting a potential for ketocarotenoid coloration to reflect metabolic capacity in the face of ongoing cellular stress (Hill et al., 2019). Observations in arthropod systems corroborate these observations in vertebrate systems. In the marine copepod *Tigriopus californicus* (Baker, 1912), interpopulation hybrids show a significant positive relationship between oxygen consumption and ketocarotenoid accumulation (Powers

and Hill, 2021) and a significant negative relationship between ATP production and ketocarotenoid coloration (Powers et al., 2021), thus indicating that ketocarotenoid accumulation may reliably indicate the capacity to withstand stress and exert energy in invertebrate species as well.

To test the Shared-Pathway Hypothesis, we experimentally exposed *T. californicus* copepods to a mitochondrially targeted compound, 2-4-dinitrophenol (DNP) and measured their accumulation of ketocarotenoid pigmentation. This copepod, native to splash-pools along the west coast of North America, accumulates astaxanthin to produce its vibrant, red coloration, using many of the same carotenoid substrates preferred by red birds and other terrestrial organisms (Prado-Cabrero et al., 2020; Weaver et al., 2018a). Moreover, *T. californicus* ketocarotenoid-based coloration has also been shown to be dependent on individual condition (Powers et al., 2021; Weaver et al., 2016; Weaver et al., 2018b). In birds, mitochondria-targeted compounds, such as synthetic ubiquinones and superoxide dismutase mimetics, have been observed to alter ketocarotenoid metabolism (Cantarero and Alonso-Alvarez, 2017; Cantarero et al., 2020a; Cantarero et al., 2020b). However, these compounds may also increase ROS levels as they alter oxidative state in the mitochondria, and this may, in turn, influence ketocarotenoid levels given their possible role in ROS scavenging (Dose et al., 2016; Naguib, 2000). In contrast, the ionophore DNP does not incur this complication (Goldgof et al., 2014; Korde et al., 2005; Leverve et al., 1998; Lozinsky et al., 2013; Rognstad and Katz, 1969; Stier et al., 2014). This compound functions as a mitochondrial "uncoupler", acting as a protonophore in the IMM of living mitochondria to dissipate the proton gradient required to make ATP (Fig. 1A) (Pinchot, 1967). At sublethal, acute doses, DNP, therefore, increases the rate of respiration in animals, including crustaceans (Caldeira da Silva et al., 2008; Cantelmo et al., 1978; Geisler et al., 2017). We predicted that, if carotenoid metabolizing enzymes share an oxidative environment with mitochondrial OXPHOS enzymes, DNP should simultaneously increase respiration rate and ketocarotenoid metabolism in *T. californicus* copepods. We tested this prediction using copepods with natural levels of astaxanthin and copepods deficient of carotenoids to test their ability to restore pigmentation from a colorless state (Fig 1B).

Materials and Methods

Copepod Culture Conditions

Tigriopus californicus cultures were maintained in a climate-controlled incubator under the following conditions: 32 psu artificial seawater (ASW) at 20 ºC on a 12:12h light: dark cycle and fed live microalgae (*Tetraselmis chuii*). The cultures used in this experiment were maintained for approximately three years as continuous cultures. Cultures were a mix of commercially obtained copepods (Reef Nutrition, Campbell, CA, USA) and admixed wild populations collected from San Diego, Bird Rock, Abalone Cove, Santa Cruz, and Bodega Bay, California. The cultures used in this experiment were admixed once a month (approx. once per generation) via transfer of water and individuals amongst all culture vessels in attempt to maintain homogeneity among culture vessels. However, we only sampled from two specific culture vessels as a source of stock copepods and one vessel as a source of colorless copepods, in an attempt to reduce variability among copepods from different sources. Regardless, the heterogeneity in the sourcing of copepods that make up our stock cultures could contribute to variability in results. Lab-reared copepods fed *Tetraselmis* retain their red coloration similar to wild populations (Davenport et al., 2004; Weaver et al., 2018a) and the *Tetraselmis* diet approximates the algae-based diet found in natural splash-pools (Powlik et al., 1997; Vittor, 1971). *Tetraselmis* algae provides dietary carotenoid precursors which *T. californicus* metabolize to synthesize astaxanthin, specifically β-carotene and hydroxyechinenone (Ahmed et al., 2014; Powers et al., 2021; Prado-Cabrero et al., 2020; Weaver et al., 2018a). We refer to copepods cultured under these conditions as “red stock copepods”.

“Colorless” *T. californicus* cultures were established by isolating gravid female copepods from the stock conditions described above and gently removing their eggs. Eggs were placed in a separate container containing only clean artificial saltwater and after hatch, were reared on ground nutritional yeast powder, but no algae (and thus, no dietary carotenoids). These nearly clear copepod cultures were kept in the dark 24 hours of the day to minimize the chance of algae growth in the culture containers. As these copepods did not have access to dietary carotenoids, they remained almost entirely clear in color throughout their entire development (Powers et al., 2020; Powers et al., 2021; Weaver et al., 2018a).

2,4-Dinitrophenol Dosages and Lethality Assay

Before experimentation, we performed survival assays to identify a maximum concentration of DNP (TCI, Portland, OR, USA) that did not produce lethality. DNP was dissolved in 32 psu ASW to make the following stock concentrations: 100µM, 50µM, 25µM, 10µM, 2µM, 1µM, 0.5µM, and 0µM (control). To assay survival at each of these concentrations, we placed five copepods in a single well of a 24-well plate with 50µL of concentrated *Tetraselmis* algae and monitored for individual death every day for four days. This was repeated five times each for each concentration, for a total of 25 copepods per DNP concentration. The highest concentration of DNP tested, 100uM, was more than 80% lethal after just 24 hours (Fig. S1). The next highest concentration, 50µM, reached 50% lethality after 48 hours (Fig. S1). 25µM DNP neared 65% lethality after four days exposure. 10µM DNP saw less than 15% lethality after four days, was comparable to the death observed in the control group (0µM, Fig. S1). The lowest concentrations of DNP tested, 2µM, 1µM and 0.5µM, all showed minimal or no lethality after four days (Fig. S1). This was less than the death observed in the control group (0µM DNP) after four days (Fig. S1). Based on these results, 10µM and 2µM DNP were chosen as the maximum acute dosages for the experiment. These two concentrations were chosen to maximize the effect of DNP on respiration over a short period of time, without reaching 50% lethality during experimental exposure. Thus, the lowest concentrations, 1uM and 0.5uM, and highest concentrations, 25µM and higher, were not tested in the subsequent respiration and carotenoid metabolism assays to minimize the risk of observing no effect on respiration and to minimize the risk of cytotoxicity, respectively.

2,4-Dinitrophenol Exposure and Carotenoid Metabolism Assay

We exposed adult copepods (three individuals per replicate) to either 0 (control), 2, or 10 µM DNP by placing them in 5 mL test solutions of ASW in six-well plates. Each replicate of three copepods was maintained through respiration and HPLC analyses (i.e., the three individuals were isolated as one unit). We separated adult copepods by sex (using morphology of forward antennules) due to observed differences in carotenoid ketolation in previous experiments (Powers et al., 2021) and maintained them under the standard rearing conditions described above for three or seven days (Table 1). At the beginning of the experiments, we added 50 µL of concentrated, live *Tetraselmis* algae as food and a source of dietary carotenoids (Table 1). This

serving of algae was *ad libitum*: at the end of the DNP exposure time, the water was still green with algal cells. We exposed red stock copepods to DNP under the following conditions: 10uM DNP for three days, 10uM DNP for seven days, and 2uM DNP for seven days. We first started with only 3-day exposure to the higher dose of DNP, 10μM, in order to minimize the risk of death. When this period of time did not result in elevated death compared to controls, we extended the exposure time to seven days to test whether oxygen consumption remained elevated at a slightly longer exposure period. We then exposed red stock copepods to 2μM DNP for the same week-long period in order to facilitate a comparison to colorless copepods exposed to DNP under the same regime (see further rationale below).

We exposed colorless copepods to DNP in a single concentration and exposure time, 2μM DNP for seven days, in order to test carotenoid metabolism from a colorless state during acute exposure to DNP. 2μM DNP was chosen over 10μM for the experiment with colorless copepods due to previous observations that yeast-fed, colorless copepods may be more susceptible to oxidative stress than red stock, algae fed copepods (Powers et al., 2021). The exposure time of seven days was chosen to ensure copepods had time to 1) respond to mitochondrial uncoupling and 2) metabolize enough astaxanthin to produce a strong signal during HPLC analyses of copepod body tissue. At a minimum, *T. californicus* needs 48 hours to produce detectible amounts of astaxanthin from a colorless state (Weaver et al., 2018a), and takes about two weeks to fully restore astaxanthin concentrations to that of red stock cultures when feeding on *Tetraselmis* algae (Davenport et al., 2004). At the end of DNP exposure, algae feeding, and carotenoid restoration, the colorless copepods regained visible red coloration; thus, we refer to these as "color-restored" in Figures displaying the carotenoid content of these copepods.

Whole Animal Respiration Rate

We measured whole animal oxygen consumption rates of control and DNP-exposed copepods using a 24-well microplate respirometer (Loligo Systems, Copenhagen, Denmark). Prior to respiration measurement, copepods were moved to a 6-well plate with clean ASW and allowed to clear their gut contents for a minimum of one hour. After this period, the three copepods from each replicate of the DNP-exposure experiment were added to separate 80 μL wells of the microrespirometer filled with 32 psu ASW. Copepods were left undisturbed in the

wells for 30 minutes prior to recording oxygen consumption. We then sealed the wells using a gel seal and weighted block. Oxygen consumption was recorded over 30 minutes after an initial burn-in period of 5 minutes. Throughout the measurement process, the temperature was 22°C. Final oxygen consumption was calculated as mmol oxygen consumed min^{-1} per mg of copepod body weight. This was calculated by calculating the slope of the raw oxygen values over time in minutes and subtracting the average slope of empty blank wells (minimum 3 per plate). Copepod body weight was measured using a precision microscale (accurate to 0.001 mg) following oxygen consumption. Prior to weighing, the copepods were dried under air flow in a fume hood for 30 minutes to obtain dry body weight. The copepods were then placed in microtubes and frozen in a -80C freezer until HPLC analysis.

HPLC Analysis of Carotenoids

Carotenoids were extracted from dried copepod tissues using sonication in acetone. We extracted from the three copepods per replicate as a single pooled sample. Centrifugation was used to pellet and remove cellular debris, saving the carotenoid extract suspended in acetone. The carotenoid extract was evaporated to dryness and resuspended in 50 µL acetone for HPLC analysis. We separated and quantified copepod carotenoids using HPLC following the methods of Weaver et al. (2018a) and Wright et al. (1991). We injected 10 µL of carotenoid extract in acetone on to a Sonoma C18 column (10 µm, 250 x 4.6 mm, ES Technologies, New Jersey, USA) fitted with a C18 guard cartridge. Carotenoids were separated using a Shimadzu Prominence HPLC system with mobile phases A 80:20 methanol: 0.5M ammonium acetate, B 90:10 acetonitrile: water, and C ethyl acetate in a tertiary gradient of 100%A to 100%B over 4 min, then to 80% C: 20% B over 14 min, back to 100% B over 3 min, and returning to 100% A over 5 min and held for 6 min [9, 52]. We visualized and detected carotenoid absorbance using a Prominence UV/Vis detector set to a wavelength of 450 nm. We identified and quantified carotenoids by comparison to authentic standards that included: astaxanthin, zeaxanthin, β-carotene, lutein, hydroxyechinenone, and canthaxanthin (Fig. S2). We normalized carotenoid concentration of each carotenoid detected by the dry weight of each copepod sample (reported as µg carotenoid per mg copepod tissue).

Statistical Analyses

All statistical analyses were performed in R (R_Core_Team 2019) using the following packages: 'lme4' (Bates et al., 2014), 'Hmisc' (Harrell Jr and Dupont, 2021), 'lmerTest' (Kuznetsova et al., 2017), 'agricolae' (De Mendiburu, 2014), 'MASS' (Venables and Ripley, 2013), 'emmeans' (Lenth et al., 2018), 'car' (Fox and Weisberg, 2018), 'sf' (Pebesma, 2018), 'MuMIn' (Barton, 2009), and 'bestNormalize' (Peterson, 2021). Data wrangling was performed in R with the help of the follow packages: 'tidyverse' (Wickham, 2017), 'dplyr' (Wickham et al., 2015), 'reshape2' (Wickham, 2007), 'plotrix' (Lemon, 2006), and 'HH' (Heiberger et al., 2015). Figures were produced using the following packages: 'ggpubr' (Kassambara, 2018), 'cowplot' (Wilke, 2016), 'RColorBrewer' (Neuwirth, 2014), and 'ggjoy' (Wilke, 2017). For a full review of our statistical analyses, please refer to the annotated R-code included in the supplemental material; we provide below a brief description of each analysis and the type of modeling used.

To test for statistically significant differences in respiration rate between DNP-treated and control copepods, we encoded a linear model with treatment (DNP vs control) as a fixed effect, an interaction with sex, and copepod mass (dry body weight) as a fixed effect. We inspected pairwise comparisons between DNP-treated and control copepods, blocked by sex, using a Tukey correction for multiple pairwise comparisons. In these models, mass-independent respiration rate was used as the dependent variable instead of mass-corrected respiration rate. As noted above, the effect of mass was controlled by including this variable as a fixed effect (Lighton, 2018; Niitepõld, 2019; Schmidt-Nielsen and Knut, 1984). We included mass a covariate for all models with respiration rate as the dependent variable. For models where respiration rate was included as a fixed effect (i.e., independent variable), we use mass-corrected respiration rate as a covariate (see below).

To test for differences in carotenoid concentration, we fit a linear model with treatment, sex, and their interaction as fixed effects. We then inspected pairwise comparisons between DNP-treated and control copepods, blocked by sex, using a Tukey correction for multiple pairwise comparisons. To model the effect of increasing respiration on carotenoid concentrations across all samples, we specified a linear mixed effects model with mass-corrected respiration rate and sex as fixed-effect covariates and diet (red stock vs colorless) as a random effect. We subset our data by "control" and "DNP-treated" copepods and used the same model for each subset to inspect the relationship between respiration rate and carotenoid concentration. Prior to analyzing

the effect of respiration rate on dietary carotenoid concentration, we transformed dietary carotenoid concentrations using an ordered quantile normalization (chosen using the 'bestNormalize' package) due to observed non-normality in the data frequency distribution. To test for any statistical relationship between dietary carotenoid and astaxanthin concentrations in our control and DNP-treated samples, we fit a mixed effects model with astaxanthin concentration as a dependent variable, transformed dietary carotenoid concentration and sex as fixed effects, and diet as a random effect.

To test whether DNP treatment altered the body mass of copepods compared to controls, we fit a linear mixed effects model with treatment and sex encoded as fixed effects and a random effect of "diet" (i.e., red stock or colorless).

Results

We found no significant difference in the mass of copepods exposed to DNP and control copepods across all treatments, after controlling for differences due to sex and diet (Fig. S3; mean tissue mass in mg±SE; DNP: 0.0453±0.0031; Control: 0.0451±0.0031).

We detected three primary carotenoids across all copepod samples. These were identified as astaxanthin, β-carotene, and hydroxyechinenone (Fig. S2). Astaxanthin was the primary, most abundant carotenoid present in the *T. californicus* tissues, making up 97.6% of the quantifiable carotenoid content in stock red copepods on average and 95.3% in color-restored copepods on average (Fig. S4; mean μg astaxanthin mg^{-1} tissue±SD; red stock: 1.29±0.45; color-restored: 0.51±0.14). The second-most abundant carotenoid in both stock red and color-restored copepods was hydroxyechinenone (Fig. S4; mean μg hydroxyechinenone mg^{-1} tissue±SD; red stock: 0.020±0.023; color-restored: 0.024±0.011). Finally, β-carotene was present and quantifiable in only some, but not all samples and was the least abundant precursor to astaxanthin (Fig. S4; mean μg β-carotene mg^{-1} tissue±SD; red stock: 0.012±0.018; color-restored: 0.0095±0.0038). In both red stock and color-restored copepods, the concentration of hydroxyechinenone was statistically greater than the concentration of β-carotene (β_1 = model estimate in μg mg^{-1} tissue, *p*-value; stock red: β_1=0.0076, p=0.0184; color-restored: β_1=0.023, p<0.001).

Red stock copepods

After three days exposure to 10μM DNP, the respiration of red stock copepods was significantly elevated in both sexes (Fig. 2A; mean mmol O_2 min^{-1} ±SE; DNP Male: 2.14±0.20; Control Male: 1.17±0.19; DNP Female: 2.06±0.17; Control Female: 1.54±0.18). Males treated with DNP consumed on average 0.53 mmol O_2 per minute more than control males (CI = confidence interval; CI = 0.036-1.01), and females treated with DNP consumed on average 0.97 more mmol O_2 per min than control females (CI = confidence interval; CI = 0.40-1.53). When 10uM DNP exposure is extended to seven days, respiration was again elevated in male copepods given DNP, but similar to control levels in females (Fig. 2B; mean mmol O_2 min^{-1} ±SE; DNP Male: 1.30±0.17; Control Male: 0.89±0.21; DNP Female: 1.29±0.20; Control Female: 1.30±0.18). However, the difference was not statistically significant in either sex (Fig. 2B). When the exposure time was kept at seven days, but the concentration of DNP was lowered to 2uM, female copepod respiration dropped significantly below that of control females (Fig. 2C; mean mmol O_2 min^{-1} ±SE; DNP Female: 0.81±0.09; Control Female: 1.12±0.11; β_1 = model estimate in mmol O_2 min^{-1}, CI = confidence interval; β_1= -0.311, CI = (-0.60)-(-0.023)). At this concentration and exposure time, male respiration was significantly elevated (Fig. 2C; mean mmol O_2 min^{-1} ±SE; DNP Male: 0.98±0.11; Control Male: 0.63±0.11; β_1 = model estimate in mmol O_2 min^{-1}, CI = confidence interval; β_1=0.35, CI=0.026-0.67).

After three days exposure to 10μM DNP, the astaxanthin concentration in male and female copepod tissues was not significantly different from that in control copepod tissues (Fig. 3A; mean μg astaxanthin mg^{-1} tissue±SE; DNP Male: 1.50±0.12; Control Male: 1.52±0.12; DNP Female: 1.24±0.13; Control Female: 1.35±0.12). However, after seven days exposure to 2μM DNP, astaxanthin concentrations were increased in both sexes, significantly in males (Fig. 3B; mean μg astaxanthin mg^{-1} tissue±SE; DNP Male: 1.64±0.16; Control Male: 0.97±0.15; DNP Female: 1.13±0.13; Control Female: 0.97±0.14). Male copepods exposed to DNP contained 0.67 more μg astaxanthin per mg of body tissue than control copepods (CI = confidence interval; CI = 0.23-1.10).

