ADAPTIVE ADVANTAGES OF CAROTENOID PIGMENTS IN ALPINE AND SUBALPINE COPEPOD RESPONSES TO POLYCYCLIC AROMATIC HYDROCARBON INDUCED PHOTOTOXICITY

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Alpine zooplankton are exposed to a variety of stressors in their natural environment including ultraviolet radiation. Physiological coping mechanisms such as the accumulation of photoprotective compounds provide these zooplankton protection from many of these stressors. Elevated levels of carotenoid compounds such as astaxanthin have been shown to help zooplankton survive longer when exposed to ultraviolet radiation presumably due to the strong antioxidant properties of carotenoid compounds. This antioxidant capacity is important because it may ameliorate natural and anthropogenic stressor-induced oxidative stress. While previous researchers have shown carotenoid compounds impart increased resistance to ultraviolet radiation in populations of zooplankton, little work has focused on the toxicological implications of PAH induced phototoxicity on zooplankton containing high levels of carotenoid compounds. This thesis discusses research studying the role that carotenoid compounds play in reducing PAH induced phototoxicity. By sampling different lakes at elevations ranging from 9,500 to 12,700 ft. in the front range of the Colorado Rocky Mountains, copepod populations containing different levels of carotenoid compounds were obtained. These populations were then challenged with fluoranthene and ultraviolet radiation. Results discussed include differences in survival and levels of lipid peroxidation among populations exhibiting different levels of carotenoid compounds, and the toxicological and ecological implications of these results.

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Introduction

Organisms native to alpine lakes are adapted to the unique conditions found in these oligotrophic ecosystems. Stressors such as ultraviolet radiation (UVR) and cold temperature play a major role in the ecology of the native flora and fauna. At the producer level are the autotrophic phytoplankton. These organisms are preyed upon by filter feeding zooplankton (France, 1995). In some fishless systems these filter feeders may occupy the highest trophic level. In many other lakes and ponds, fish, predatory invertebrates, and other predators feed on these zooplankton (McNaught et al., 1999).

The freshwater macrozooplankton can be classified into two main groups – the cladocerans and the copepods. Both of these groups are invertebrates within the phylum Arthropoda, subphylum Crustacea. Cladocerans (class Branchiopoda, order Cladocera) consist of the daphnids, including such well known genera as *Ceriodaphnia* and *Daphnia*. In alpine lakes and ponds, cladocerans are present though less abundant relative to other taxa (Knapp et al., 2001). Copepods (class Maxillopoda) are the other major category of freshwater zooplankton. The copepods can be divided into two orders – Cyclopoida and Calanoida. Calanoid copepods make up the largest proportion of the pelagic macroinvertebrate community in many alpine lakes, and are the group that this thesis is focused on.

All of the zooplankton found within alpine lakes have particular adaptations that allow them to cope with the stressors found in these environments. UVR is a common natural stressor found in both terrestrial and aquatic ecosystems. The term UVR encompasses three different bands: UV-A, UV-B, and UV-C. UV-C (wavelength 220-290nm) is essentially nonexistent in most ecosystems because it is absorbed rapidly by the atmosphere. The compounds that make up the atmosphere, particularly ozone, absorb UV-B (wavelength 290-320nm), but less effectively

than UV-C. This results in UV-B being present in the environment in measurable levels, as opposed to UV-C. UV-A (wavelength 320-400nm is the most prevalent of the UVR bands, being absorbed by the atmosphere less effectively than UV-C or UV-B and thus being the most common type of UVR found in ecosystems.

Though much of the UVR that enters our atmosphere is deflected or absorbed by the atmosphere itself, the portion that reaches the surface of the earth can be damaging to organisms. With an increase in elevation, and thus a decrease in overhead atmospheric thickness, UV-B and UV-A strength increases. This results in alpine ecosystems being exposed to much higher levels of UVR than lower-elevation ecosystems.

Water, and the materials suspended and dissolved therein (such as dissolved organic carbon, phytoplankton, etc.), can both absorb and reflect UVR. In the water column, as depth increases, UVR intensity decreases. Due to their oligotrophic nature, these water bodies are commonly low in both primary productivity as well as dissolved organic carbon, resulting in greater water transparency compared to lower-elevation environments (Sommaruga, 2001).

When photons of UV wavelength (primarily in the UV-B range) hit biological tissue, damage is often in the form of the generation of reactive oxygen species (ROS) which readily react with biological tissues (Hansson et al., 2007). If the UVR-induced ROS is produced in or near lipid-rich tissues like lipid stores or cellular membranes, lipid peroxidation may result (Ito, 1978). Another major cause of damage to biological tissue is through UVR induced DNA damage (Zagarese et al., 1997). DNA can be damaged by UVR via the formation of thymine dimers between adjacent base-pairs, which, depending upon the location may be carcinogenic or result in heritable implications for offspring (Ito, 1978).