After three days exposure to 10μM DNP, we observed no clear or significant difference between the concentration of dietary carotenoids in the tissues of DNP-treated copepods compared to tissues of control copepods (Fig. S5A; mean μg dietary carotenoids mg^{-1} tissue±SE; DNP Male: 0.050±0.012; Control Male: 0.040±0.12; DNP Female: 0.047±0.013; Control Female: 0.046±0.012). Similarly, we found no significant difference between the concentration of dietary carotenoids in copepods treated with 2μM DNP for three days compared to control copepods (Fig. S5B; mean μg dietary carotenoids mg^{-1} tissue±SE; DNP Male: 0.028±0.0067; Control Male: 0.018±0.0063; DNP Female: 0.0081±0.0054; Control Female: 0.0095±0.0059).

Color-restored copepods

In colorless copepods, DNP increased the rate of respiration after seven days in both males and females (Fig. 4A; mean mmol O_2 min^{-1} ±SE; DNP Male: 1.02±0.062; Control Male: 0.82±0.073; DNP Female: 0.84±0.076; Control Female: 0.68±0.080). This was statistically significant in males (β_1 = model estimate in mmol O_2 min^{-1}; CI = confidence interval; β_1=0.20, CI=0.014-0.39).

When allowed to restore red coloration through supplementation with *Tetraselmis* algae, male copepods treated with DNP produced only slightly more astaxanthin than controls (Fig. 4B; mean μg astaxanthin mg^{-1} tissue±SE; DNP Male: 0.54±0.025; Control Male: 0.52±0.028). However, female copepods treated with DNP accumulated significantly more astaxanthin compared to controls (Fig. 4B; mean μg astaxanthin mg^{-1} tissue±SE; DNP Female: 0.53±0.032; Control Female: 0.43±0.032; β_1 = model estimate in μg astaxanthin mg^{-1} tissue, CI = confidence interval; β_1=0.098, CI=0.0091-0.19). There were no significant differences between the average concentration of dietary carotenoids in copepods treated with DNP and in control copepods (Fig. S6; mean μg dietary carotenoids mg^{-1} tissue±SE; DNP Male: 0.021±0.0020; Control Male: 0.020±0.0022; DNP Female: 0.033±0.0025; Control Female: 0.030±0.0025). Both DNP-treated and control copepods of both sexes contained significantly more astaxanthin than colorless males and females never given access to algae to restore their coloration ($p<0.0001$ for each comparison; Fig. 4B; mean μg astaxanthin mg^{-1} tissue±SE; colorless males: 0.133±0.052; colorless females; 0.128±0.052).

Astaxanthin, dietary carotenoids, and respiration

Across all experiments, there was a positive relationship between respiration rate and astaxanthin concentration after controlling for sex and diet (Figs 5A, 5B), significant only in DNP-treated copepods (Fig. 5B; *df*=74; *t*=0.52; β_1 = model estimate in µg astaxanthin mg^{-1} tissue, CI = confidence interval; $B_1 = 4.4e^{-3}$; CI=$6.1e^{-4}$ - $8.5e^{-3}$). This same effect was observed between dietary carotenoids and respiration rate in DNP-treated copepods (Fig. 5C, 5D), again controlling for sex and diet (Fig. 5D; *df*=55; *t*=2.49; β_1 = model estimate in ordered quantile of dietary carotenoid concentration, CI = confidence interval; B_1 = 0.014; CI=$7.8e^{-4}$ – 0.024). However, there was a significant relationship between the concentration of dietary carotenoids and the concentration of astaxanthin in the tissues of control copepods only (Fig. 6A; *df*=79; *t*=3.41; β_1 = model estimate in µg astaxanthin mg^{-1} tissue, CI = confidence interval; B_1 = 0.12; CI=0.050 – 0.18). We observed no significant effect of increasing dietary carotenoid concentration on astaxanthin concentration in the tissues in DNP-exposed copepods (Fig. 6B).

Discussion

When treated with the mitochondria uncoupler and protonophore, 2,4-dinitrophenol, *T. californicus* copepods produced more of the red carotenoid pigment astaxanthin after seven days, but not after three days. These experiments provide further support for the hypothesis that metabolism of ketocarotenoids from yellow dietary pigments is intimately linked to energetic processes in the mitochondria. Here, we observed that acute exposure to DNP increased the metabolic rate in *T. californicus* copepods similar to observations in other taxa (Caldeira da Silva et al., 2008; Cantelmo et al., 1978; Geisler et al., 2017; Goldgof et al., 2014; Pinchot, 1967). As predicted if carotenoid metabolism is tied to mitochondrial function, we then observed that DNP-treated copepods produced more astaxanthin than did control copepods. This study provides the first experimental demonstration of a relationship between DNP-induced changes to mitochondrial metabolism and the accumulation of metabolized carotenoid pigments. Critically, it is the first chemical manipulation of mitochondrial function to affect metabolism of ketocarotenoids where results should not be confounded by an increase in the production of reactive oxygen species in the mitochondria.

An alternative hypothesis is that carotenoid-based coloration is a signal of condition because there are tradeoffs in the allocation of carotenoid resources between coloration and stress responses (Alonso-Alvarez et al., 2007; Alonso- Alvarez et al., 2008; Moller et al., 2000; Simons et al., 2012) This is the Resource Tradeoff Hypothesis which proposes that carotenoids such as astaxanthin may serve a defensive role against oxidative stress such that carotenoid pigments may be a key, but limited, metabolic resource (Hill, 1999; Lozano, 1994; Moller et al., 2000). The shared pathway hypothesis and the resource tradeoff hypothesis are not mutually exclusive; carotenoid resource availability and mitochondrial energetics might both affect coloration (see Fig. 2 of Powers and Hill (2021)). For instance, our observation that copepods increased the concentration of astaxanthin in their tissues could indicate that the DNP, in stabilizing the mitochondrial membranes via uncoupling, freed up carotenoid resources that would normally be consumed during antioxidant activities. (Caramujo et al., 2012; Davenport et al., 2004; Weaver et al., 2018b).

Our experiments cannot fully refute the resource tradeoff hypothesis. However, for several reasons we think a carotenoid resource tradeoff alone cannot fully explain our results. Differences in astaxanthin concentrations between DNP-treated and control copepods did not appear to be due to differences in a greater uptake of dietary carotenoids. In both red stock copepods and colorless copepods and for both males and females, individuals exposed to DNP did not accumulate more dietary carotenoids than control copepods (Figs S6, S7). Moreover, we observed no relationship between dietary carotenoid concentration and astaxanthin concentrations in DNP-treated copepods (Fig. 6). Thus, the positive relationship between dietary carotenoids and respiration rate (Fig. 5D) may have been driven by hydroxyechinenone, which is also a metabolic intermediate between β-carotene and astaxanthin (Weaver et al., 2018a). Further, DNP exposure did not result in an increase in mass compared in DNP treated copepods compared to controls, countering the idea that differences in astaxanthin concentrations might simply be due to a difference in algae consumption and, by extension, dietary carotenoid resources.

Several lines of evidence support the hypothesis that DNP treatment stimulated increased metabolism of astaxanthin via increased mitochondrial metabolism. We observed an increase in the rate of oxygen consumption, and hence respiration, in copepods exposed to DNP compared to controls (Fig. 2, 4). This effect was present in both sexes after 3 days exposure to a moderate

dose of 10μM DNP. Excluding red stock female copepods (addressed below), the effect persisted in both red stock and colorless copepods when exposure was extended to seven days and the DNP dosage was lowered to 2μM (Figs 2, 4). We observed that, on average, copepods exposed to DNP over seven days accumulated more astaxanthin in their tissues compared to control copepods (Figs 3, 4), even though this effect was statistically significant in red stock males and colorless females only. This indicates that the red stock copepods exposed to DNP increased astaxanthin concentrations in their tissues beyond the normal levels found in copepods in routine culture conditions. Again, this effect was strongest in male red stock copepods (Fig. 3B). In comparison, colorless copepods exposed to DNP replenished their tissue concentrations of astaxanthin at a faster rate than control individuals, significantly in the case of the colorless females tested. We suspect that we did not see a significant increase in astaxanthin concentrations after three days exposure to DNP (Figure 3A) due to the base carotenoid conversion rate in the species requiring a full two weeks to reach levels comparable to wild individuals (Davenport et al., 2004). Three days may not have been enough time to detect measurable differences due to DNP exposure, a limitation addressed through the inclusion of 7-day exposure trials.

It previously was shown that hybridization had a greater negative impact on astaxanthin metabolism in colorless male *T. californicus* than in colorless females (Powers et al., 2021). Colorless copepods supplemented with DNP also replenished more astaxanthin over seven days than did colorless copepods not provided DNP. However, this effect was observed to be stronger in colorless females (Fig. 4B). Colorless females—but not red stock females— supplemented with 2μM DNP for seven days showed an elevated oxygen consumption on average compared to controls (Figs 3B, 3C, 4). However, red stock females exposed to DNP showed oxygen consumption rates that had, on average, fallen below that of controls (Figure 2C). This creates an interesting pattern, because at higher concentrations of DNP (10μM) over a shorter period (3 days), red stock females showed significantly elevated respiration rates compared to controls (Figure 2A). When exposure to 10μM DNP was extended to one week, the respiration rate of red stock females was not different from controls (Figure 2B). These observations could indicate a hormetic effect of DNP in red stock females fed algae or may also be explained by the observations that female *T. californicus* copepods are more resistant to changes due to oxidative stress (Foley et al., 2019; Harada et al., 2019; Willett, 2010; Willett and Son, 2018). It is possible

that colorless females raised on yeast were less able to compensate for the effects of DNP, possibly due to the relatively poor nutrition provided by yeast compared to algae (Powers et al., 2021). We think it unlikely that this result was due to DNP degrading on its own over the course of seven days. The half-life of DNP in water is over a year (Tratnyek and Hoigne, 1991), and even powerful bacterial degradation of DNP can still takes up to 68 days (Capel and Larson, 1995).

It has been observed that in low doses or relatively short exposures, DNP acts to increase the rate of respiration during oxidative phosphorylation, speeding up the rate at which redox reactions occur to compensate for the lack of ATP produced (Goldgof et al., 2014; Leverve et al., 1998; Rognstad and Katz, 1969; Stier et al., 2014). This uncoupling does not result in an increase of ROS (Goldgof et al., 2014; Korde et al., 2005; Lozinsky et al., 2013; Stier et al., 2014), and thus, should not have required the consumption of astaxanthin to combat oxidative stress, as has been observed in *T. californicus* exposed to pro-oxidants (Weaver et al., 2018b). We propose that the acute doses of DNP that we tested uncoupled oxidative phosphorylation from ATP production, briefly increasing the rate at which redox reactions occurred in copepod mitochondria in compensation. In turn, this shift in redox environment may have also stimulated an increase in the rate of redox reactions required to metabolize β-carotene and hydroxyechinenone to astaxanthin. In hybrid inbred lines of *T. californicus*, we also observed an inverse relationship between astaxanthin accumulation and ATP production in colorless copepods (Powers et al., 2021); although in that previous study, respiration rate was not measured. In a separate analysis of *T. californicus* hybrids, however, increased respiration was associated with increased ketocarotenoid accumulation (Powers and Hill, 2021).

It appears that across diverse animal taxa, the enzymes responsible for carotenoid metabolism belong to the cytochrome p450 superfamily of oxygenases (Lopes et al., 2016; Mojib et al., 2014; Mundy et al., 2016; Twyman et al., 2018; Twyman et al., 2016; Weaver et al., 2020). The predicted cellular location of carotenoid oxygenases places them either directly in the IMM, or in the mitochondrially associated membranes (MAM) in the cytosol (Hill et al., 2019). However, a full description of the mechanism and subcellular location of carotenoid metabolizing enzymes in *T. californicus* is needed before we can fully understand the role of mitochondrial redox state in influencing the expression of ketocarotenoid-based coloration in copepods (Mojib et al., 2014; Weaver et al., 2020). These oxygenases perform their functions via

redox reactions with coupled reductases that utilize electron donors such as NADH, NADPH, or FADH in the same fashion as the enzymes of the mitochondrial ETS (Cojocaru et al., 2007; Munro et al., 2007; Strohmaier et al., 2019). Low doses of DNP result in an increase in electron donors, like NADH or NADPH (Leverve et al., 1998; Rognstad and Katz, 1969). This increase is observed to occur first in the cytosol, and then, subsequently in the mitochondria itself via the influx of NADH and ATP back into the mitochondria (Leverve et al., 1998; Rognstad and Katz, 1969). These changes lead to a decrease in the ratios of electron donors to acceptors in both the cytosol and mitochondria but an increase in reducing power (Leverve et al., 1998). An increase in the redox reaction rate of the mitochondria in the copepods is critically important given the predicted biochemistry of the enzymatic reactions required to metabolize dietary carotenoids to ketocarotenoids.

Recent evidence suggests that changes to mitochondrial energetics can affect carotenoid metabolism. It has been demonstrated that other mitochondria-targeted compounds, besides DNP, can affect the expression of ketocarotenoid-based coloration or the expression of carotenoid metabolizing enzymes. Cantarero et al. (2020a) demonstrated that exposure to the IMM-targeted superoxide dismutase mimetic, mitoTEMPO, successfully altered avian ketolase expression in zebra finches (*Taeniopygia guttata,* Reichenbach, 1862). These same researchers also successfully documented an effect of stabilizing or destabilizing the mitochondrial redox environment with mitoQ and dTTP, respectively, on the visible redness of bills of these same birds (Cantarero and Alonso-Alvarez, 2017). These compounds may have altered the normal ratios of ubiquinol and ubiquinone, redox cyclers responsible for the shuttling of electrons in the ETS, thereby altering the redox state of carotenoid metabolizing enzymes (Cantarero and Alonso-Alvarez, 2017). Caution should be taken to extrapolate these results to our observations in *T. californicus* considering the biological differences between terrestrial and aquatic organism physiology. Moreover, the effect of mitochondria targeted compounds is likely highly contextual and, perhaps, species specific. Indeed, when Red Crossbills (*Loxia curvirostra,* Linnaeus, 1758) were exposed to mitoQ and mitoTEMPO, each compound affected the levels of ketocarotenoids in the blood and feathers differently (Cantarero et al., 2020b).

Our observations of an association between aerobic respiration and metabolism of carotenoids in copepods supports the hypothesis of an intimate link between ketocarotenoid metabolism and mitochondrial function and corroborates previous studies. In wild-caught house

finches (*Haemorhous mexicanus*, Müller, 1776), Hill et al. (2019) observed a positive relationship between state 4 respiration (resting rate) and ketocarotenoid-based coloration as well as an inverse relationship between lipid damage, mitochondrial replacement, red coloration. In this study, the authors hypothesized that redder individuals that accumulated more ketocarotenoids in their feathers may have been more capable of withstanding higher levels of metabolic damage and energetic output before needing to replace old mitochondria with new copies. Similarly, in *T. californicus* hybrids fed a restrictive diet supplemented with *ad libitum* carotenoids, tissue concentrations of ketocarotenoids was negatively correlated with ATP production and not significantly related to offspring development time (Powers et al., 2021), indicating that redder individuals may have needed less ATP to maintain homeostasis. When the dietary stress was alleviated in this copepod study via reintroduction of algal nutrients, the negative correlation between ATP production and ketocarotenoid accumulation disappeared, and there was a positive correlation between ketocarotenoid concentrations and offspring development times (Powers et al., 2021). As noted previously, experimentation with *T. californicus* fed algae ad libitum also resulted in a significant positive relationship between ketocarotenoid biosynthesis and respiration rate (Powers and Hill, 2021).

Observations in the house finch and *T. californicus* lend support to an evolving understanding of how metabolic rate relates to "quality" or "fitness", and an increasing understanding that metabolic rate itself may be highly context dependent (Koch et al., 2021). Indeed, it has been suggested that under conditions in which food is not limiting, it may be beneficial to operate at a higher metabolic rate (Norin and Metcalfe, 2019). Likewise, quantitative analyses of over one-hundred published studies have revealed that it is common to find positive relationships between metabolic rate and many components of fitness are observed (Arnold et al., 2021).

In the house finch and *T. californicus* studies discussed above, it was unclear how the interaction between reactive oxygen species produced via normal oxidative phosphorylation might influence the relationship between ketocarotenoid concentrations and mitochondrial function at a mechanistic level. In our study, however, we observe an overall relationship between respiration rate and ketocarotenoid biosynthesis without the confounding effects of increased ROS, despite a potential additional effect of sex on the relationship. A critical point is that we did not measure natural differences in metabolic efficiency in this study; the chemically

induced uncoupling that we used forced an increase in respiration regardless of natural efficiency. With this design, we did not test for a correlation between mitochondrial efficiency and carotenoid ketolation; rather, we tested for a correlation between the rates of mitochondrial redox reactions and carotenoid ketolation, regardless of ATP production. As we extend conclusions to systems like the wild house finches or the interpopulation hybrids of *T. californicus*, we make the assumption that ATP production scales with respiratory rate naturally, such that faster respiring individuals generally make more ATP. In this experiment we did not measure ATP production. Thus, we cannot make explicit conclusions about the ratio of energy produced to oxygen consumed or the incurrence of any compensatory mechanisms to restore ATP production in response to DNP exposure in the copepods. Future studies may measure both ATP production and respiration rate to assess relationships between carotenoid ketolation and mitochondrial efficiency. Regardless, the results presented in this study suggest that a propensity for ketocarotenoid coloration to signal metabolic capacity is independent of a mediating influence of ROS, even though we cannot conclude that ROS has no effect on tissue carotenoid concentrations in wild populations.

Our observations of potential links between cellular respiration and carotenoid coloration in copepods hold important implications for the evolution of condition-dependent traits. Unlike with house finches, *T. californicus* copepods do not choose mates based on red carotenoid coloration (Powers et al., 2020). Thus, the condition-dependent carotenoid signal in these copepods appears to exist without a receiver, and it seems unlikely to have evolved in copepods because it is a condition-dependent signal. Rather, our observations with copepods may speak to a fundamental biochemical link between ketocarotenoid-based coloration and mitochondrial function—red carotenoid coloration in copepods evolved for UV protection (Weaver et al. 2018b), and its condition dependency is simply a property of the enzymatic pathways that produce the red pigments. The implications of this observation is that in some systems, such as songbirds, metabolically modified carotenoids have been co-opted as a social signal of individual condition (Twyman et al., 2016) and that condition dependency of red carotenoid coloration does not evolve via sexual selection.