While UVR on its own can result in damage to tissues, there is a class of phototoxic polycyclic-aromatic-hydrocarbon (PAH) pollutants that can, in the presence of UVR, exhibit synergistic effects resulting in increased levels of biological tissue damage (Arfsten et al., 1996). Fluoranthene (Figure 1) is one of these compounds. It is a highly

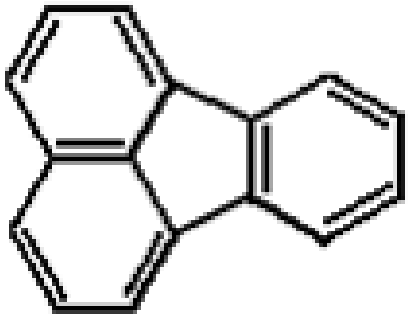

Figure 1. Structure of fluoranthene molecule.

lipophilic compound and thus accumulates readily in biological tissue such as lipid membranes. Fluoranthene can absorb electromagnetic energy in the range of UV-A wavelengths, creating excited singlet and triplet state molecules, which can result in the production of ROS (Newsted and Giesy, 1987). As fluoranthene readily accumulates in lipid-rich biological membranes, generated ROS can easily result in lipid peroxidation (Choi and Oris, 2000).

One behavioral mechanism zooplankton utilize to cope with UVR stress is diel vertical migration (DVM). Phytoplankton densities are typically highest in the uppermost, warmest depths of most lakes, correlating with the highest in-lake levels of solar irradiance and temperatures most beneficial to autotrophic phytoplankton (Loose and Dawidowicz, 1994). These shallow depths also tend to harbor higher concentrations of visual predators, such as fish, that actively predate on zooplankton. By migrating up into the surface waters at dusk, zooplankton are able to feed in surface waters while decreasing the probability that they will be consumed by visual predators. At the same time, the zooplankton are able to decrease the amount

of UVR they are exposed to if they were feeding at the surface during the daytime (Figure 2; Santer, 1998; Rhode et al., 2001).

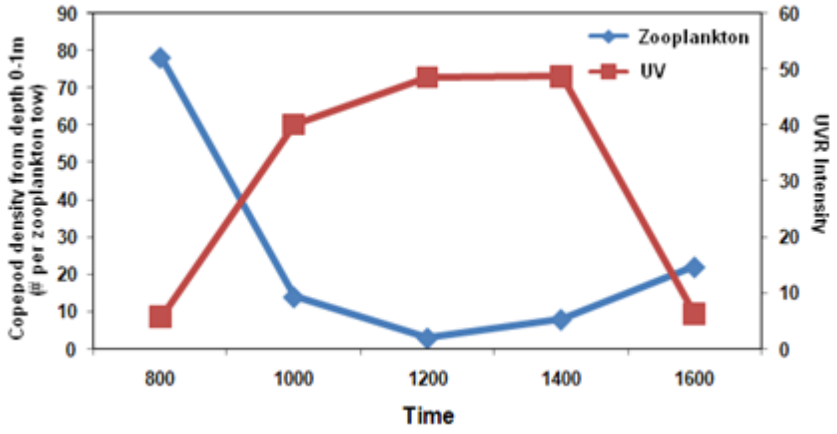

Figure 2. UVR induced diel vertical migration in copepods from Crater Lake, Mt. Blanca, CO on August 2, 2008. Adapted from Roberts et al. unpublished data.

While migrating deeper in the water column is an adequate method to reduce UVR and predation stressors, it is not without costs. Temperature plays an important role in zooplankton DVM. Zooplankton are poikilotherms and their metabolic rate is directly correlated with their surrounding temperature. By staying in the colder, deeper waters in the lake during the daytime, a zooplankton's metabolic rate is slowed enough to significantly reduce growth and reproduction compared to a zooplankton exposed to the warmer surface water temperature (Loose and Dawidowicz, 1994). Though DVM is an extremely effective UVR avoidance mechanism, it is only available to those organisms that live in relatively deep lakes. For organisms that live in shallower environments, UVR exposure may be unavoidable by DVM. Even in very deep lakes, a small fraction of ambient UVR may reach the deepest waters.

Zooplankton also possess physiological adaptations for ameliorating the effects of UVR including the accumulation of photoprotective compounds such as mycosporine-like amino acids

(MAAs). MAAs are well documented UVR absorbing compounds. These compounds are acquired through the diet of zooplankton, as animals lack the synthetic pathway required for *de novo* MAA synthesis (Shick and Dunlap, 2002). Once acquired, they have been shown to provide UVR protection to freshwater copepods (Moeller et al., 2005; Sommaruga, 2001; Persaud et al., 2007). While MAAs are effective UVR screening compounds, some harmful UVR may still be absorbed by sensitive biological tissue – including DNA and lipids.

Another physiological UVR-coping strategy in freshwater zooplankton is DNA repair. In the event that DNA experiences oxidative stress and is subsequently damaged, organisms have mechanisms to identify and repair or replace the damaged section of DNA (Karentz et al., 1994). Photorepair via longer wavelength light (380-450 nm) dependent enzymes have been shown to repair UVR-damaged DNA in copepods. DNA repair mechanisms seem to play a critical role in freshwater zooplankton surviving and coping with UVR (Zagarese et al., 1997).

While MAAs and photorepair have been shown to be effective at absorbing some UVR that hits an organism and repairing UVR damage to DNA, the threat of lipid peroxidation has not been extensively studied in alpine zooplankton. Antioxidant compounds are vital to all organisms that undergo aerobic metabolism as ROS are regularly produced through the electron transport chain. For alpine zooplankton, the potential threat that ROS pose is much greater than that for lower elevation zooplankton, both due to increased UVR strength as well as increased water clarity.

Another physiological mechanism which alpine copepods employ to ameliorate UVR stress is the accumulation of carotenoid pigments such as astaxanthin (Figure 3; Hairston, 1976). Astaxanthin is a naturally occurring carotenoid compound, and the source of the reddish

coloration found in many crustaceans and salmonids (Higuera-Ciapara et al., 2006; Matsuno, 2001). It is also a potent antioxidant, having stronger

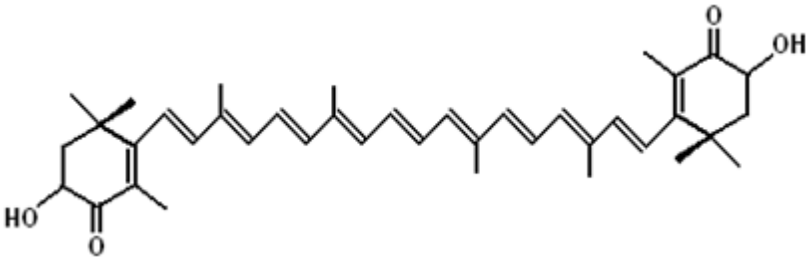

Figure 3. Structure of astaxanthin molecule.

antioxidant properties than beta-carotene and alpha-tocopherol (Miki, 1991). Though animals cannot synthesize carotenoid compounds *de novo*, plants and bacteria can. Copepods in alpine lakes tend to have high concentrations of carotenoid compounds, which they presumably acquire through their diet. Once the source carotenoid is acquired, zooplankton can modify the parent compound into other carotenoid compounds which may be more useful (Higuera-Ciapara et al., 2006).

Studies by Byron (1982) and Hairston (1977) have shown very high levels of carotenoid compounds in subalpine and alpine lake copepods. Copepods found in many high altitude lakes contain such high concentrations of carotenoids that their bodies appear bright red. The high levels of carotenoids bequeath photoprotectant properties to the organisms (Hairston, 1976). It has been suggested by Sommaruga (2001) that the mechanism by which these compounds function is likely not direct photoprotection (via reflectance or absorbance of UVR) but rather indirect photoprotection (via scavenging reactive oxygen species formed by UVR). Both mechanisms of protection result in the same outcome – increased survival when exposed to high levels of UVR. As these carotenoid compounds are strong antioxidants, their presence may also

impart a higher xenobiotic tolerance than less-heavily pigmented individuals, as many xenobiotics induce oxidative stress.

Though carotenoid compounds are beneficial to alpine copepods by reducing the effects of UVR, the presence of high concentrations of carotenoid compounds also exposes the copepod to an increased risk of predation by visual predators such as fish. The pigmentation levels of copepods show phenotypic plasticity when exposed to caged fish (Hansson, 2004; Vestheim and Kaartvedt, 2006). In addition to fish, UVR itself can also cause copepods to exhibit phenotypic plasticity in terms of carotenoid content (Hansson et al., 2007).

The carotenoid compounds are a diverse group of chemicals, with different antioxidant properties (Edge et al., 1997). The most common carotenoid compound found in alpine zooplankton is also one of the most potent antioxidants – astaxanthin. Within both marine and freshwater crustaceans, astaxanthin can exist either as free, unbound astaxanthin, as astaxanthin esterified to fatty acids, or as protein-bound astaxanthin. Observations by Sommer et al., (2006) have shown that the amounts of astaxanthin found within marine copepods vary greatly on both a daily and depth scale. Sommer also found that the relative amount of esterified astaxanthin to free astaxanthin also changed throughout the day, though the esterified form typically made up the majority of the copepods' total carotenoid content. According to Kobayashi and Sakamoto (1999), esterified astaxanthin has a higher lipophilicity in relation to free astaxanthin.

When astaxanthin is in its free or esterified form, it appears as a bright red color. When free astaxanthin is bound to particular proteins such as a α- or β-crustacyanin complex, however, these compounds can take on different colors such as a deep purple or blue (Cianci et al., 2002). In some copepods, the accumulation of this protein-bound form of astaxanthin can cause the entire copepod to appear as bluish color (Goodwin and Srisukh, 1949; Figure 4). Though some

researchers (Herring, Hairston and others) have mentioned the occurrence of these unique, blue copepods in fresh and saltwater, little

research has focused on the implications of this drastic change in appearance. While the compound responsible for the coloration is still astaxanthin, as a protein bound compound, it may play a significantly different role than its free or esterified counterparts.