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Competing interests

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Data Availability

All relevant data are presented within this manuscript or in the online supplementary materials.

References

Ahmed, F., Fanning, K., Netzel, M., Turner, W., Li, Y. and Schenk, P. M. (2014). Profiling of carotenoids and antioxidant capacity of microalgae from subtropical coastal and brackish waters. *Food Chem* **165**, 300-6.

Alonso-Alvarez, C., Bertrand, S., Faivre, B., Chastel, O. and Sorci, G. (2007). Testosterone and oxidative stress: the oxidation handicap hypothesis. *Proceedings of the Royal Society B: Biological Sciences* **274**, 819-825.

Alonso-Alvarez, C., Pérez-Rodríguez, L., Mateo, R., Chastel, O. and Vinuela, J. (2008). The oxidation handicap hypothesis and the carotenoid allocation trade-off. *Journal of Evolutionary Biology* **21**, 1789-1797.

Arnold, P. A., Delean, S., Cassey, P. and White, C. R. (2021). Meta-analysis reveals that resting metabolic rate is not consistently related to fitness and performance in animals. *Journal of Comparative Physiology B* **191**, 1097-1110.

Barton, K. (2009). MuMIn: multi-model inference. *http://r-forge. r-project. org/projects/mumin/*.

Baruah, P., Goswami, U. and Baruah, P. (2012). Characterization of carotenoid pigments in amphibian, rhacophorous bipunctatus. *Journal of research in Biology* **2**, 114-118.

Bates, D., Mächler, M., Bolker, B. and Walker, S. (2014). Fitting linear mixed-effects models using lme4. *arXiv preprint arXiv:1406.5823*.

Biernaskie, J. M., Grafen, A. and Perry, J. C. (2014). The evolution of index signals to avoid the cost of dishonesty. *Proc Biol Sci* **281**.

Blount, J. D. and McGraw, K. J. (2008). Signal functions of carotenoid colouration. In *Carotenoids*, pp. 213-236: Springer.

Bonduriansky, R. (2007). The evolution of condition-dependent sexual dimorphism. *The American Naturalist* **169**, 9-19.

Caldeira da Silva, C. C., Cerqueira, F. M., Barbosa, L. F., Medeiros, M. H. and Kowaltowski, A. J. (2008). Mild mitochondrial uncoupling in mice affects energy metabolism, redox balance and longevity. *Aging cell* **7**, 552-560.

Cantarero, A. and Alonso-Alvarez, C. (2017). Mitochondria-targeted molecules determine the redness of the zebra finch bill. *Biol Lett* **13**.

Cantarero, A., Andrade, P., Carneiro, M., Moreno-Borrallo, A. and Alonso-Alvarez, C. (2020a). Testing the carotenoid-based sexual signalling mechanism by altering CYP2J19 gene expression and colour in a bird species. *Proceedings of the Royal Society B* **287**, 20201067.

Cantarero, A., Mateo, R., Camarero, P. R., Alonso, D., Fernandez-Eslava, B. and Alonso-Alvarez, C. (2020b). Testing the shared-pathway hypothesis in the carotenoid-based coloration of red crossbills. *Evolution* **74**, 2348-2364.

Cantelmo, A. C., Conklin, P. J., Fox, F. R. and Rao, K. R. (1978). Effects of sodium pentachlorophenate and 2, 4-dinitrophenol on respiration in crustaceans. In *Pentachlorophenol*, pp. 251-263: Springer.

Capel, P. D. and Larson, S. J. (1995). A chemodynamic approach for estimating losses of target organic chemicals from water during sample holding time. *Chemosphere* **30**, 1097-1107.

Caramujo, M. J., de Carvalho, C. C., Silva, S. J. and Carman, K. R. (2012). Dietary carotenoids regulate astaxanthin content of copepods and modulate their susceptibility to UV light and copper toxicity. *Mar Drugs* **10**, 998-1018.

Cojocaru, V., Winn, P. J. and Wade, R. C. (2007). The ins and outs of cytochrome P450s. *Biochimica et Biophysica Acta (BBA)-General Subjects* **1770**, 390-401.

Czeczuga, B. (1980). Carotenoids in some parts of certain species of lizards. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry* **65**, 755-757.

Davenport, J., Healy, A., Casey, N. and Heffron, J. J. (2004). Diet-dependent UVAR and UVBR resistance in the high shore harpacticoid copepod Tigriopus brevicornis. *Marine ecology progress series* **276**, 299-303.

De Mendiburu, F. (2014). Agricolae: statistical procedures for agricultural research. *R package version* **1**.

Dose, J., Matsugo, S., Yokokawa, H., Koshida, Y., Okazaki, S., Seidel, U., Eggersdorfer, M., Rimbach, G. and Esatbeyoglu, T. (2016). Free Radical Scavenging and Cellular Antioxidant Properties of Astaxanthin. *Int J Mol Sci* **17**.

Foley, H. B., Sun, P. Y., Ramirez, R., So, B. K., Venkataraman, Y. R., Nixon, E. N., Davies, K. J. A. and Edmands, S. (2019). Sex-specific stress tolerance, proteolysis, and lifespan in the invertebrate Tigriopus californicus. *Exp Gerontol* **119**, 146-156.

Fox, J. and Weisberg, S. (2018). An R companion to applied regression: Sage publications.

Ge, Z., Johnson, J. D., Cobine, P. A., McGraw, K. J., Garcia, R. and Hill, G. E. (2015). High concentrations of ketocarotenoids in hepatic mitochondria of Haemorhous mexicanus. *Physiological and Biochemical Zoology* **88**, 444-450.

Geisler, J. G., Marosi, K., Halpern, J. and Mattson, M. P. (2017). DNP, mitochondrial uncoupling, and neuroprotection: A little dab'll do ya. *Alzheimer's & Dementia* **13**, 582-591.

Goldgof, M., Xiao, C., Chanturiya, T., Jou, W., Gavrilova, O. and Reitman, M. L. (2014). The chemical uncoupler 2, 4-dinitrophenol (DNP) protects against diet-induced obesity and improves energy homeostasis in mice at thermoneutrality. *Journal of Biological Chemistry* **289**, 19341-19350.

Harada, A. E., Healy, T. M. and Burton, R. S. (2019). Variation in thermal tolerance and its relationship to mitochondrial function across populations of Tigriopus californicus. *Frontiers in physiology* **10**, 213.

Harrell Jr, F. E. and Dupont, C. (2021). Hmisc: Harrell Miscelleneous.

Havird, J. C., Weaver, R. J., Milani, L., Ghiselli, F., Greenway, R., Ramsey, A. J., Jimenez, A. G., Dowling, D. K., Hood, W. R., Montooth, K. L. et al. (2019). Beyond the Powerhouse: Integrating Mitonuclear Evolution, Physiology, and Theory in Comparative Biology. *Integr Comp Biol* **59**, 856-863.

Heiberger, R. M., Heiberger, R. M. and Burt Holland, B. H. (2015). Statistical Analysis and Data Display An Intermediate Course with Examples in R: Springer.

Heine, K. B. and Hood, W. R. (2020). Mitochondrial behaviour, morphology, and animal performance. *Biological Reviews* **95**, 730-737.

Higham, J. P. (2014). How does honest costly signaling work? *Behavioral Ecology* **25**, 8-11.

Hill, G. E. (1999). Is there an immunological cost to carotenoid-based ornamental coloration? *The American Naturalist* **154**, 589-595.

Hill, G. E. (2002). A red bird in a brown bag: the function and evolution of colorful plumage in the house finch: Oxford University Press on Demand.

Hill, G. E. (2011). Condition-dependent traits as signals of the functionality of vital cellular processes. *Ecol Lett* **14**, 625-34.

Hill, G. E. (2014a). Cellular respiration: the nexus of stress, condition, and ornamentation. *Integrative and comparative biology* **54**, 645-657.

Hill, G. E. (2014b). Stress, condition, and ornamentation. *American Zoologist* **54**, 533-538.

Hill, G. E., Hood, W. R., Ge, Z., Grinter, R., Greening, C., Johnson, J. D., Park, N. R., Taylor, H. A., Andreasen, V. A. and Powers, M. J. (2019). Plumage redness signals mitochondrial function in the house finch. *Proceedings of the Royal Society B* **286**, 20191354.

Hill, G. E. and Johnson, J. D. (2012). The vitamin A–redox hypothesis: a biochemical basis for honest signaling via carotenoid pigmentation. *The American Naturalist* **180**, E127-E150.

Hill, G. E. and McGraw, K. J. (2006). Bird coloration: mechanisms and measurements: Harvard University Press.

Kassambara, A. (2018). ggpubr:"ggplot2" based publication ready plots. *R package version 0.1* **7**.

Kayser, H. (1982). Carotenoids in insects. *Carotenoid chemistry and biochemistry*, 195-210.

Koch, R. E., Buchanan, K. L., Casagrande, S., Crino, O., Dowling, D. K., Hill, G. E., Hood, W. R., McKenzie, M., Mariette, M. M. and Noble, D. W. (2021). Integrating Mitochondrial Aerobic Metabolism into Ecology and Evolution. *Trends in Ecology & Evolution*.

Koch, R. E., Josefson, C. C. and Hill, G. E. (2017). Mitochondrial function, ornamentation, and immunocompetence. *Biological Reviews* **92**, 1459-1474.

Korde, A. S., Pettigrew, L. C., Craddock, S. D. and Maragos, W. F. (2005). The mitochondrial uncoupler 2, 4-dinitrophenol attenuates tissue damage and improves mitochondrial homeostasis following transient focal cerebral ischemia. *Journal of neurochemistry* **94**, 1676-1684.

Kuznetsova, A., Brockhoff, P. B. and Christensen, R. H. (2017). lmerTest package: tests in linear mixed effects models. *Journal of statistical software* **82**, 1-26.

Lemon, J. (2006). Plotrix: a package in the red light district of R. R-News 6: 8–12.

Lenth, R., Singmann, H., Love, J., Buerkner, P. and Herve, M. (2018). Emmeans: Estimated marginal means, aka least-squares means. *R package version* **1**, 3.

Leverve, X., Sibille, B., Devin, A., Piquet, M.-A., Espié, P. and Rigoulet, M. (1998). Oxidative phosphorylation in intact hepatocytes: quantitative characterization of the mechanisms of change in efficiency and cellular consequences. In *Bioenergetics of the Cell: Quantitative Aspects*, pp. 53-65: Springer.

Lighton, J. R. (2018). Measuring metabolic rates: a manual for scientists: Oxford University Press.

Lopes, R. J., Johnson, J. D., Toomey, M. B., Ferreira, M. S., Araujo, P. M., Melo-Ferreira, J., Andersson, L., Hill, G. E., Corbo, J. C. and Carneiro, M. (2016). Genetic Basis for Red Coloration in Birds. *Curr Biol* **26**, 1427-34.

Lozano, G. A. (1994). Carotenoids, parasites, and sexual selection. *Oikos*, 309-311.

Lozinsky, O. V., Lushchak, V., Storey, J. M., Storey, K. B. and Lushchak, V. I. (2013). The mitochondrial uncoupler 2, 4-dinitrophenol attenuates sodium nitroprusside-induced toxicity in Drosophila melanogaster: Potential involvement of free radicals. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* **158**, 244-252.

Maoka, T. (2011). Carotenoids in marine animals. *Mar Drugs* **9**, 278-93.

Meléndez-Martínez, A. J., Britton, G., Vicario, I. M. and Heredia, F. J. (2007). Relationship between the colour and the chemical structure of carotenoid pigments. *Food Chemistry* **101**, 1145-1150.

Mojib, N., Amad, M., Thimma, M., Aldanondo, N., Kumaran, M. and Irigoien, X. (2014). Carotenoid metabolic profiling and transcriptome-genome mining reveal functional equivalence among blue-pigmented copepods and appendicularia. *Mol Ecol* **23**, 2740-56.

Moller, A., Biard, C., Blount, J., Houston, D., Ninni, P., Saino, N. and Surai, P. (2000). Carotenoid-dependent signals: indicators of foraging efficiency, immunocompetence or detoxification ability? *Poultry and Avian Biology Reviews* **11**, 137-160.

Mundy, N. I., Stapley, J., Bennison, C., Tucker, R., Twyman, H., Kim, K.-W., Burke, T., Birkhead, T. R., Andersson, S. and Slate, J. (2016). Red carotenoid coloration in the zebra finch is controlled by a cytochrome P450 gene cluster. *Current Biology* **26**, 1435-1440.

Munro, A. W., Girvan, H. M. and McLean, K. J. (2007). Cytochrome P450–redox partner fusion enzymes. *Biochimica et Biophysica Acta (BBA)-General Subjects* **1770**, 345-359.

Naguib, Y. M. (2000). Antioxidant activities of astaxanthin and related carotenoids. *Journal of agricultural and food chemistry* **48**, 1150-1154.

Neuwirth, E. (2014). RColorBrewer: ColorBrewer palettes. R package version 1.1-2.

Niitepõld, K. (2019). Effects of flight and food stress on energetics, reproduction, and lifespan in the butterfly Melitaea cinxia. *Oecologia* **191**, 271-283.

Norin, T. and Metcalfe, N. B. (2019). Ecological and evolutionary consequences of metabolic rate plasticity in response to environmental change. *Philosophical Transactions of the Royal Society B* **374**, 20180180.

Pebesma, E. J. (2018). Simple features for R: standardized support for spatial vector data. *R J.* **10**, 439.

Peterson, R. A. (2021). Finding Optimal Normalizing Transformations via bestNormalize. *The R Journal*.

Picard, M. and Sandi, C. (2020). The social nature of mitochondria: Implications for human health. *Neurosci Biobehav Rev* **120**, 595-610.

Pinchot, G. B. (1967). The mechanism of uncoupling of oxidative phosphorylation by 2, 4-dinitrophenol. *Journal of Biological Chemistry* **242**, 4577-4583.

Powers, M. J. and Hill, G. E. (2021). A review and assessment of the Shared-Pathway Hypothesis for the maintenance of signal honesty in red ketocarotenoid-based coloration. *Integrative and comparative biology*.

Powers, M. J., Hill, G. E. and Weaver, R. J. (2020). An experimental test of mate choice for red carotenoid coloration in the marine copepod Tigriopus californicus. *Ethology* **126**, 344-352.

Powers, M. J., Martz, L. D., Burton, R. S., Hill, G. E. and Weaver, R. J. (2021). Evidence for hybrid breakdown in production of red carotenoids in the marine invertebrate Tigriopus californicus. *PLoS One* **16**, e0259371.

Powlik, J. J., Lewis, A. G. and Spaeth, M. (1997). Development, body length, and feeding of Tigriopus californicus (Copepoda, Harpacticoida) in laboratory and field populations. *Crustaceana*, 324-343.

Prado-Cabrero, A., Saefurahman, G. and Nolan, J. M. (2020). Stereochemistry of Astaxanthin Biosynthesis in the Marine Harpacticoid Copepod Tigriopus Californicus. *Mar Drugs* **18**.

Rognstad, R. and Katz, J. (1969). The effect of 2, 4-dinitrophenol on adipose-tissue metabolism. *Biochemical Journal* **111**, 431-444.

Rowe, L. and Houle, D. (1996). The lek paradox and the capture of genetic variance by condition dependent traits. *Proceedings of the Royal Society of London. Series B: Biological Sciences* **263**, 1415-1421.

Schmidt-Nielsen, K. and Knut, S.-N. (1984). Scaling: why is animal size so important?: Cambridge university press.

Seabra, L. M. A. J. and Pedrosa, L. F. C. (2010). Astaxanthin: structural and functional aspects. *Revista de Nutrição* **23**, 1041-1050.

Searcy, W. A. and Nowicki, S. (2010). The evolution of animal communication: Princeton University Press.

Simons, M. J., Cohen, A. A. and Verhulst, S. (2012). What does carotenoid-dependent coloration tell? Plasma carotenoid level signals immunocompetence and oxidative stress state in birds–a meta-analysis. *PLoS One* **7**, e43088.

Smith, J. M. and Harper, D. (2003). Animal signals: Oxford University Press.

Stier, A., Bize, P., Roussel, D., Schull, Q., Massemin, S. and Criscuolo, F. (2014). Mitochondrial uncoupling as a regulator of life-history trajectories in birds: an experimental study in the zebra finch. *Journal of Experimental Biology* **217**, 3579-3589.

Strohmaier, S. J., Huang, W., Baek, J. M., Hunter, D. J. and Gillam, E. M. (2019). Rational evolution of the cofactor-binding site of cytochrome P450 reductase yields variants with increased activity towards specific cytochrome P450 enzymes. *The FEBS journal* **286**, 4473-4493.

Svensson, P. A. and Wong, B. (2011). Carotenoid-based signals in behavioural ecology: a review. *Behaviour* **148**, 131-189.

Tratnyek, P. G. and Hoigne, J. (1991). Oxidation of substituted phenols in the environment: a QSAR analysis of rate constants for reaction with singlet oxygen. *Environmental science & technology* **25**, 1596-1604.

Twyman, H., Andersson, S. and Mundy, N. I. (2018). Evolution of CYP2J19, a gene involved in colour vision and red coloration in birds: positive selection in the face of conservation and pleiotropy. *BMC evolutionary biology* **18**, 1-10.

Twyman, H., Valenzuela, N., Literman, R., Andersson, S. and Mundy, N. I. (2016). Seeing red to being red: conserved genetic mechanism for red cone oil droplets and co-option for red coloration in birds and turtles. *Proceedings of the Royal Society B: Biological Sciences* **283**, 20161208.

Venables, W. N. and Ripley, B. D. (2013). Modern applied statistics with S-PLUS: Springer Science & Business Media.

Vittor, B. A. (1971). Effects of the environment on fitness related life history characters in Tigriopus californicus: Thesis (Ph. D.)--Oregon, Dept. of Biology.

Von Lintig, J. (2010). Colors with functions: elucidating the biochemical and molecular basis of carotenoid metabolism. *Annual review of nutrition* **30**, 35-56.

Weaver, R. J., Cobine, P. A. and Hill, G. E. (2018a). On the bioconversion of dietary carotenoids to astaxanthin in the marine copepod, Tigriopus californicus. *Journal of Plankton Research* **40**, 142-150.

Weaver, R. J., Gonzalez, B. K., Santos, S. R. and Havird, J. C. (2020). Red Coloration in an Anchialine Shrimp: Carotenoids, Genetic Variation, and Candidate Genes. *The Biological Bulletin* **238**, 119-130.

Weaver, R. J., Hill, G. E., Kuan, P.-L. and Tseng, Y.-C. (2016). Copper exposure reduces production of red carotenoids in a marine copepod. *Ecological Indicators* **70**, 393-400.