Figure 4. Image of copepods showing red and blue forms of astaxanthin.

For alpine zooplankton exposed to UVR induced ROS, one of the most important parts of the body that are at greatest risk from damage would be lipids, both in lipid stores and cellular membranes. Esterified astaxanthin is highly lipophilic and is often found in high concentrations in both lipid reserves and cellular membranes – both locations which are prone to extremely damaging lipid peroxidation. Ringelberg (1980) noted that carotenoid compounds are found within copepod fat globules, carapace epidermis, ovaries, and eggs – all of which are areas critical to survival yet highly prone to the damaging effects of UVR induced ROS. Fluoranthene greatly enhances the amount of ROS produced by UVR in lipid-rich areas, and, thus, carotenoids may play an important role in amelioriating the effects of PAH-induced phototoxicity.

The overarching goal of this study was to examine the antioxidant capacity of carotenoid compounds in alpine and subalpine calanoid copepods and their role in protection from PAH photoinduced toxicity. Though it's been shown that carotenoid compounds play a protective role in copepod survival when exposed to UVR, the actual role of protection has yet to be fully understood. The major hypothesis of this research is that copepod carotenoid compounds act as more than just general photoprotectants and may offer protection from environmental contaminants via antioxidant functioning. To test this hypothesis, a series of objectives were developed:

Objective 1: Develop a method for quantification of free and esterified astaxanthin in alpine copepods.

Hypothesis 1: Chromatography-based methods will allow for separation and quantification of free and esterified astaxanthin.

Astaxanthin is found in both free and esterified forms. It is unknown whether certain forms are more or less protective of the organisms. Thus, it is essential to have a method to quantify the relative amounts of free and esterified astaxanthin within the zooplankton.

Though many researchers have shown that the red pigments found in alpine zooplankton are carotenoid compounds, the bulk of this work has been done using absorbance of bulk extracts on a UV-VIS spectrophotometer. While this provides a measure of the overall astaxanthin in an organism, there exists little characterization and quantification data for the different components of alpine zooplankton astaxanthin. Using reverse phase high pressure liquid chromatography (HPLC), relative amounts of free astaxanthin and astaxanthin esters were quantified and characterized.

Objective 2: Generate high and low carotenoid content copepods from alpine lake ecosystems.

Researchers have shown that carotenoid compounds act as strong antioxidants (Edge et al., 1997), while Byron, Hairston and others have shown that elevated carotenoid levels result in better survival when exposed to UVR. To study the relationship between carotenoid levels and sensitivity to oxidative stress, populations of copepods containing a range of astaxanthin concentrations were obtained. A series of phototoxicity experiments designed to induce elevated levels of ROS production were conducted using each population.

Hypothesis 2: Caging copepods under varying UV conditions will produce differential carotenoid profiles. Alternatively, copepods may be collected from different lakes with differing UV conditions to obtain populations with differing carotenoid profiles.

To determine the role that carotenoid compounds play in ameliorating the stressors associated with UVR, a gradient of carotenoid containing cultures of copepods must be produced. Though cladocerans are easily reared and maintained under laboratory conditions, culturing copepods is much more difficult. Moraine ponds found near the University of Colorado's Mountain Research Station, however, provide an ideal setting for *in situ* culturing of test organisms under conditions described in the literature which result in variable astaxanthin levels. Alternatively, if *in situ* culturing was unsuccessful, different populations of copepods would be used which have been shown in preliminary studies to contain different concentrations of astaxanthin (Kovach, unpublished data). Copepods from the moraine ponds (Mountain Research Station) exhibit low levels of total carotenoid compared to copepods from Crater and Emerald Lakes. By capturing copepods from these different locations, separate populations displaying different levels of carotenoid compound could be used in subsequent toxicity testing.

Hypothesis 3: Increased levels of astaxanthin will reduce lipid peroxidation in UVR-PAH exposed copepods thus decreasing their sensitivity to phototoxicity.

Once the different populations of copepods are collected which exhibit different levels of carotenoid content, phototoxicity experiments were conducted. These tests involved placing the different copepod populations into varying concentrations of fluoranthene, a known phototoxic PAH. The organisms were then placed outside in a high UVR environment. Mortality and time to death were monitored.

After the conclusion of the phototoxicity experiments, the overall amount of lipid peroxidation within the given organisms was quantified in each treatment by measuring malondialdehyde (MDA) production. MDA production is commonly used as an indicator of lipid peroxidation (Roberts and Oris, 2004).

Materials and Methods

Study Sites

Test organisms were collected from a range of different high elevation ponds and lakes in Colorado (Table 1 and Figure 5). These different systems all contain heavily pigmented copepods and are high in elevation and UVR.

Name	Copepod Color	Temp (°C)	Cond. (µmhos/cm)	pH	Hardness (mg/L $CaCO_3$)	Alkalinity (mg/L $CaCO_3$)	Lattitude	Longitude	Elevation (feet asl)
Pond 0A	Blue	18	28.3	5.9	12	20	N 40 01.827	W 105 33.110	10,000
Pond 5C	Blue	19	19.8	5.3	8	20	N 40 01.803	W 105 33.676	10,000
Pond 5A	Mixed	19	19.6	5.7	8	20	N 40 01.809	W 105 33.636	10,000
Green Lake 4	Red	10	10.2	6.9	-	-	N 40 03.307	W 105 37.230	11,500
Emerald Lake	Red	9	10.1	5.7	-	-	N 40 18.588	W 105 40.005	10,080
Crater Lake	Red	6	22.0	-	16	-	N 37 34.554	W 105 29.716	12,700
Como Creek	N/A	10	15.3	5.5	8	20	N 40 01.893	W 105 32.279	9,500

Table 1. Descriptions of the sample sites and associated organisms and water quality parameters.

Site 1: Rocky Mountain National Park – Emerald Lake

Straddling the continental divide, Rocky Mountain National Park contains almost 100,000 acres of alpine ecosystem. One of the most picturesque lakes found within the park is Emerald Lake (elevation 10,080 feet). Emerald Lake sits in a bowl between two 12,000 foot peaks – Flattop Mountain and Hallett's Peak. This ultra-oligotrophic lake contains heavily pigmented *Diaptomus* sp.

Figure 5. Locations of research sites in Colorado, USA.

Site 2 - Colorado University's Mountain Research Station – Moraine Ponds

A series of glacial moraine ponds are located within the Niwot Ridge Long Term Ecological Research (LTER) area of Roosevelt National Forest. This LTER site is located in north central Colorado, just west of Boulder. The ponds are the location of much of Earl Byron's

previous work on copepods (Byron, 1982). The ponds are shallow (<2 meters) and are fairly productive; some are ephemeral. All are fishless, with the main zooplankton predators including the invertebrate *Chaoborus* spp. and tiger salamanders (*Ambystoma tigrinum*, Green, 1825). The ponds are found at an elevation of approximately 10,000 feet.

Site 3 - Mount Blanca - Crater Lake

Crater Lake is located in south central Colorado is the Sangre de Cristo mountain range. One of the largest mountains, Mt. Blanca, contains a series of alpine lakes found between 11,000 and 12,700 feet in elevation. Crater Lake (elevation 12,700 feet) contains large copepods with extremely high levels of astaxanthin. It is also home to a population of cutthroat trout (*Oncorhynchus clarki*, Richardson, 1836) whose flesh contains high concentrations of carotenoid pigment.

In situ Copepod Culturing

Using ½" PVC pipe, 6 box-shap ed frames were built. Each was 1 meter wide by 1 meter long and ½ meter deep. 250µm polypropylene mesh was used to cover the entire frame, leaving only the top face open. This mesh size was large enough to allow phytoplankton and water to pass freely through the enclosures, but small enough to keep the larger copepods enclosed. Styrofoam pipe insulation was placed around the top face such that the tops of the enclosures would float approximately 5 cm over the surface of the water. A single layer of plastic sheeting (Cortguard or Aclar) was then fastened onto the top of each enclosure. Courtgard (manufactured by CPFilms, Inc.), though visibly transparent, is almost completely UVR opaque. Approximately 90% of UVR is blocked while visible light passes through. Conversely, Aclar (manufactured by Honeywell International, Inc.) is transparent to both visible light and UVR. A single layer of

Aclar sheeting blocks approximately 25% of ambient UVR. Three of the enclosures were covered with Aclar, while the remaining three were covered in Cortguard.

Two enclosures (one with Aclar, one with Cortguard) were then floated in three separate ponds (Ponds 0A, 5A, 5C). Approximately 200 copepods were collected via zooplankton net and placed into each enclosure. The enclosures were then anchored in the middle of the pond, away from the shading effects of trees. These enclosures were left to incubate for 2 weeks, which, according to Hansson (1997), was sufficient enough to have elicited a drop in copepod carotenoid content of 22% in the UVR-shaded enclosures.

Toxicity Testing

Copepods were collected from each sample location by hand. A 12" diameter, 250µm mesh zooplankton tow net was pulled through the surface waters of each pond in the morning, the day prior to the toxicity assays. Samples were kept in water from their respective pond and transported back to the lab. All samples were left to equilibrate to room temperature (~20°C) until the evening. Water was also collected from Como Creek – a creek found on the site of the UC-MRS. This water was filtered with a glass fiber filter and also left to equilibrate to room temperature.

At approximately 6pm on the same day of collection, the copepods were placed into wide-mouthed 250mL glass crystallizing dishes containing the filtered water with an addition of acetone-dissolved fluoranthene (new stocks were made weekly and stored refrigerated in aluminum foil-wrapped glassware to keep dark). Fluoranthene treatments used were 0, 0.125, 0.5, 2, and 8µg/L (nominal) – with three replicates per treatment (as seen in figure 6). A series of UVR control treatments were run simultaneously, at 0 and 8µg/L. These UVR control treatments

were treated identically, with the exception of being left inside of the lab away from any windows.