Weaver, R. J., Koch, R. E. and Hill, G. E. (2017). What maintains signal honesty in animal colour displays used in mate choice? *Philos Trans R Soc Lond B Biol Sci* **372**.

Weaver, R. J., Wang, P., Hill, G. E. and Cobine, P. A. (2018b). An in vivo test of the biologically relevant roles of carotenoids as antioxidants in animals. *Journal of Experimental Biology* **221**.

Wickham, H. (2007). Reshaping data with the reshape package. *Journal of statistical software* **21**, 1-20.

Wickham, H. (2017). Tidyverse: Easily install and load the 'tidyverse'. *R package version* **1**, 2017.

Wickham, H., Francois, R., Henry, L. and Müller, K. (2015). dplyr: A grammar of data manipulation. *R package version 0.4* **3**, p156.

Wilke, C. (2017). ggjoy: joyplots in 'ggplot2': R package version 0.3. 0.

Wilke, C. O. (2016). cowplot: streamlined plot theme and plot annotations for 'ggplot2'. *CRAN Repos* **2**, R2.

Willett, C. S. (2010). Potential fitness trade-offs for thermal tolerance in the intertidal copepod Tigriopus californicus. *Evolution: International Journal of Organic Evolution* **64**, 2521-2534.

Willett, C. S. and Son, C. (2018). The evolution of the thermal niche across locally adapted populations of the copepod Tigriopus californicus. *Bulletin, Southern California Academy of Sciences* **117**, 150-156.

Wright, S., Jeffrey, S., Mantoura, R., Llewellyn, C., Bjørnland, T., Repeta, D. and Welschmeyer, N. (1991). Improved HPLC method for the analysis of chlorophylls and carotenoids from marine phytoplankton. *Marine ecology progress series*, 183-196.

Figures

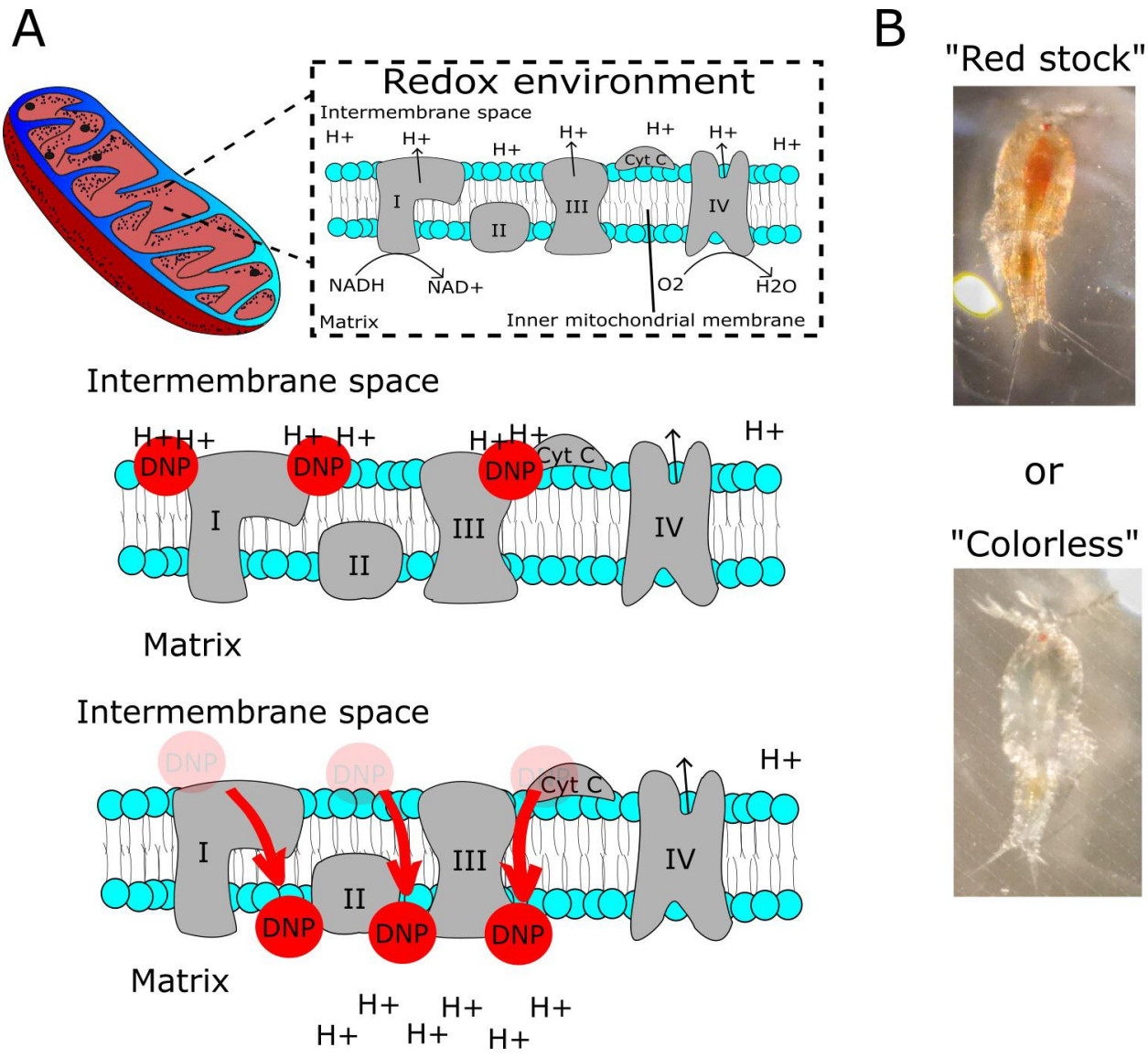

Fig. 1: A simple representation of the mechanism by which DNP acts as a protonophore in the mitochondria. A) DNP binds binds to hydrogen protons that have been pumped into the intermembrane space during the process of oxidative phosphorylation in the electron transport system. DNP moves across the inner mitochondrial membrane and releases the hydrogen protons back into the mitochondrial matrix, thereby dissipating the proton gradient required to produce ATP via ATP synthase (not pictured). B) Photographs of the red stock copepods and "colorless" copepods cultured in our laboratory.

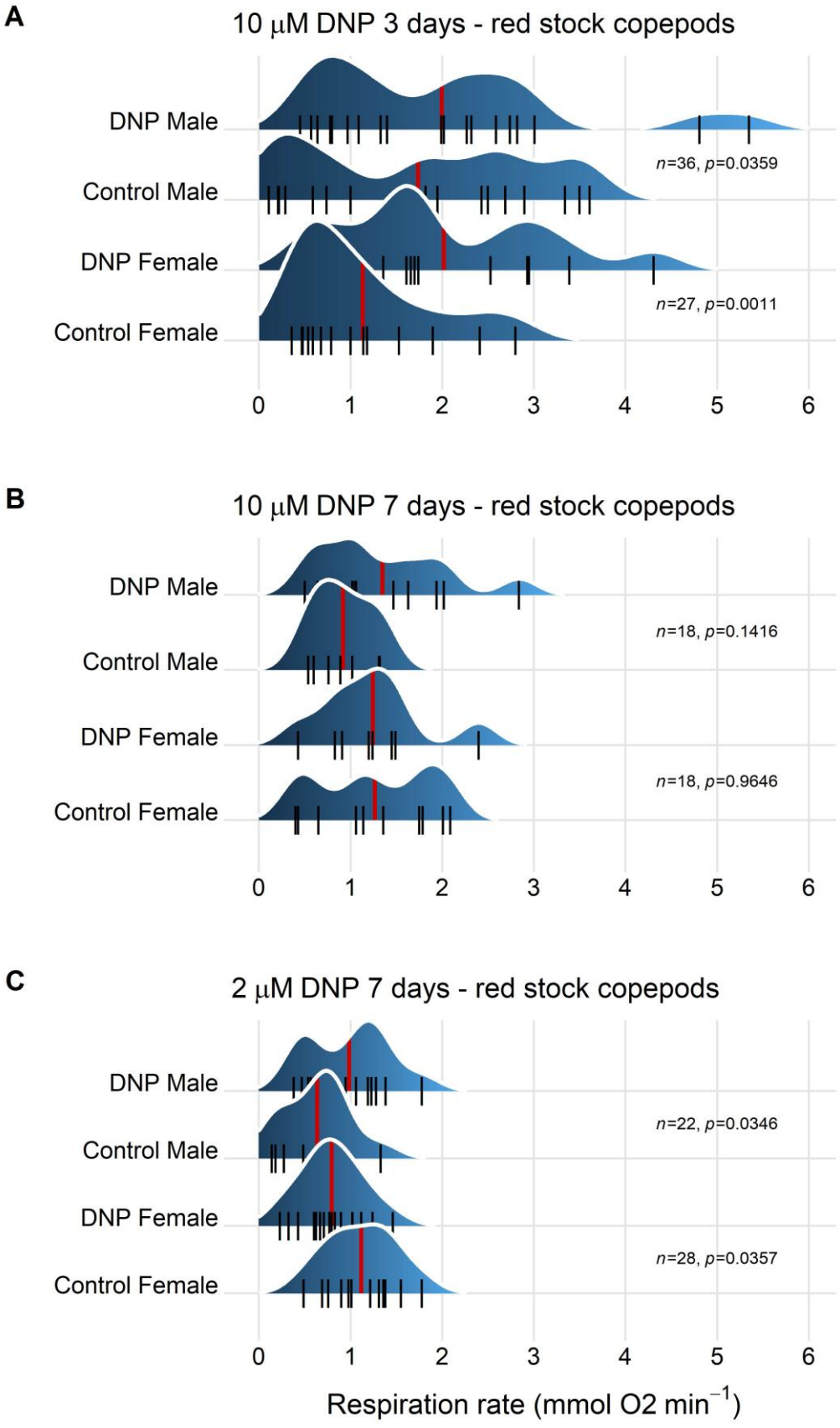

Fig. 2: Ridgeline plot showing the distribution of oxygen consumption measurements for DNP-treated and control red stock copepods, separated by sex. The label at the top of each panel indicates the concentration of DNP tested and the exposure time. The red line represents the group average. Small black lines indicate individual trial replicates of 3 copepods each. The p-value shown is reported from a linear model comparing the two treatment groups that included the interaction with sex and mass as a fixed effect. The samples sizes indicated by *n* represents the number of experimental replicates analyzed in each model, with each replicate containing 3 individual copepods.

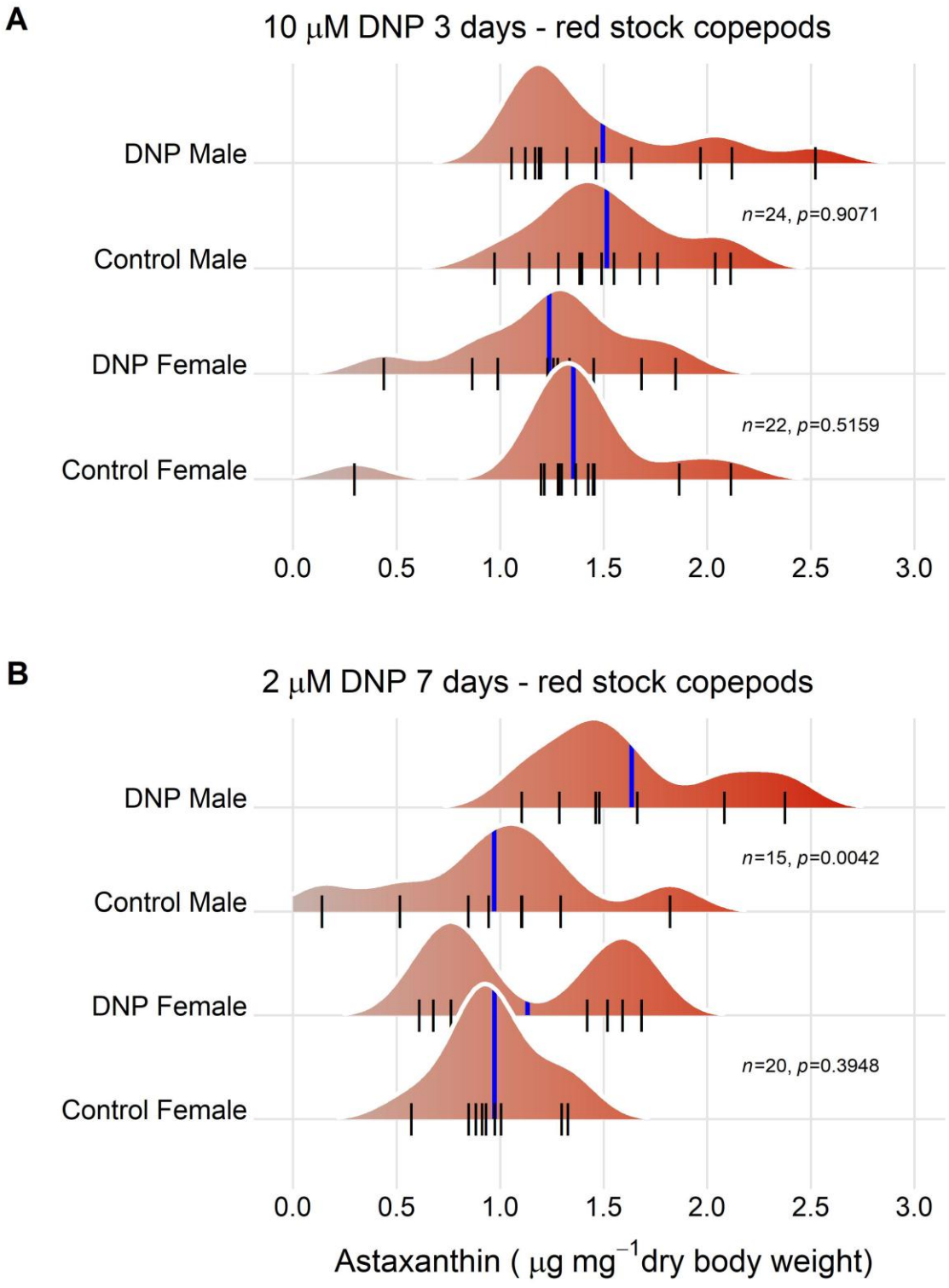

Fig. 3: Ridgeline plot showing the distribution of astaxanthin concentration measurements for DNP-treated and control red stock copepods, separated by sex. The label at the top of each panel indicates the concentration of DNP tested and the exposure time. The blue line represents the group average. Small black lines indicate individual trial replicates of 3 copepods each. The p-value shown is reported from a linear model comparing the two treatment groups that included the interaction with sex. The samples sizes indicated by *n* represents the number of experimental replicates analyzed in each model, with each replicate containing 3 individual copepods.

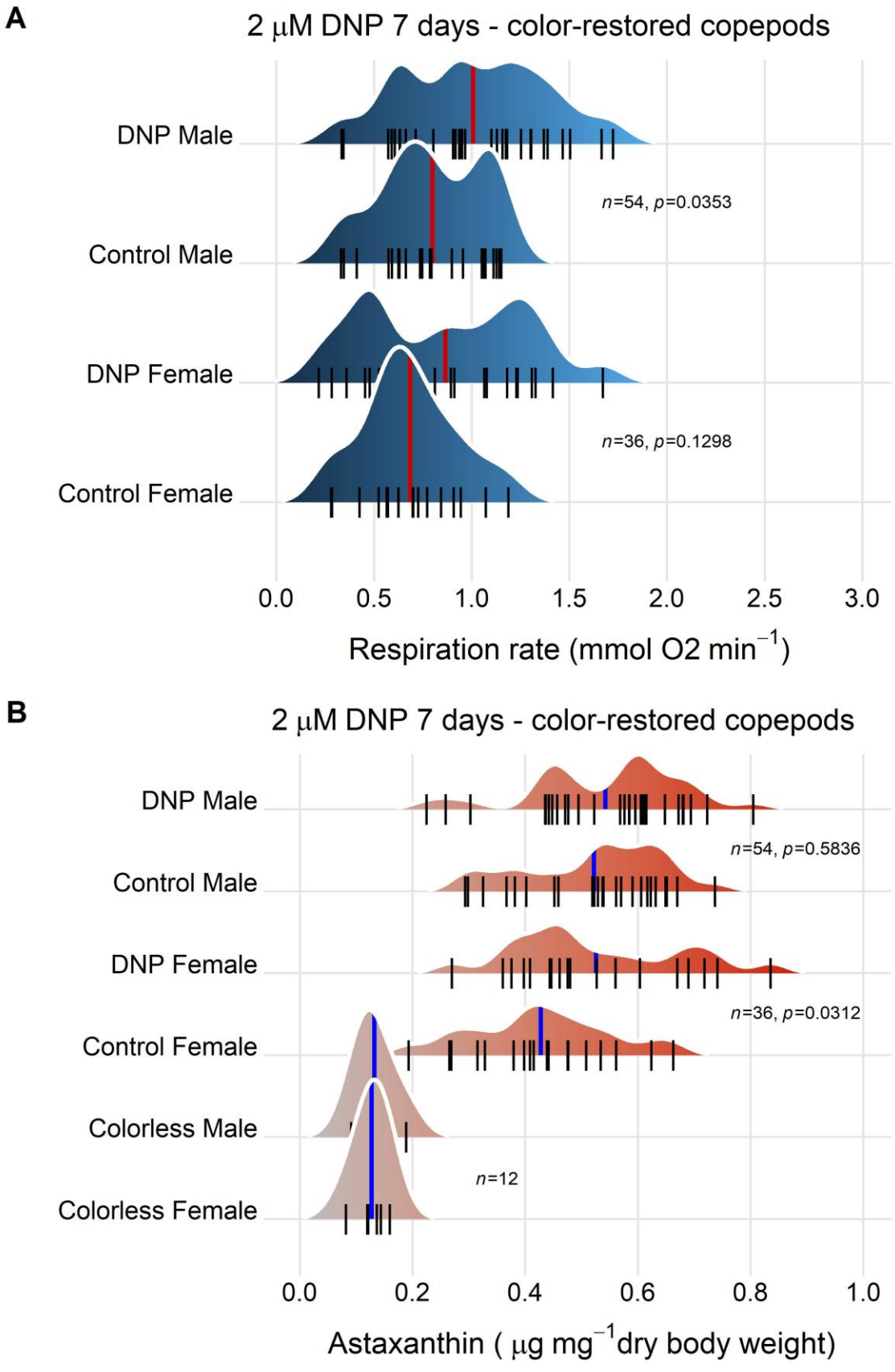

Fig. 4: **Ridgeline plot showing the distribution of oxygen consumption measurements for DNP-treated and control color-restored copepods, separated by sex (A) and the distribution of astaxanthin concentration measurements for DNP-treated and control color-restored copepods, separated by sex (B).** The astaxanthin concentration of colorless copepods are included in panel B for visual comparison. The label at the top of each graph indicates the concentration of DNP tested and the exposure time. The blue or red line represents the group average. Small black lines indicate individual trial replicates of 3 copepods each. The p-value shown is reported from a linear model comparing the two treatment groups that included the interaction with sex and, in the case of analyzing the respiration data, mass as a fixed effect. The models analyzing astaxanthin concentration did not include mass because astaxanthin concentrations were already standardized by mass. The samples sizes indicated by *n* represents the number of experimental replicates analyzed in each model, with each replicate containing 3 individual copepods.