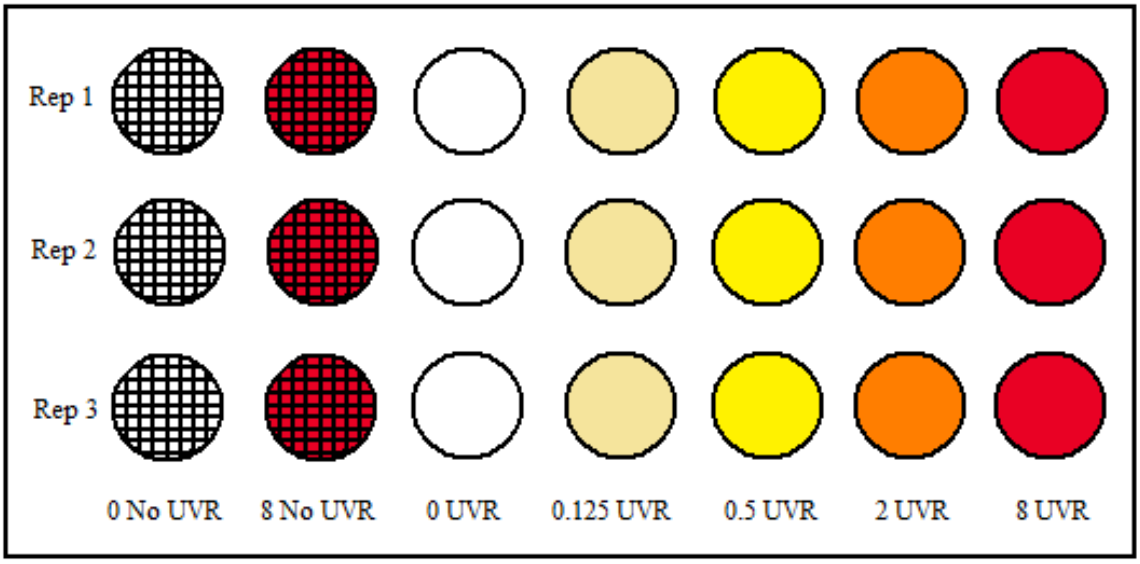

Figure 6. Schematic diagram of experimental treatments. Number correlates with nominal fluoranthene concentration (in µg/L). Screened enclosures (columns 1 and 2) were not exposed to UVR.

Each test solution was mixed immediately prior to dosing, and copepods were placed into these solutions at a concentration of 1 individual per 10mL solution and left to incubate overnight. For each test, approximately 30 individuals from each population, pre-exposure, were flash frozen in liquid nitrogen in marked micro centrifuge tubules. At the conclusion of all testing, all samples were transported back to the laboratory at the University of North Texas for carotenoid analysis.

At 8am the next morning, all solutions were renewed. At approximately 10am (or immediately upon favorable weather conditions), all dishes were placed in direct sunlight in a shallow water bath designed to maintain a constant temperature. Mortality was assessed every 15 minutes, and tests were run either until 2pm or inclement weather set in. An organism was

considered dead when they elicited no visible movement after 5 seconds of gentle prodding with a transfer pipette and/or upon having their first antennae folded in close to their body (as seen in the organisms in Figure 4). Throughout each test, water bath temperature was checked every 15 minutes and, as needed, either hot or cold water was added to the water bath to maintain a constant temperature between 17-20°C.

As UV-A is the range of light at which fluoranthene is phototoxic, light intensity was recorded in the UV-A range (340nm) every 5 minutes using a BioSpherical multi-wavelength radiometer (BioSpherical Instruments, San Diego, CA). In order to calculate a cumulative light dose, the raw data (expressed as $\mu W/cm^2 \cdot nm$) were converted into $nJoules/cm^2 \cdot sec$. By multiplying the light intensity by the time between each measurement, an estimate of actual dose was obtained. These estimated doses were added together to obtain the cumulative dose of UV-A light for each light measurement. It was then possible to estimate the total exposure of UV-A that each organism was exposed to during the test before death.

Actual "phototoxic dose-to-death" was then calculated using a formula similar to that found in Oris and Giesy (1987). Nominal fluoranthene concentration was multiplied by UV-A dose to obtain the phototoxic dose. For example, if an individual exposed to 2µg/L fluoranthene was exposed to $500nJ/cm^2$ during the experiment before dying, its effective phototoxic dose would be 1000.

Due to the highly variable nature of the sunlight during the experiments, the responses of those organisms exposed to the lower concentrations of fluoranthene were highly variable. Oftentimes, doses 0 through 2µg/L would exhibit little to no partial mortality over the entirety of the test, while the highest concentration (8µg/L) always elicited at least 80% mortality (typically 100%). To directly compare the different populations' sensitivities, two methods were utilized.

First, using only the 8µg/L data (as lower doses resulted in little to no mortality), a phototoxic "dose to death" was calculated for each individual within each replicate. Those doses were pooled by replicate to generate three mean doses to death for each fluoranthene concentration. This procedure was calculated for each population, and the phototoxic doses to death were then compared via single factor ANOVA with "population" as a factor followed by a Student-Newman-Keulls multiple range comparison test (α=0.05).

The second method utilized all fluoranthene treatments. Using probit regression, phototoxic dose-to-death was correlated to mortality. This method allowed for the comparison of populations in terms of dose-to-death at different levels of mortality. The phototoxic dose that resulted in 50% mortality (LD50) was used to compare the different populations.

Lipid Peroxidation Analysis

Oxidative stress is the predominant mechanism by which UVR induced fluoranthene toxicity is manifested. Due to the highly variable nature of the cloud cover at the UC-Mountain Research Station in the summer, I was concerned about directly comparing lipid peroxidation among different dose-response experiments. To avoid this problem, a specific toxicity test was run to compare different populations' levels of lipid peroxidation following exposure to UVR and fluoranthene. This particular toxicity test was run using each population of zooplankton available at the time, with a range of both carotenoid compounds as well as visible colors. This test was run identically to the other toxicity tests with the exception of having only a single concentration of fluoranthene: 2µg/L. This intermediate concentration was chosen due to the high level of mortality seen at higher doses. If 8µg/L (the next highest concentration I used) had been selected, it is likely that the more sensitive individuals would have experienced significant mortality. This test was exposed to peak UVR for two hours only.

To determine the relationship between carotenoid compounds and antioxidant function, it was necessary to have some measurement of oxidative stress. To measure oxidative stress, a thiobarbituric acid reactive substances (TBARS) assay was used. TBARS is a colorimetric assay which utilizes a byproduct of lipid peroxidation, malondialdehyde, to measure oxidative stress in lipids. When present, malondialdehyde and thiobarbituric acid bind together to form a pink-colored adduct.

The assay was performed using methods modified from Cayman Chemical (Catalog No. 10009055). From each population, 3 replicates, each containing 25 individual zooplankton were homogenized in 250µl of 100mM phosphate buffer (pH 7.0) using a bead beater for 15 seconds. Samples were then centrifuged for 10 minutes at 1,600g at 4°C. A 50µl fraction of the supernatant was combined with 50µl of an 8.1% SDS solution in a glass vial. 2ml of a thiobarbituric acid dissolved in mixture of sodium hydroxide and acetic acid was added to the sample. Vials were placed into a boiling hot water bath for one hour. The vials were then cooled immediately in an ice water bath to halt the reaction and kept on ice for 10 minutes. Upon warming to room temperature, duplicate 200µl volumes of the solution were pipetted into individual wells on a 96-well plate and absorbance was read at 535nm on a UV-VIS spectrophotometer (BioTek Instruments). A malondialdehyde standard curve was obtained using the same methods as above. By comparing the sample absorbance values to the standard curve, the amount of lipid peroxidation (measured as µM malondialdehyde) in each sample was obtained.

The raw lipid peroxidation values were then standardized to total protein to account for differences in sample mass. To do this, a Lowry total protein assay was utilized. The Lowry's total protein assay (Thermo Scientific No. 23240) is a colorimetric assay. To perform the assay,

40µl of homogenized sample (left over from the TBARS assay) was placed, in duplicate, into a microplate well. A 200µl aliquot of modified Lowry reagent (an alkaline copper-containing solution) was added to each well. The plate was then mixed on a plate mixer for 30 seconds, covered and incubated at room temperature. During this time, a copper-protein complex was formed. After 10 minutes, 20µl of a Folin-Ciocalteu reagent was pipetted into each well followed by the plate being mixed for another 30 seconds and incubating at room temperature for 30 minutes. The Folin-Ciocalteu reagent is reduced in proportion to the copper-protein complexes, resulting in the formation of a bluish colored, water soluble product. After the incubation, the absorbance of the wells was measured at 750nm. The samples' absorbances were compared to a standard curve composed of a series of standard solutions of bovine serum albumin, diluted using the same 100mM phosphate buffer used in the sample preparation.

Carotenoid Analysis

Method Development

Both lipid peroxidation (via TBARS assay) and total protein (via Lowry assay) were measured using methods common enough that 96-well plate assays were available for them. Measuring carotenoid compounds is much less common. Of the research that has analyzed carotenoid content, the majority use a spectrophotometer to measure bulk carotenoid. By extracting the carotenoid compounds from the organism and then comparing the sample's absorbance to the standard's absorbance at ~474nm (the absorbance peak for free astaxanthin) to a series of diluted astaxanthin standards, it is possible to estimate the total amount of astaxanthin within a sample but not differences in free and esterified forms

Knowing that copepod extract likely contains a mix of carotenoid compounds, an accurate method was needed to be able to measure those. To accomplish that, HPLC was

utilized. Using HPLC to measure different types of astaxanthin is not new per se but it has not been applied to the study of alpine copepods. To start, a similar method by Wade et al. (2005) used to analyze different types of astaxanthin (free and esterified) in lobster epithelia and shell was attempted. After attempting to prepare and analyze samples identically (save for the actual HPLC machine), no peaks were found in the resulting chromatographs.