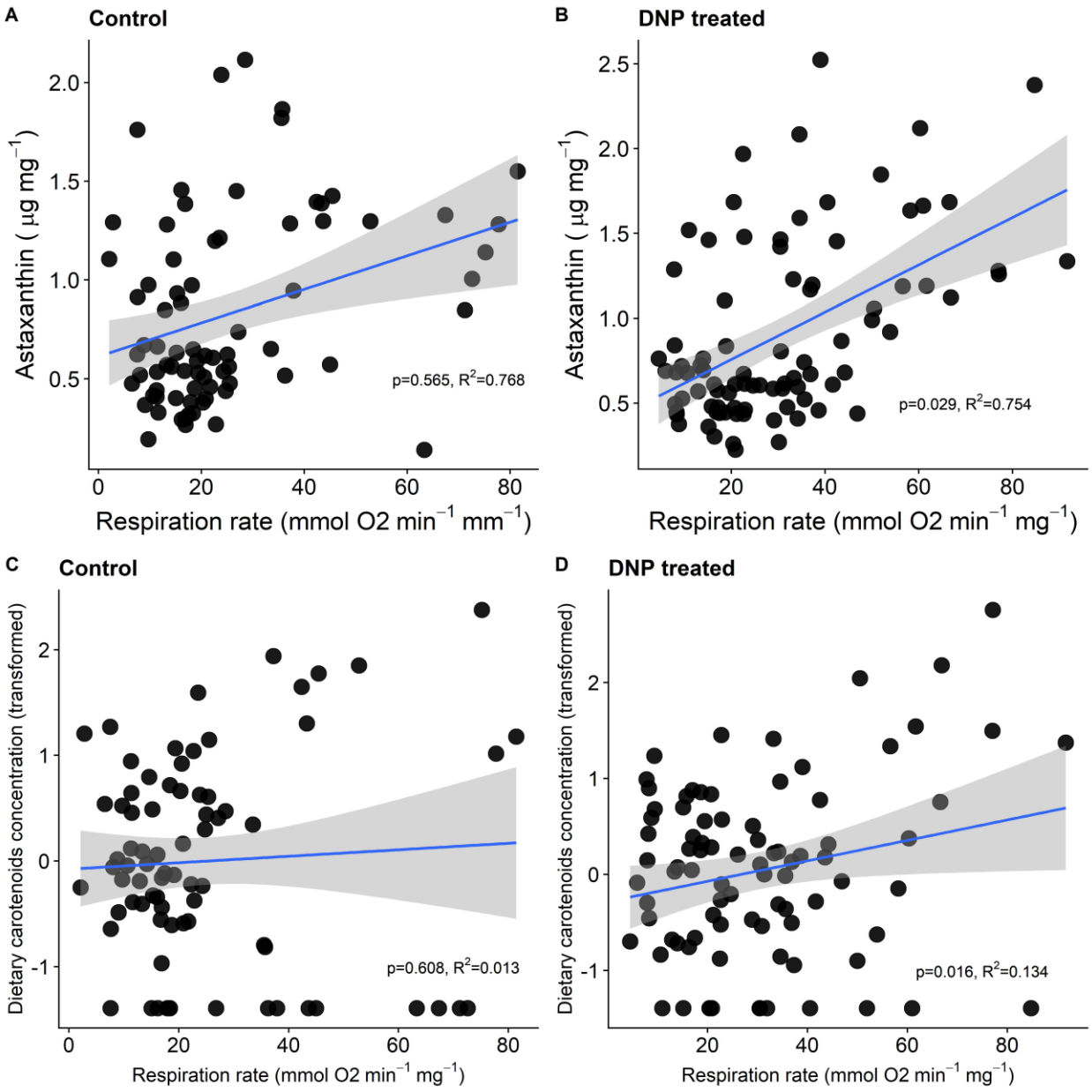

Fig. 5: Scatterplots showing the relationship between respiration rate (x-axis, all panels) and either A) astaxanthin in control copepods, B) astaxanthin in DNP-treated copepods, C) dietary carotenoids in control copepods, and D) dietary carotenoids in DNP-treated copepods. P-values are reported from linear mixed effects models with sex included as a fixed effect and diet (i.e., red stock or color-restored) coded as a random effect. Using raw dietary carotenoids concentration value, model residuals were initially skewed and non-normal, so we transformed dietary carotenoid values using an ordered quantile transformation. R^2 values are

adjusted values, accounting for the additional terms encoded in the model. The blue line is a smoothed line of best fit and the grey shading represents the 95% confidence interval around this line. Each black dot represents a single experimental trial, with 3 copepods per trial.

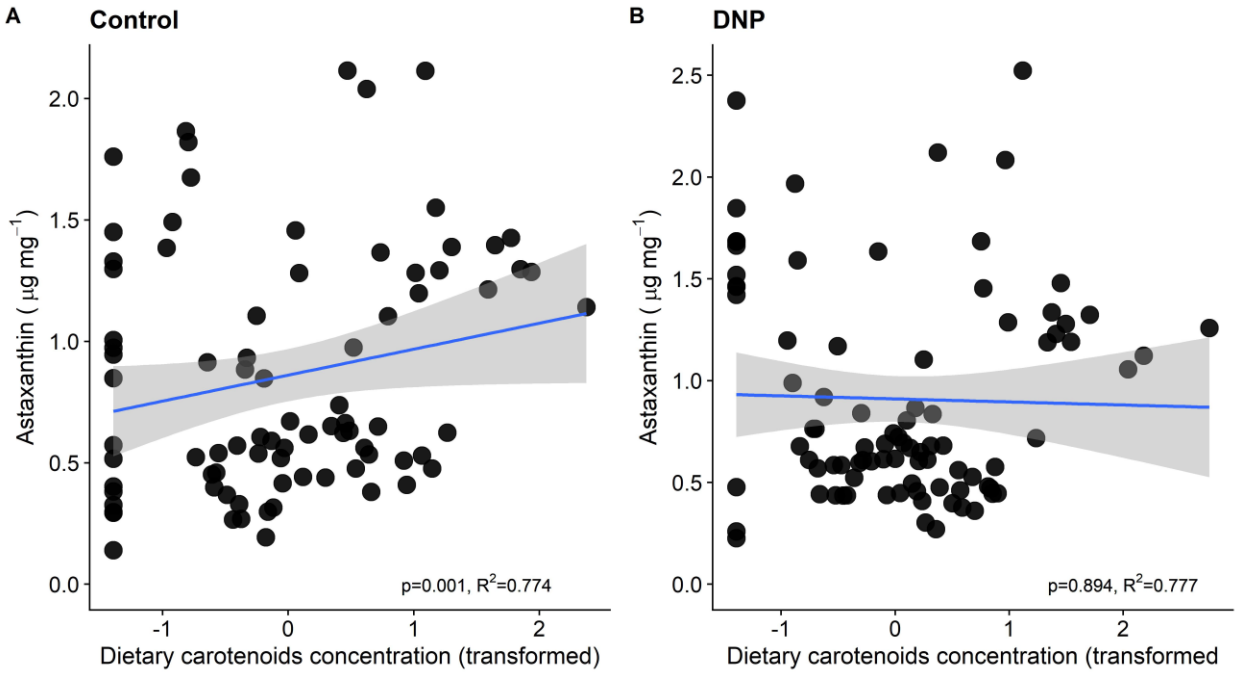

Fig. 6: Scatterplots showing the relationship between dietary carotenoid data (x-axis, all panels) and either A) astaxanthin in control copepods, or B) astaxanthin in DNP-treated copepods. P-values are reported from linear mixed effects models with sex included as a fixed effect and diet (i.e., red stock or color-restored) coded as a random effect. Using raw dietary carotenoids concentration value, model residuals were initially skewed and non-normal, so we transformed dietary carotenoid values using an ordered quantile transformation. R^2 values are adjusted values, accounting for the additional terms encoded in the model. The blue line is a smoothed line of best fit and the grey shading represents the 95% confidence interval around this line. Each black dot represents a single experimental trial, with three copepods per trial.

Table 1: A summary of experimental trials and treatments used in this study.

Treatment	**Copepod type**	**Days Exposure**	**Culture food source**	**Measurements**
10µM DNP	Red stock	3	*Tetraselmis chuii*	Respiration + HPLC
10µM DNP	Red stock	7	*Tetraselmis chuii*	Respiration*
2µM DNP	Red stock	7	*Tetraselmis chuii*	Respiration + HPLC
2µM DNP	Colorless	7	Yeast powder	Respiration + HPLC

*These tissues were not stored at -80°C following respiration. Thus, we could not perform HPLC analysis.

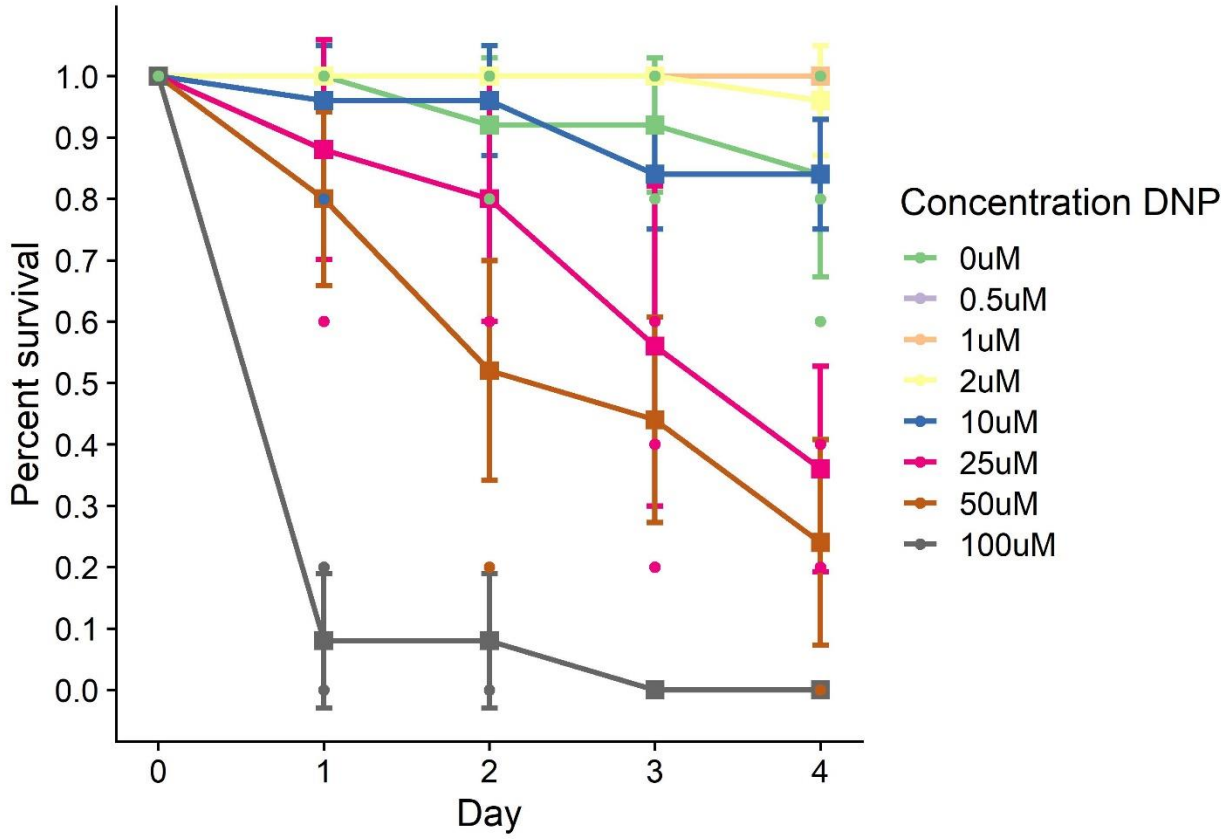

Fig. S1. Average survival out of 5 copepods over 4 days exposure time to different DNP concentrations. Each average value (indicated by the squares) represents the average of 5 trials (25 copepods total for each tested concentration). Bars represent standard errors around the averages for each day. Small circles indicate individual trials, with most falling on the average (thus, hidden visually). The lilac (0.5µM), orange (1µM), and yellow lines (2µM) overlap.

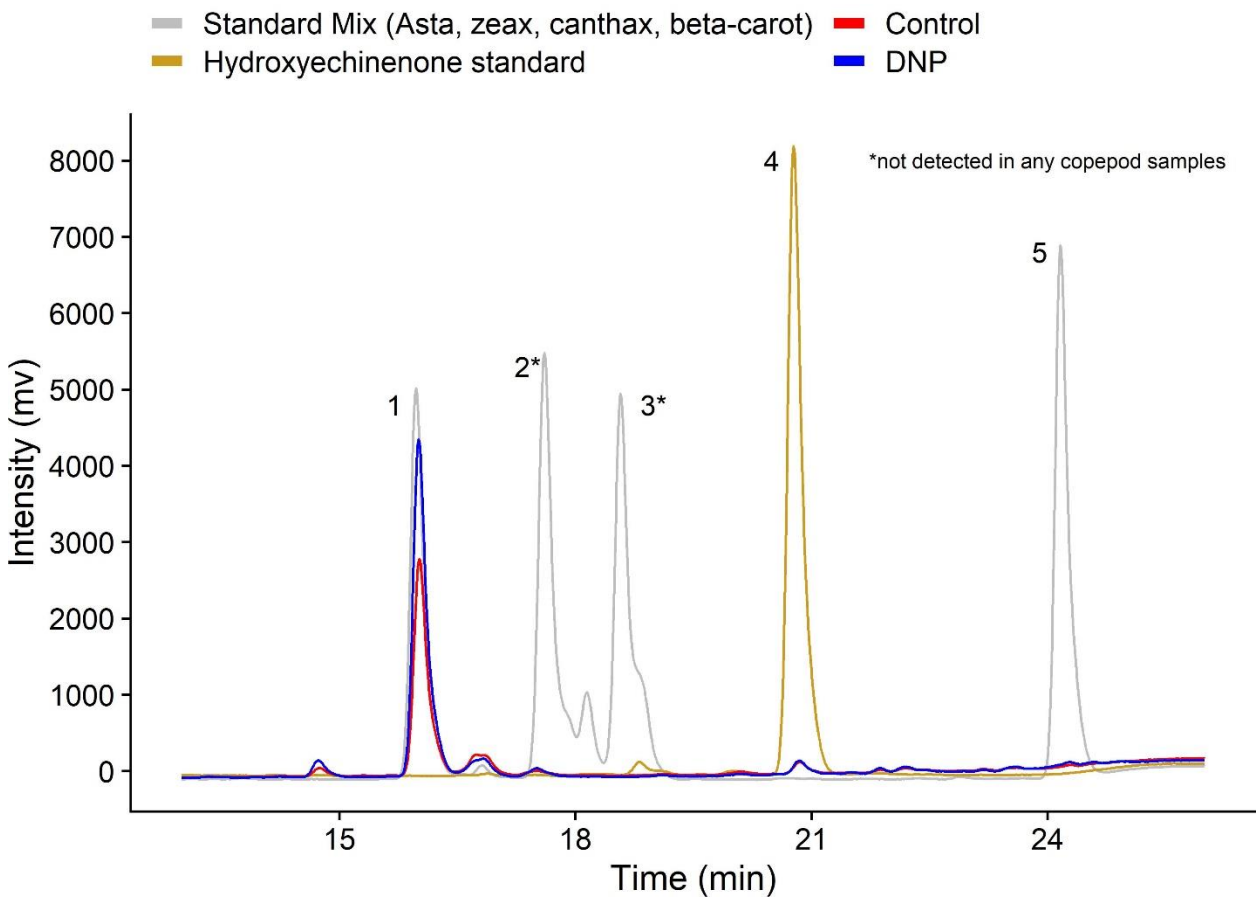

Fig. S2. Representative HPLC chromatogram showing carotenoid standards (grey and yellow), a DNP-treated copepod sample (blue), and a control copepod sample (red). Peaks are 1) astaxanthin, 2) zeaxanthin, 3) canthaxanthin, 4) hydroxyechinenone, 5) β-carotene. β-carotene and hydroxyechinenone are present in the *Tetraselmis* algae used to feed copepods. In many samples, like the representative graph shown here, β-carotene was only detectable in trace amounts.

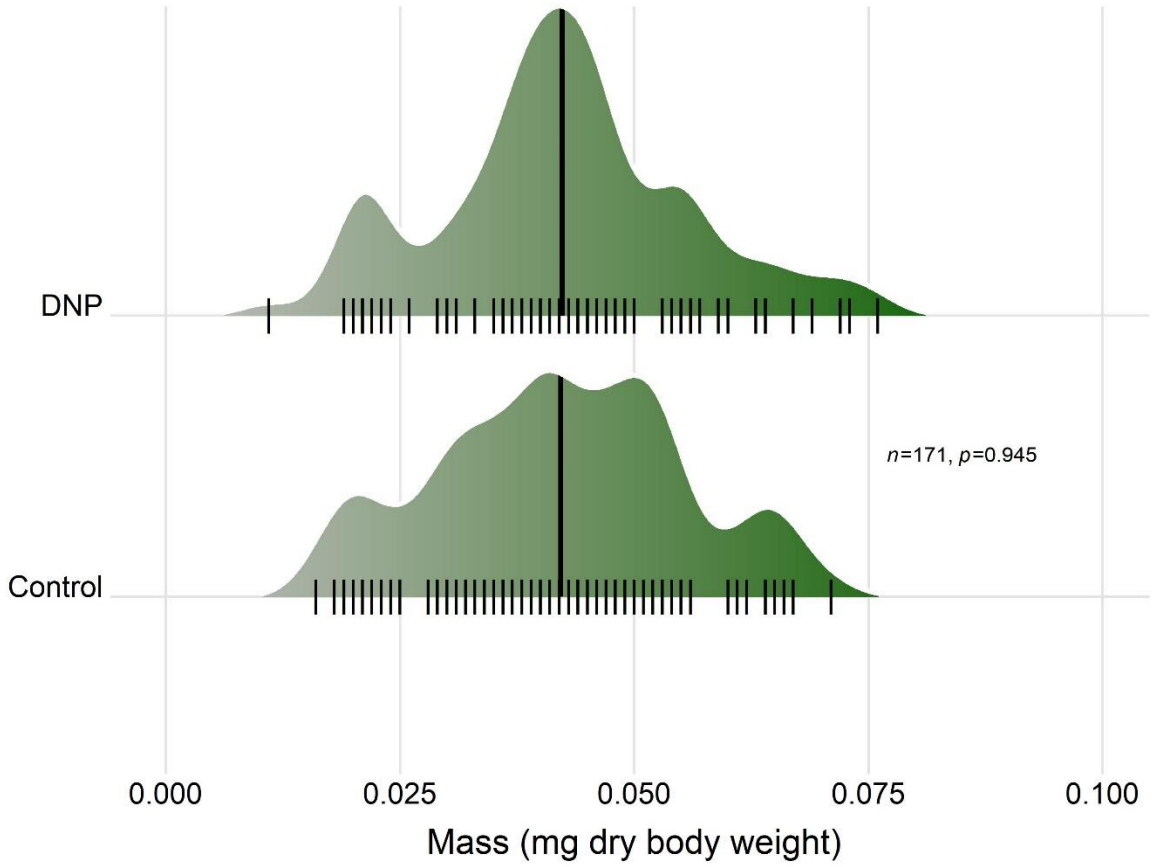

Fig. S3. Ridgeline plot showing the distribution of dry mass measurements for DNP-treated and control copepods. The tall black line represents the group average. Small black lines indicate individual trial replicates of 3 copepods each. The p-value shown is reported from a linear mixed effects model comparing the two treatment groups that included a fixed effect of sex and a random effect of diet (i.e., red stock or color-restored). The samples sizes indicated by *n* represents the number of experimental replicates analyzed in the model, with each replicate containing 3 individual copepods.

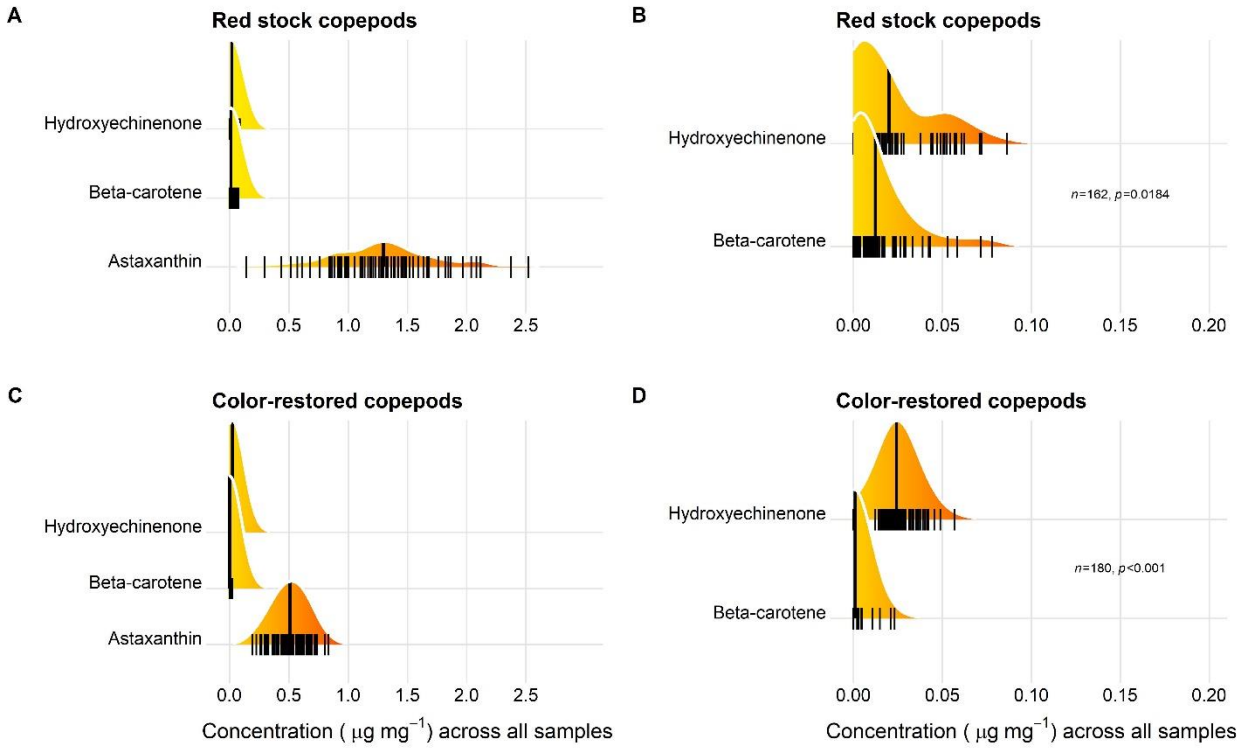

Fig. S4. Ridgeline plots showing the distribution of carotenoid concentrations for all copepod samples in red stock copepods (AB) and color-restored copepods (CD). The same data is shown twice: panels C and D are the same hydroxyechinenone and β-carotene data shown without the swamping effect of plotting astaxanthin on the same graph. The concentration of astaxanthin was significantly higher than either hydroxyechinenone or β-carotene (p<0.0001 for each comparison). The tall black line represents the average for each carotenoid. Small black lines indicate individual trial replicates of 3 copepods each.

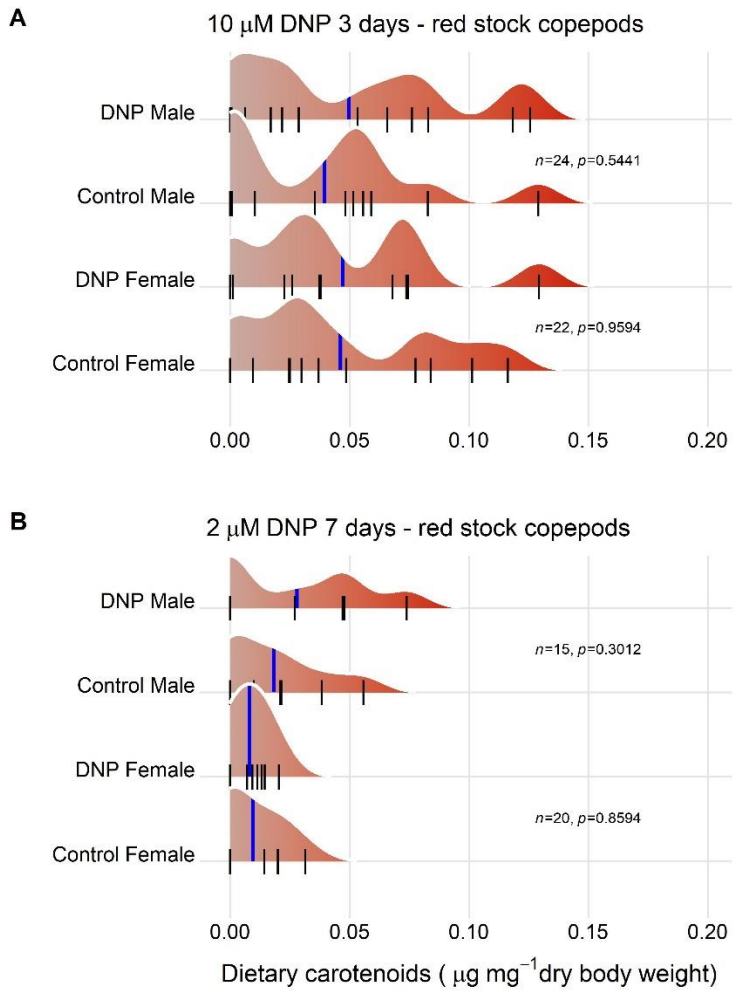

Fig. S5. Ridgeline plot showing the distribution of dietary carotenoid concentration measurements for DNP-treated and control red stock copepods, separated by sex. Panels are separated by A) the dietary carotenoid concentrations after 3 days exposure to 10μM DNP and B) dietary carotenoid concentrations after 7 days exposure to 2μM DNP. The label at the top of each panel indicates the concentration of DNP tested and the exposure time. The blue line represents the group average. Small black lines indicate individual trial replicates of 3 copepods each. The p-value shown is reported from a linear model comparing the two treatment groups that included the interaction with sex. The samples sizes indicated by *n* represents the number of experimental replicates analyzed in each model, with each replicate containing 3 individual copepods.

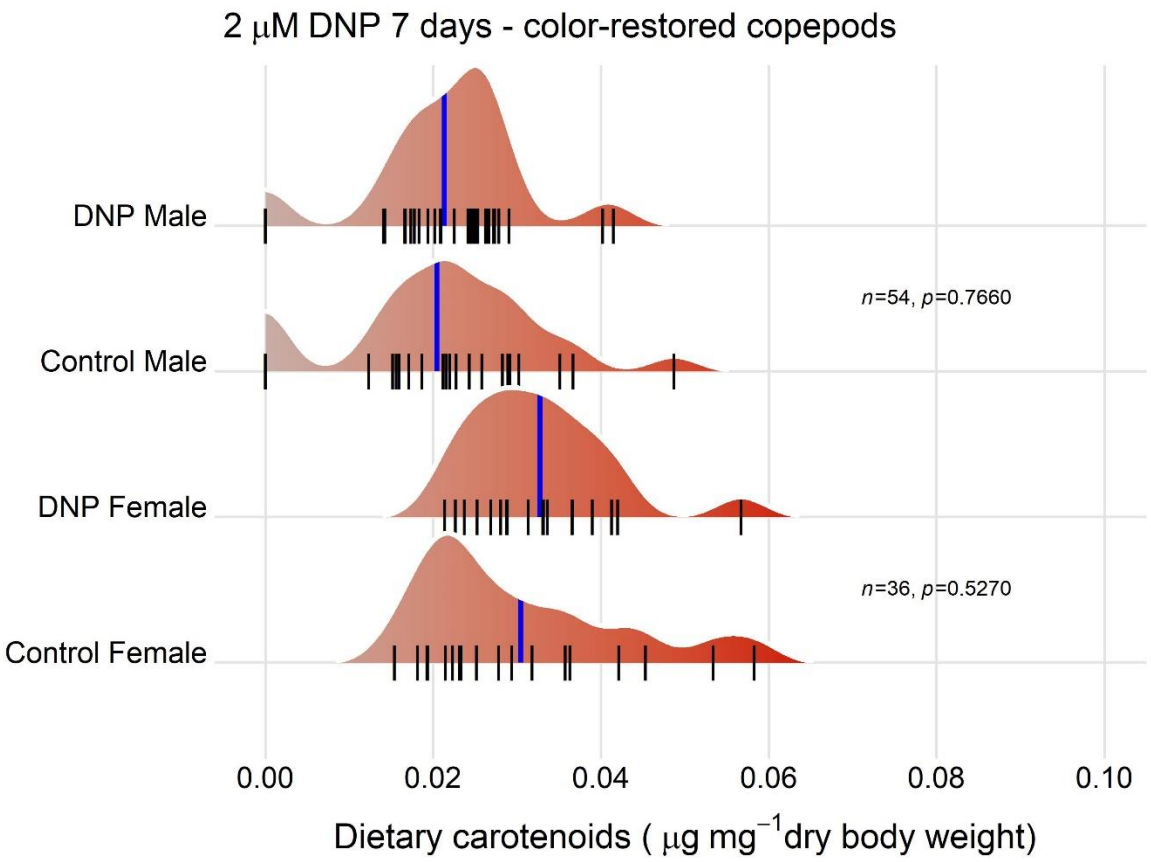

Fig. S6. Ridgeline plot showing the distribution of dietary carotenoid concentration measurements for DNP-treated and control color-restored copepods, separated by sex. The blue line represents the group average. Small black lines indicate individual trial replicates of 3 copepods each. The p-value shown is reported from a linear model comparing the two treatment groups that included the interaction with sex. The samples sizes indicated by *n* represents the number of experimental replicates analyzed in each model, with each replicate containing 3 individual copepods. We detected no dietary carotenoids in the tissues of colorless males and females never given access to algae to restore their coloration.

```
#load packages
library(Hmisc)
library(tidyverse)
library(lme4)
library(cowplot)
library(lmerTest)
library(agricolae)
library(MASS)
library(emmeans)
library(ggpubr)
library(dplyr)
library(RColorBrewer)
library(reshape2)
library(car)
library(sf)
library(ggjoy)
library(MuMIn)
library(plotrix)
library(HH)
library(bestNormalize)
#Set theme globally
theme_set(theme_cowplot())

#####Assessing survival####

##Lethal dose analysis
datum = read.csv(file = "DNP_survival.csv")

datum2 = melt(datum[,c(2, 7, 8, 9, 10, 11)], id="ID")

datum2.5 <- as.data.frame(str_split_fixed(datum2$ID, "_", 2))
datum2$dose = as.factor(datum2.5$V1)
datum2$variable = as.numeric(datum2$variable)
datum2$variable = as.numeric(datum2$variable-1)

mod.1 = lm(value~dose*variable, data = datum2)
mod.1.comp <- emmeans(mod.1, pairwise~dose | variable)

datum2$dose <- factor(datum2$dose, levels = c("0uM", "0.5uM", "1uM", "2uM", "10uM", "25uM",
"50uM", "100uM"))

jpeg(file = "survival curves.jpg", units = "in", width = 6.5, height = 4.5, res = 500)
ggplot(data=datum2, aes(x=variable, y=value, col = dose)) +
  stat_summary(fun.data = mean_sdl, fun.args = list(mult = 1), geom = "errorbar", size =1, width=0.1) +
  stat_summary(fun = mean, geom = "line", size =1) +
  stat_summary(fun = mean, geom ="point", size = 3, shape =15, show.legend = FALSE)+
  geom_point()+
  scale_x_continuous(name="Day", breaks = seq(0, 4, by = 1))+
```

```
 scale_y_continuous(name="Percent survival", breaks = seq(0, 1, by = 0.1))+
 guides(color = guide_legend(title="Concentration DNP"))+
 scale_color_brewer(palette = "Accent")
dev.off()



############### DNP oxygen consumption  ##############################

####10uM 3 day trials
datum3 = read.csv(file = "InhibitorData10uM.csv")

 datum3$ID <- factor(datum3$ID, levels = c("Control Female", "DNP Female", "Control Male", "DNP
Male"))


mod.2 = lm(abs(Slope_ppm_per_min)~Group * Sex + Weight_mg, data = datum3)
emmeans(mod.2, pairwise ~ Group | Sex)
confint(emmeans(mod.2, pairwise ~ Group | Sex))

#Ridgeline plot
resp.10uM.3days <- ggplot(datum3, aes(x=abs(Slope_ppm_per_min), y=ID, fill = ..x..)) +
  geom_density_ridges_gradient(scale=1.7, bandwidth = 0.3, rel_min_height =0.01,
   jittered_points = TRUE, position = position_points_jitter(width = 0.000001, height = 0), point_shape =
"|", point_size = 5, point_color = "black",
                       quantile_lines = TRUE, quantile_fun = mean, size =1, vline_color = "red3", color =
'white')+
  scale_x_continuous(breaks = seq(0,6, by =1), limits = c(0,6)) +
  theme_ridges(center_axis_labels = TRUE)+
  xlab("") +ylab("")+
  ggtitle(label =  bquote('10'~mu*'M'~'DNP 3 days - red stock copepods'))+
  theme(legend.position = "non")+
  annotate("text", x = 5, y = 3.5, label = expression(italic(n)*"=36,"~italic(p)*"=0.0359"), size = 3)+
  annotate("text", x = 5, y = 1.5, label = expression(italic(n)*"=27,"~italic(p)*"=0.0011"), size = 3)

jpeg(file = "Resp_10uM_3day.jpg", units = "in", width = 7, height = 5, res = 500)
resp.10uM.3days
dev.off()


####10uM 7 day trials
datum4 = read.csv(file = "InhibitorData7Day10uM.csv")

datum4$ID <- factor(datum4$ID, levels = c("Control Female", "DNP Female", "Control Male", "DNP
Male"))


mod.3 = lm(abs(Slope_ppm_per_min)~Group * Sex + Weight_mg, data = datum4)
```

```
emmeans(mod.3, pairwise ~ Group | Sex)

#Ridgeline plot
resp.10uM.7days <- ggplot(datum4, aes(x=abs(Slope_ppm_per_min), y=ID, fill = ..x..)) +
 geom_density_ridges_gradient(scale=1.2, bandwidth = 0.2, rel_min_height =0.01,
   jittered_points = TRUE, position = position_points_jitter(width = 0.00000101, height = 0), point_shape
= "|", point_size = 5, point_color = "black",
   quantile_lines = TRUE, quantile_fun = mean, size =1, vline_color = "red3", color = 'white')+
 scale_x_continuous(breaks = seq(0,6, by =1), limits = c(0,6)) +
 theme_ridges(center_axis_labels = TRUE)+
 xlab("") +ylab("")+
 ggtitle(label =  bquote('10'~mu*'M'~'DNP 7 days - red stock copepods'))+
 theme(legend.position = "non")+
 annotate("text", x = 5, y = 3.5, label = expression(italic(n)*"=18,"~italic(p)*"=0.1416"), size = 3)+
 annotate("text", x = 5, y = 1.5, label = expression(italic(n)*"=18,"~italic(p)*"=0.9646"), size = 3)

jpeg(file = "Resp_10uM_7day.jpg", units = "in", width = 7, height = 5, res = 500)
resp.10uM.7days
dev.off()


###2uM analysis
datum5 = read.csv(file = "InhibitorData7Day2uM.csv")

datum5$ID <- factor(datum5$ID, levels = c("Control Female", "DNP Female", "Control Male", "DNP
Male"))

mod.4 = lm(abs(Slope_ppm_per_min)~Group * Sex + Weight_mg, data = datum5)
emmeans(mod.4, pairwise ~ Group | Sex)
confint(emmeans(mod.4, pairwise ~ Group | Sex))

#Ridgeline plot

 resp.2uM.7day<- ggplot(datum5, aes(x=abs(Slope_ppm_per_min), y=ID, fill = ..x..)) +
   geom_density_ridges_gradient(scale=1.3, bandwidth = 0.2, rel_min_height =0.01,
    jittered_points = TRUE, position = position_points_jitter(width = 0.000001, height = 0), point_shape =
"|", point_size = 5, point_color = "black",
                        quantile_lines = TRUE, quantile_fun = mean, size =1, vline_color = "red3", color =
'white')+
  scale_x_continuous(breaks = seq(0,6, by =1), limits = c(0,6)) +
   theme_ridges(center_axis_labels = TRUE)+
   xlab(bquote('Respiration rate (mmol O2'~min^-1*')')) +ylab("")+
   ggtitle(label =  bquote('2'~mu*'M'~'DNP 7 days - red stock copepods'))+
   theme(legend.position = "non")+
   annotate("text", x = 5, y = 3.5, label = expression(italic(n)*"=22,"~italic(p)*"=0.0346"), size = 3)+
   annotate("text", x = 5, y = 1.5, label = expression(italic(n)*"=28,"~italic(p)*"=0.0357"), size = 3)

 jpeg(file = "Resp_2uM_7day.jpg", units = "in", width = 7, height = 5, res = 500)
```

```
  resp.2uM.7day
  dev.off()



#####Yeast 2 um 7 day #####
datum5yeast = read.csv(file = "Yeast.data.csv")
str(datum5yeast)
#Remove erroneous data point
datum5yeast = datum5yeast[-c(18),]

#Make respiration data absolute
datum5yeast$resp.final = abs(datum5yeast$resp.final)

#order ID factor
datum5yeast$ID <- factor(datum5yeast$ID, levels = c("Control Female", "DNP Female", "Control Male",
"DNP Male"))

#Run model and check results
mod.5 = lm(abs(Slope_mmol_per_min)~Group * Sex +Weight_mg, data = datum5yeast)
emmeans(mod.5, pairwise ~ Group | Sex)
confint(emmeans(mod.5, pairwise ~ Group | Sex))

#Ridgeline plot

resp.2uM.yeast <- ggplot(datum5yeast, aes(x=abs(Slope_mmol_per_min), y=ID, fill = ..x..)) +
  geom_density_ridges_gradient(scale=1.3, bandwidth = 0.1, rel_min_height =0.01,
  jittered_points = TRUE, position = position_points_jitter(width = 0.000001, height = 0), point_shape =
"|", point_size = 5, point_color = "black",
                       quantile_lines = TRUE, quantile_fun = mean, size =1, vline_color = "red3", color =
'white')+
  theme_ridges(center_axis_labels = TRUE)+
  ggtitle(label = bquote('2'~mu*'M'~'DNP 7 days - color-restored copepods'))+
  scale_x_continuous(breaks = seq(0,3, by =0.5), limits = c(0,3)) +
  xlab(bquote('Respiration rate (mmol O2'~min^-1*')')) +ylab("")+
  theme(legend.position = "non")+
  annotate("text", x = 2, y = 3.5, label = expression(italic(n)*"=54,"~italic(p)*"=0.0353"), size = 3)+
  annotate("text", x = 2, y = 1.5, label = expression(italic(n)*"=36,"~italic(p)*"=0.1298"), size = 3)

jpeg(file = "Resp_Yeast_7day_2uM.jpg", units = "in", width = 7, height = 5, res = 500)
resp.2uM.yeast
dev.off()



#Combine algae respiration figures into one three-panel figure
Fig.resp.ridgelines <- ggarrange(resp.10uM.3days, resp.10uM.7days, resp.2uM.7day,
                                 labels = c("A", "B", "C"),
```

```
                    ncol=1, nrow=3, common.legend = FALSE, legend = "none")

jpeg(file="Algae resp ridgelines.jpg", units="in", width=6, height=10, res=500)
Fig.resp.ridgelines
dev.off()

############ HPLC data analysis ############

carotenoid.datum = read.csv(file = "carotenoid.data.csv")

carotenoid.datum$log.resp.min = log(abs(carotenoid.datum$resp.min))

#Split data set into separate data frames
#Plot data and run models for each group comparing across males and females
#algae fed copepods
carotenoid.datum.algae = subset(carotenoid.datum, diet == "algae" )
carotenoid.datum.algae = droplevels(carotenoid.datum.algae)

#Compare all identifiable carotenoids in algae dataset
#Subset desired samples from data frame
datum.algae_sub = carotenoid.datum.algae[,c(1,13,14,15)]
##Then rearrange your data frame
dd.algae = melt(datum.algae_sub, id=c("HPLC.ID"))

mod.carot.comparison <- lm(value~variable, data = dd.algae)
summary(mod.carot.comparison)
emmeans(mod.carot.comparison, pairwise ~ variable)

#Repeat for just beta-carot vs hydroxy
datum.algae_sub.2 = carotenoid.datum.algae[,c(1,14,15)]
##Then rearrange your data frame
dd.algae.2 = melt(datum.algae_sub.2, id=c("HPLC.ID"))

mod.carot.comparison.2 <- lm(value~variable, data = dd.algae.2)
summary(mod.carot.comparison.2)
confint(mod.carot.comparison.2)


#Ridgeline plot for all carots
all.carots.algae <- ggplot(dd.algae, aes(x=value, y=variable, fill = ..x..)) +
  geom_density_ridges_gradient(scale=1.3, bandwidth = 0.1, rel_min_height =0.01,
  jittered_points = TRUE, position = position_points_jitter(width = 0.000001, height = 0), point_shape =
"|", point_size = 5, point_color = "black",
                       quantile_lines = TRUE, quantile_fun = mean, size =1, vline_color = "black", color =
'white')+
  scale_x_continuous(breaks = seq(0,2.5, by =0.5), limits = c(0,3)) +
  theme_ridges(center_axis_labels = TRUE)+
  scale_fill_gradient(low="yellow", high="red")+
```

```
  xlab(bquote('')) +ylab("")+
  ggtitle(label = "Red stock copepods")+
  scale_y_discrete(labels = c('Astaxanthin','Beta-carotene', 'Hydroxyechinenone'))+
  theme(legend.position = "non")

jpeg(file = "carotenoid comparison ridgeline.jpg", units = "in", width = 6, height = 4, res = 500)
all.carots.algae
dev.off()

#Ridgeline plot for just beta carot and hydroxy
dietary.carots.algae <- ggplot(dd.algae.2, aes(x=value, y=variable, fill = ..x..)) +
  geom_density_ridges_gradient(scale=1.3, bandwidth = 0.01, rel_min_height =0.01,
                               jittered_points = TRUE, position = position_points_jitter(width = 0.000001, height = 0),
point_shape = '|', point_size = 5, point_color = "black",
                               quantile_lines = TRUE, quantile_fun = mean, size =1, vline_color = "black", color =
'white')+
  scale_x_continuous(breaks = seq(0,0.2, by =0.05), limits = c(0,0.2)) +
  theme_ridges(center_axis_labels = TRUE)+
  scale_fill_gradient(low="yellow", high="red")+
  xlab(bquote('')) +ylab("")+
  ggtitle(label = "Red stock copepods")+
  scale_y_discrete(labels = c('Beta-carotene', 'Hydroxyechinenone'))+
  theme(legend.position = "non")+
  annotate("text", x = 0.15, y = 1.5, label = expression(italic(n)*"=162,"~italic(p)*"=0.0184"), size = 3)




####Yeast fed copepods
carotenoid.datum.yeast = subset(carotenoid.datum, diet == "yeast" )
carotenoid.datum.yeast = droplevels(carotenoid.datum.yeast)
carotenoid.datum.yeast$ID <- factor(carotenoid.datum.yeast$ID, levels = c("Colorless Female",
"Colorless Male","Control Female", "DNP Female", "Control Male", "DNP Male"))

#Compare all identifiable carotenoids in color restored copepods only (subset out colorless)
#Subset desired samples from data frame
datum.yeast_sub = carotenoid.datum.yeast[c(1:90),c(1,13,14,15)]
##Then rearrange your data frame
dd.yeast = melt(datum.yeast_sub, id=c("HPLC.ID"))

mod.carot.comparison.yeast <- lm(value~variable, data = dd.yeast)
summary(mod.carot.comparison.yeast)
emmeans(mod.carot.comparison.yeast, pairwise ~ variable)

#Repeat for just beta-carot and hydroxy
datum.yeast_sub.2 = carotenoid.datum.yeast[c(1:90),c(1,14,15)]
##Then rearrange your data frame
```

```
dd.yeast.2 = melt(datum.yeast_sub.2, id=c("HPLC.ID"))

mod.carot.comparison.yeast.2 <- lm(value~variable, data = dd.yeast.2)
summary(mod.carot.comparison.yeast.2)
confint(mod.carot.comparison.yeast.2)

#Ridgeline plot of all carotenoids
all.carots.yeast <- ggplot(dd.yeast, aes(x=value, y=variable, fill = ..x..)) +
  geom_density_ridges_gradient(scale=2, bandwidth = 0.1, rel_min_height =0.01,
  jittered_points = TRUE, position = position_points_jitter(width = 0.000001, height = 0), point_shape =
"|", point_size = 5, point_color = "black",
                       quantile_lines = TRUE, quantile_fun = mean, size =1, vline_color = "black", color =
'white')+
  scale_x_continuous(breaks = seq(0,2.5, by =0.5), limits = c(0,3)) +
  theme_ridges(center_axis_labels = TRUE)+
  scale_fill_gradient(low="yellow", high="red")+
  xlab(bquote('Concentration ('~mu*'g'~'mg'^-1*') across all samples')) +ylab("")+
  ggtitle(label = "Color-restored copepods")+
  scale_y_discrete(labels = c('Astaxanthin','Beta-carotene', 'Hydroxyechinenone'))+
  theme(legend.position = "non")

jpeg(file = "carotenoid comparison ridgeline yeast.jpg", units = "in", width = 6, height = 4, res = 500)
all.carots.yeast
dev.off()

#Ridgeline plot for just beta carot and hydroxy
dietary.carots.yeast <- ggplot(dd.yeast.2, aes(x=value, y=variable, fill = ..x..)) +
  geom_density_ridges_gradient(scale=1.3, bandwidth = 0.01, rel_min_height =0.01,
  jittered_points = TRUE, position = position_points_jitter(width = 0.000001, height = 0), point_shape =
"|", point_size = 5, point_color = "black",
                       quantile_lines = TRUE, quantile_fun = mean, size =1, vline_color = "black", color =
'white')+
  scale_x_continuous(breaks = seq(0,0.2, by =0.05), limits = c(0,0.2)) +
  theme_ridges(center_axis_labels = TRUE)+
  scale_fill_gradient(low="yellow", high="red")+
  xlab(bquote('Concentration ('~mu*'g'~'mg'^-1*') across all samples')) +ylab("")+
  ggtitle(label = "Color-restored copepods")+
  scale_y_discrete(labels = c('Beta-carotene', 'Hydroxyechinenone'))+
  theme(legend.position = "non")+
  annotate("text", x = 0.15, y = 1.5, label = expression(italic(n)*"=180,"~italic(p)*"<0.001"), size = 3)

#Combine yeast and algae carotenoids data into one figure
Fig.all.carots <- ggarrange(all.carots.algae,  dietary.carots.algae, all.carots.yeast, dietary.carots.yeast,
                            labels = c("A", "B", "C", "D"),
                            ncol=2, nrow=2, common.legend = FALSE, legend = "none")

jpeg(file="All carots.jpg", units="in", width=11.5, height=7, res=500)
Fig.all.carots
```

```
dev.off()



#Comparing astaxanthin concentration (subset out colorless copepods)
mod.yeast = lm(asta.conc~treatment * sex, data = carotenoid.datum.yeast[1:90,])
summary(mod.yeast)
emmeans(mod.yeast, pairwise ~ treatment | sex)
confint(emmeans(mod.yeast, pairwise ~ treatment | sex))

#Comparing astaxanthin concentration in colorless males and females
mod.yeast.b = lm(asta.conc~ID, data = carotenoid.datum.yeast[91:103,])
summary(mod.yeast.b)

#Compare astaxanthin concentration in colorless to control and DNP copepods
mod.yeast.c = lm(asta.conc~ID * sex, data = carotenoid.datum.yeast)
emmeans(mod.yeast.c, pairwise ~ ID | sex)


#Ridgeline plot
asta.2uM.yeast <- ggplot(carotenoid.datum.yeast, aes(x=asta.conc, y=ID, fill = ..x..)) +
  geom_density_ridges_gradient(scale=1.7, bandwidth = 0.03, rel_min_height =0.01,
  jittered_points = TRUE, position = position_points_jitter(width = 0.000001, height = 0), point_shape =
"|", point_size = 5, point_color = "black",
                       quantile_lines = TRUE, quantile_fun = mean, size =1, vline_color = "blue", color =
'white')+
  scale_x_continuous(breaks = seq(0,1, by =0.2), limits = c(0,1)) +
  theme_ridges(center_axis_labels = TRUE)+
  scale_fill_gradient(low="grey", high="red3")+
  xlab(bquote('Astaxanthin ('~mu*'g'~'mg'^-1*'dry body weight)')) +ylab("")+
  ggtitle(label = bquote('2'~mu*'M'~'DNP 7 days - color-restored copepods'))+
  theme(legend.position = "non")+
  annotate("text", x = 0.88, y = 3.5, label = expression(italic(n)*"=36,"~italic(p)*"=0.0312"), size = 3)+
  annotate("text", x = 0.88, y = 5.5, label = expression(italic(n)*"=54,"~italic(p)*"=0.5836"), size = 3)+
  annotate("text", x = 0.35, y = 1.5, label = expression(italic(n)*"=12"), size = 3)

jpeg(file = "Asta_Yeast_7day_2uM.jpg", units = "in", width = 7, height = 5, res = 500)
asta.2uM.yeast
dev.off()



#Combine 2uM yeast respiration figure and asta figure into one
Fig.2uM.yeast.resp.and.asta <- ggarrange(resp.2uM.yeast, asta.2uM.yeast,
                       labels = c("A", "B"),
                       ncol=1, nrow=2, common.legend = FALSE, legend = "none")

jpeg(file="Resp and asta figure 2uM yeast.jpg", units="in", width=6, height=9, res=500)
```

```
Fig.2uM.yeast.resp.and.asta
dev.off()



#Comparing dietary carotenoid concentration
mod.yeast.dietary = lm(dietary.conc~treatment * sex, data = carotenoid.datum.yeast)
summary(mod.yeast.dietary)
emmeans(mod.yeast.dietary, pairwise ~ treatment | sex)
#Ridgeline plot

dietary.2uM.yeast<- ggplot(carotenoid.datum.yeast[1:90,], aes(x=dietary.conc, y=ID, fill = ..x..)) +
  geom_density_ridges_gradient(scale=1.1, bandwidth = 0.003, rel_min_height =0.01,
   jittered_points = TRUE, position = position_points_jitter(width = 0.00000001, height = 0), point_shape
= "|", point_size = 5, point_color = "black",
                       quantile_lines = TRUE, quantile_fun = mean, size =1, vline_color = "blue", color =
'white')+
  scale_x_continuous(breaks = seq(0,0.1, by =0.02), limits = c(0,0.1)) +
  theme_ridges(center_axis_labels = TRUE)+
  scale_fill_gradient(low="grey", high="red3")+
  xlab(bquote('Dietary carotenoids ('~mu*'g'~'mg'^-1*'dry body weight)')) +ylab("")+
  ggtitle(label = bquote('2'~mu*'M'~'DNP 7 days - color-restored copepods'))+
  theme(legend.position = "non")+
  annotate("text", x = 0.08, y = 1.5, label = expression(italic(n)*"=36,"~italic(p)*"=0.5270"), size = 3)+
  annotate("text", x = 0.08, y = 3.5, label = expression(italic(n)*"=54,"~italic(p)*"=0.7660"), size = 3)

jpeg(file = "Dietary_Yeast_7day_2uM.jpg", units = "in", width = 7, height = 5, res = 500)
dietary.2uM.yeast
dev.off()



####10uM copepods for 3 days
carotenoid.datum.10uM = subset(carotenoid.datum.algae, conc.dnp == "10uM")
carotenoid.datum.10uM = droplevels(carotenoid.datum.10uM)
carotenoid.datum.10uM$ID <- factor(carotenoid.datum.10uM$ID, levels = c("Control Female", "DNP
Female", "Control Male", "DNP Male"))

#Comparing astaxanthin concentration
mod.10uM = lm(asta.conc~treatment * sex , data = carotenoid.datum.10uM)
summary(mod.10uM)
emmeans(mod.10uM, pairwise ~ treatment | sex)
#Ridgeline plot

asta.10uM.3day <- ggplot(carotenoid.datum.10uM, aes(x=asta.conc, y=ID, fill = ..x..)) +
  geom_density_ridges_gradient(scale=1.1, bandwidth = 0.15, rel_min_height =0.01,
```

```
  jittered_points = TRUE, position = position_points_jitter(width = 0.000001, height = 0), point_shape =
"|", point_size = 5, point_color = "black",
                      quantile_lines = TRUE, quantile_fun = mean, size =1, vline_color = "blue", color =
'white')+
  scale_x_continuous(breaks = seq(0,3, by =0.5), limits = c(0,3)) +
  theme_ridges(center_axis_labels = TRUE)+
  scale_fill_gradient(low="grey", high="red3")+
  xlab(bquote('')) +ylab("")+
  ggtitle(label = bquote('10'~mu*'M'~'DNP 3 days - red stock copepods'))+
  theme(legend.position = "non")+
  annotate("text", x = 2.5, y = 1.5, label = expression(italic(n)*"=22,"~italic(p)*"=0.5159"), size = 3)+
  annotate("text", x = 2.5, y = 3.5, label = expression(italic(n)*"=24,"~italic(p)*"=0.9071"), size = 3)


jpeg(file = "Asta_10uM_3day.jpg", units = "in", width = 7, height = 5, res = 500)
asta.10uM.3day
dev.off()

#Comparing dietary carotenoid concentration
mod.10uM.dietary = lm(dietary.conc~treatment * sex, data = carotenoid.datum.10uM)
summary(mod.10uM.dietary)
emmeans(mod.10uM.dietary, pairwise ~ treatment | sex)
#Ridgeline plot

dietary.10uM.3day <- ggplot(carotenoid.datum.10uM, aes(x=dietary.conc, y=ID, fill = ..x..)) +
  geom_density_ridges_gradient(scale=1.1, bandwidth = 0.009, rel_min_height =0.01,
   jittered_points = TRUE, position = position_points_jitter(width = 0.000001, height = 0), point_shape =
"|", point_size = 5, point_color = "black",
                      quantile_lines = TRUE, quantile_fun = mean, size =1, vline_color = "blue", color =
'white')+
  scale_x_continuous(breaks = seq(0,0.2, by =0.05), limits = c(0,0.2)) +
  theme_ridges(center_axis_labels = TRUE)+
  scale_fill_gradient(low="grey", high="red3")+
  xlab(bquote('')) +ylab("")+
  ggtitle(label = bquote('10'~mu*'M'~'DNP 3 days - red stock copepods'))+
  theme(legend.position = "non")+
  annotate("text", x = 0.15, y = 1.5, label = expression(italic(n)*"=22,"~italic(p)*"=0.9594"), size = 3)+
  annotate("text", x = 0.15, y = 3.5, label = expression(italic(n)*"=24,"~italic(p)*"=0.5441"), size = 3)

jpeg(file = "Dietary_10uM_3day.jpg", units = "in", width = 7, height = 5, res = 500)
dietary.10uM.3day
dev.off()
```

```
#2uM copepods for 7 days
carotenoid.datum.2uM = subset(carotenoid.datum.algae, conc.dnp == "2uM")
carotenoid.datum.2uM = droplevels(carotenoid.datum.2uM)
carotenoid.datum.2uM$ID <- factor(carotenoid.datum.2uM$ID, levels = c("Control Female", "DNP
Female", "Control Male", "DNP Male"))

#Comparing astaxanthin concentration
mod.2uM = lm(asta.conc~treatment * sex, data = carotenoid.datum.2uM)
summary(mod.2uM)
emmeans(mod.2uM, pairwise ~ treatment | sex)
confint(emmeans(mod.2uM, pairwise ~ treatment | sex))
#Ridgeline plot

asta.2uM.7days <- ggplot(carotenoid.datum.2uM, aes(x=asta.conc, y=ID, fill = ..x..)) +
  geom_density_ridges_gradient(scale=1.3, bandwidth = 0.15, rel_min_height =0.01,
  jittered_points = TRUE, position = position_points_jitter(width = 0.000001, height = 0), point_shape =
"|", point_size = 5, point_color = "black",
                       quantile_lines = TRUE, quantile_fun = mean, size =1, vline_color = "blue", color =
'white')+
  scale_x_continuous(breaks = seq(0,3, by =0.5), limits = c(0,3)) +
  theme_ridges(center_axis_labels = TRUE)+
  scale_fill_gradient(low="grey", high="red3")+
  xlab(bquote('Astaxanthin ('~mu*'g'~'mg'^-1*'dry body weight)')) +ylab("")+
  ggtitle(label = bquote('2'~mu*'M'~'DNP 7 days - red stock copepods'))+
  theme(legend.position = "non")+
  annotate("text", x = 2.5, y = 1.5, label = expression(italic(n)*"=20,"~italic(p)*"=0.3948"), size = 3)+
  annotate("text", x = 2.5, y = 3.5, label = expression(italic(n)*"=15,"~italic(p)*"=0.0042"), size = 3)

jpeg(file = "Asta_2uM_7day.jpg", units = "in", width = 7, height = 5, res = 500)
asta.2uM.7days
dev.off()

#Comparing dietary carotenoid concentration
mod.2uM.dietary = lm(dietary.conc~treatment * sex, data = carotenoid.datum.2uM)
summary(mod.2uM.dietary)
emmeans(mod.2uM.dietary, pairwise ~ treatment | sex)
#Ridgeline plot

dietary.2uM.7days <- ggplot(carotenoid.datum.2uM, aes(x=dietary.conc, y=ID, fill = ..x..)) +
  geom_density_ridges_gradient(scale=1.1, bandwidth = 0.009, rel_min_height =0.01,
  jittered_points = TRUE, position = position_points_jitter(width = 0.000001, height = 0), point_shape =
"|", point_size = 5, point_color = "black",
                       quantile_lines = TRUE, quantile_fun = mean, size =1, vline_color = "blue", color =
'white')+
  scale_x_continuous(breaks = seq(0,0.2, by =0.05), limits = c(0,0.2)) +
  theme_ridges(center_axis_labels = TRUE)+
  scale_fill_gradient(low="grey", high="red3")+
  xlab(bquote('Dietary carotenoids ('~mu*'g'~'mg'^-1*'dry body weight)')) +ylab("")+
```

```
  ggtitle(label = bquote('2'~mu*'M'~'DNP 7 days - red stock copepods'))+
  theme(legend.position = "non")+
  annotate("text", x = 0.15, y = 1.5, label = expression(italic(n)*"=20,"~italic(p)*"=0.8594"), size = 3)+
  annotate("text", x = 0.15, y = 3.5, label = expression(italic(n)*"=15,"~italic(p)*"=0.3012"), size = 3)

jpeg(file = "Dietary_2uM_7day.jpg", units = "in", width = 7, height = 5, res = 500)
dietary.2uM.7days
dev.off()



#Combine astaxanthin figures for 10uM 3day and 2uM 7day into one
Fig.asta.10uM.and.2uM <- ggarrange(asta.10uM.3day, asta.2uM.7days,
                          labels = c("A", "B"),
                          ncol=1, nrow=2, common.legend = FALSE, legend = "none")

jpeg(file="Asta 10uM and 2uM.jpg", units="in", width=6, height=8, res=500)
Fig.asta.10uM.and.2uM
dev.off()

#Combine dietary carotenoids figures for 10uM 3day and 2uM 7day into one
Fig.dietary.10uM.and.2uM <- ggarrange(dietary.10uM.3day, dietary.2uM.7days,
                          labels = c("A", "B"),
                          ncol=1, nrow=2, common.legend = FALSE, legend = "none")

jpeg(file="Dietary 10uM and 2uM.jpg", units="in", width=6, height=8, res=500)
Fig.dietary.10uM.and.2uM
dev.off()



####Comparing astaxanthin vs respiration controlling for effect of diet and sex####
#DNP copepods
mod.asta.vs.resp.DNP = lmer(asta.conc~abs(resp.min.mg) + sex + (1|diet),
                    data = subset(carotenoid.datum, treatment == "DNP"))
summary(mod.asta.vs.resp.DNP)
confint(mod.asta.vs.resp.DNP)
r.squaredGLMM(mod.asta.vs.resp.DNP)
#plot asta vs resp
asta.vs.resp.dnp <- subset(carotenoid.datum, treatment == "DNP") %>%
  ggplot(aes(x = abs(resp.min.mg), y=asta.conc)) +
  geom_point(aes(y = asta.conc),size = 5, alpha =0.9)+
  scale_x_continuous(breaks = seq(0, 80, by =20))+
  geom_smooth(method='lm')+
  xlab(bquote('Respiration rate (mmol O2'~min^-1~'mg'^-1*')'))+
  ylab(bquote('Astaxanthin ('~mu*'g'~'mg'^-1*')'))  +
```

```
  ggtitle(label = "DNP treated")+
  theme(axis.text = element_text(size = 16),
        axis.title = element_text(size = 18))+
  annotate("text", 70, 0.5, label =  bquote('p=0.029, R'^2*'=0.754'), size=4)

#Control copepods
mod.asta.vs.resp.control = lmer(asta.conc~abs(resp.min.mg)+ sex+(1|diet),
                        data = subset(carotenoid.datum, treatment == "Control"))
summary(mod.asta.vs.resp.control)
r.squaredGLMM(mod.asta.vs.resp.control)
#plot asta vs resp
asta.vs.resp.control <- subset(carotenoid.datum, treatment == "Control") %>%
  ggplot(aes(x = abs(resp.min.mg), y=asta.conc)) +
  geom_point(aes(y = asta.conc),size = 5, alpha =0.9)+
  geom_smooth(method='lm')+
  xlab(bquote('Respiration rate (mmol O2'~min^-1~'mm'^-1*')'))+
  ylab(bquote('Astaxanthin ('~mu*'g'~'mg'^-1*')'))  +
  ggtitle(label = "Control")+
  theme(axis.text = element_text(size = 16),
        axis.title = element_text(size = 18))+
  annotate("text", 70, 0.5, label =  bquote('p=0.565, R'^2*'=0.768'), size=4)


###Comparing dietary carotenoids vs respiration controlling for effect of diet
xtrans <- bestNormalize(carotenoid.datum$dietary.conc)
carotenoid.datum$tf.dietary = xtrans$x.t

#DNP copepods
mod.dietary.vs.resp.DNP = lmer(tf.dietary~abs(resp.min.mg) + sex + (1|diet),
                        data = subset(carotenoid.datum, treatment == "DNP"))
summary(mod.dietary.vs.resp.DNP)
confint(mod.dietary.vs.resp.DNP)
r.squaredGLMM(mod.dietary.vs.resp.DNP)
#plot asta vs resp
dietary.vs.resp.dnp <- subset(carotenoid.datum, treatment == "DNP") %>%
  ggplot(aes(x = abs(resp.min.mg), y=tf.dietary)) +
  geom_point(aes(y = tf.dietary),size = 5, alpha =0.9)+
  scale_x_continuous(breaks = seq(0, 80, by =20))+
  geom_smooth(method='lm')+
  xlab(bquote('Respiration rate (mmol O2'~min^-1~'mg'^-1*')'))+
  ylab(bquote('Dietary carotenoids concentration (transformed)'))  +
  ggtitle(label = "DNP treated")+
  theme(axis.text = element_text(size = 15),
        axis.title = element_text(size = 14))+
  annotate("text", 75, -1, label =  bquote('p=0.016, R'^2*'=0.134'), size=4)

#Control copepods
mod.dietary.vs.resp.control = lmer(tf.dietary~abs(resp.min.mg)+ sex+(1|diet),
```

```
                        data = subset(carotenoid.datum, treatment == "Control"))
summary(mod.dietary.vs.resp.control)
confint(mod.dietary.vs.resp.control)
r.squaredGLMM(mod.dietary.vs.resp.control)
#plot asta vs resp
dietary.vs.resp.control <- subset(carotenoid.datum, treatment == "Control") %>%
  ggplot(aes(x = abs(resp.min.mg), y=tf.dietary)) +
  geom_point(aes(y = tf.dietary),size = 5, alpha =0.9)+
  geom_smooth(method='lm')+
  xlab(bquote('Respiration rate (mmol O2'~'min^-1~'mg'^-1*')'))+
  ylab(bquote('Dietary carotenoids concentration (transformed)'))  +
  ggtitle(label = "Control")+
  theme(axis.text = element_text(size = 15),
        axis.title = element_text(size = 14))+
  annotate("text", 70, -1, label =  bquote('p=0.608, R'^2*'=0.013'), size=4)


#Combine ALL scatterplots (astaxanthin and dietary) into one four-panel figure
Fig.carotenoids.vs.resp.overall <- ggarrange(asta.vs.resp.control, asta.vs.resp.dnp,
dietary.vs.resp.control, dietary.vs.resp.dnp,
                                    labels = c("A", "B", "C", "D"),
                                  ncol=2, nrow=2, common.legend = TRUE, legend = "top")

jpeg(file="carotenoids.vs.resp.scatters.jpg", units="in", width=11, height=11, res=500)
Fig.carotenoids.vs.resp.overall
dev.off()



##Is there a relationship between astaxanthin and dietary carotenoids?
#DNP copepods
mod.dietary.vs.asta = lmer(asta.conc~tf.dietary+ sex+(1|diet),
                           data = subset(carotenoid.datum, treatment == "DNP"))
summary(mod.dietary.vs.asta)
r.squaredGLMM(mod.dietary.vs.asta)

#plot asta vs dietary
dietary.vs.asta.dnp <- subset(carotenoid.datum, treatment == "DNP") %>%
  ggplot(aes(x = tf.dietary, y=asta.conc)) +
  geom_point(aes(y = asta.conc),size = 5, alpha =0.9)+
  geom_smooth(method='lm')+
  xlab(bquote('Dietary carotenoids concentration (transformed'))+
  ylab(bquote('Astaxanthin ('~mu*'g'~'mg'^-1*')'))  +
  ggtitle(label = "DNP")+
  theme(axis.text = element_text(size = 14),
        axis.title = element_text(size = 15))+
  annotate("text", 2, 0, label =  bquote('p=0.894, R'^2*'=0.777'), size=4)
```

```
#Control copepods
mod.dietary.vs.asta.control = lmer(asta.conc~tf.dietary+ sex+(1|diet),
                    data = subset(carotenoid.datum, treatment == "Control"))
summary(mod.dietary.vs.asta.control)
confint(mod.dietary.vs.asta.control)
r.squaredGLMM(mod.dietary.vs.asta.control)

#plot asta vs dietary
dietary.vs.asta.control <- subset(carotenoid.datum, treatment == "DNP") %>%
 ggplot(aes(x = tf.dietary, y=asta.conc)) +
 geom_point(aes(y = asta.conc),size = 5, alpha =0.9)+
 geom_smooth(method='lm')+
 xlab(bquote('Dietary carotenoids concentration (transformed)'))+
 ylab(bquote('Astaxanthin ('~mu*'g'~'mg'^-1*')'))  +
 ggtitle(label = "Control")+
 theme(axis.text = element_text(size = 14),
      axis.title = element_text(size = 15))+
 annotate("text", 1.5, 0, label =  bquote('p=0.001, R'^2*'=0.774'), size=4)

#Combine scatterplots into one figure
Fig.dietary.vs.asta <- ggarrange(dietary.vs.asta.control, dietary.vs.asta.dnp,  labels = c("A", "B"),
                          ncol=2, nrow=1, common.legend = TRUE, legend = "top")

jpeg(file="dietary.vs.asta.scatters.jpg", units="in", width=11, height=6, res=500)
Fig.dietary.vs.asta
dev.off()



#Is there a significant difference in mass between DNP-treated and control copepods?
mod.mass = lmer(mass~treatment + sex + (1|diet), data = carotenoid.datum[1:171,])
summary(mod.mass)
emmeans(mod.mass, pairwise~ treatment)

#Try with fixed effect of diet to see if difference between diets
mod.mass2 = lm(mass~treatment + sex + diet, data = carotenoid.datum[1:171,])
summary(mod.mass2)


#Ridgeline plot (subset out colorless data, irrelevant)
jpeg(file = "Mass_vs_treatment.jpg", units = "in", width = 7, height = 5, res = 500)
ggplot(carotenoid.datum[1:171,], aes(x=mass, y=treatment, fill = ..x..)) +
 geom_density_ridges_gradient(scale=1.1, bandwidth = 0.003, rel_min_height =0.01,
 jittered_points = TRUE, position = position_points_jitter(width = 0.000001, height = 0), point_shape =
"|", point_size = 5, point_color = "black",
                    quantile_lines = TRUE, quantile_fun = mean, size =1, vline_color = "black", color =
'white')+
 scale_x_continuous(breaks = seq(0,0.1, by =0.025), limits = c(0,0.1)) +
```

```
  theme_ridges(center_axis_labels = TRUE)+
  scale_fill_gradient(low="grey", high="darkgreen")+
  xlab(bquote('Mass (mg dry body weight)')) +ylab("")+
  theme(legend.position = "non")+
  annotate("text", x = 0.085, y = 1.5, label = expression(italic(n)*"=171,"~italic(p)*"=0.945"), size = 3)
dev.off()


#Is there a significant difference in respiration rate between algae raised and yeast raised copepods?
mod.resp.diet = lm(abs(resp.min)~diet + mass + sex, data = carotenoid.datum)
summary(mod.resp.diet)
emmeans(mod.resp.diet, pairwise~ treatment)


#Ridgeline plot (subset out colorless data, irrelevant)
jpeg(file = "resp.vs.diet.jpg", units = "in", width = 7, height = 5, res = 500)
ggplot(carotenoid.datum, aes(x=abs(resp.min.mg), y=diet, fill = ..x..)) +
  geom_density_ridges_gradient(scale=1.7, bandwidth = 5, rel_min_height =0.01,
                               jittered_points = TRUE, position = position_points_jitter(width = 0.000001, height = 0),
point_shape = "|", point_size = 5, point_color = "black",
                               quantile_lines = TRUE, quantile_fun = mean, size =1, vline_color = "black", color =
'white')+
  scale_x_continuous(breaks = seq(0,100, by =20), limits = c(0,100)) +
  theme_ridges(center_axis_labels = TRUE)+
  scale_fill_gradient(low="lightblue", high="darkblue")+
  xlab(bquote('Respiration rate (mmol O2'~'min^-1~'mg'^-1*')')) +ylab("")+
  theme(legend.position = "non")+
  annotate("text", x = 80, y = 1.8, label = expression(italic(n)*"=183,"~italic(p)*"<0.001"), size = 3.5)
dev.off()



####Chromatogram figure####
datumhplc = read.csv(file = "hplc.chromatograms.csv")

#Trim first 13 minutes off to remove solvent front peak and reduce white space of graph
#trim off last 5 minutes to remove equilibration period and reduce white space of graph
datumhplctrim = subset(datumhplc, time >=13 & time <=26)

#Subset desired samples from data frame
dd_sub = datumhplctrim[,c(1,3,4,7,8)]
dd_subtetra = datumhplctrim[,c(1,2,3,4)]

#You will notice numbers after the Y variable call for the standard mix. This is just an adjustment to
#all of the values of the intensity column for that standard to correct for the baseline drift
#of the HPLC system

#Adjust y values for baseline drift during hplc
```

```
dd_sub$X3HE = dd_sub$X3HE+200
dd_subtetra$X3HE = dd_subtetra$X3HE+200

##Then rearrange your data frame
dd = melt(dd_sub, id=c("time"))
ddtetra = melt(dd_subtetra, id=c("time"))

#Make plot comparing DNP and control copepod chromatograms representatives
chromatogram.yeast <- ggplot(dd) + geom_line(aes(x=time, y=value, colour=variable)) +
  scale_colour_manual(name="",values=c("grey","goldenrod3", "red", "blue"),
                      labels=c("Standard Mix (Asta, zeax, canthax, beta-carot)","Hydroxyechinenone standard",
"Control", "DNP"))+
  scale_y_continuous(breaks=seq(0,8000,1000))+
  scale_x_continuous(breaks=seq(15,27,3))+
  ylab("Intensity (mv)")+ xlab("Time (min)")+
  annotate("text", x = 15.7, y = 4800, label = "1",size = 4, color ="black")+
  annotate("text", x = 17.4, y = 5300, label = "2*", size = 4, color ="black")+
  annotate("text", x = 19, y = 4800, label = "3*", size = 4, color ="black")+
  annotate("text", x = 20.5, y = 8000, label = "4", size = 4, color ="black")+
  annotate("text", x = 23.9, y = 6800, label = "5",  size = 4, color ="black")+
  annotate("text", x = 24, y = 8000, label = "*not detected in any copepod samples",  size = 3, color
="black")+
  guides(colour = guide_legend(override.aes = list(size=2), nrow = 2, ncol = 2))+
  theme(legend.position = "top", legend.direction = "horizontal")+
  theme(plot.margin = unit(c(0,0,0,0), "cm"))

#Print plot
jpeg(filename = "representative chromatogram.jpg", units = "in", width = 7, height = 5, res=500)
chromatogram.yeast
dev.off()
```