After examining many other methods of analyzing carotenoid compounds using HPLC, it was found that each method utilizes a different approach, different solvents, and different sizes and types of columns, because each method was geared towards identifying different levels and types of carotenoid compounds based on their representative samples. It was decided that the best approach would be to develop a new method tailored to look at the particular compounds of interest desired at levels relevant to this study.

Extraction

After obtaining an astaxanthin standard and zooplankton samples from the research sites, a few organic solvents were tested to determine an ideal extraction solvent. Based on literature studies, acetone and ethanol were mentioned in previous studies, so both were tested. While astaxanthin was soluble in both acetone and ethanol, and it was extracted from copepods readily in both, acetone was chosen for its ease in obtaining, as well as its rapid evaporation rate. By homogenizing samples with a bead-beater, extraction rates and efficiency were much improved over non-homogenized samples.

Wavelength Selection

A spectrophotometer was utilized to obtain the peak absorbance of astaxanthin. Extract from both astaxanthin and copepod samples were compared on a spectrophotometer, and it was found that both samples had peaks centered around 474nm ±2nm (Figure 7). All further analyses (both spectrophotometrically and via HPLC) were conducted at this wavelength.

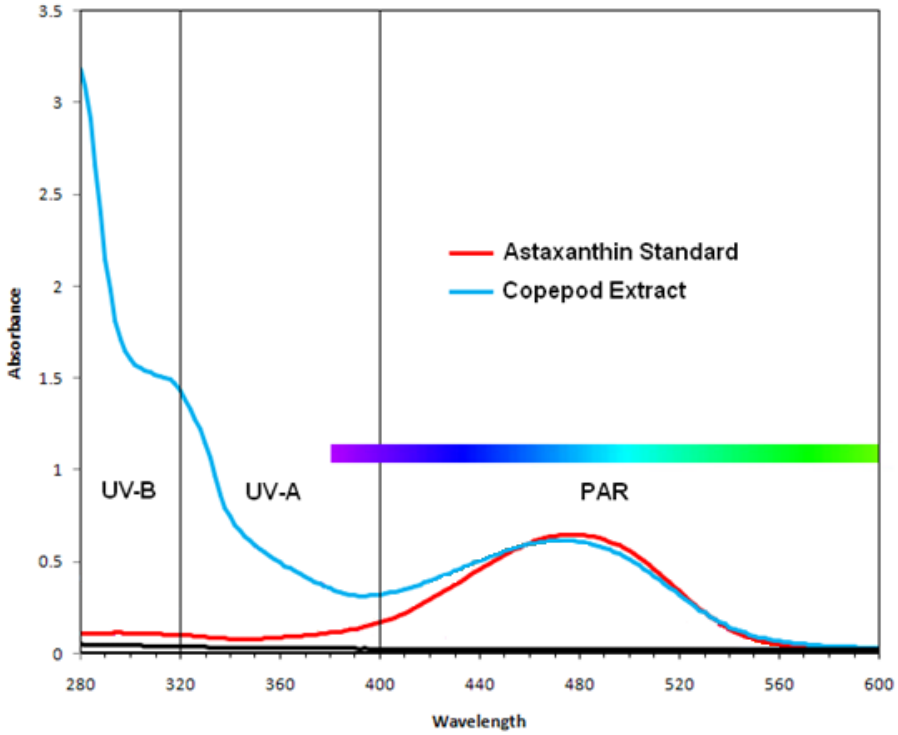

Figure 7. Absorbance spectra for both free astaxanthin standard and copepod extract.

Sample Preparation

While acetone was shown to be an effective extraction solvent, the HPLC methods that were being used had no acetone in the solvent profiles, so it was dried off under inert gas (nitrogen). As carotenoid compounds are light sensitive, care was taken to prevent light from reaching the samples throughout this step. As the HPLC method was to be reverse-phase, numerous mixtures of solvents were tested that would provide a mixture non-polar enough that

all sample extracts and standards were totally soluble in it, yet as polar as possible to keep the sample from “streaking” through the very non-polar C18 column being used. This led to better peak sharpness and resolution. The mixture eventually selected for this task was 75:25 v:v MeOH:Ethylacetate.

HPLC Analysis

Due to differences in the polarities between free and esterified astaxanthin, it was decided that a gradient elution profile was required. By adjusting the relative abundance of both solvent mixtures being used, the polarity of the mobile phase could be adjusted. The initial runs included injection of 10-20µL of sample followed by a gradient profile starting with 25% ethylacetate and increasing up to 100% ethylacetate. It was found by very slowly and gradually increasing the more non-polar solvent, it was possible to estimate the approximate strength of the non-polar phase that was required to elute all compounds of interest. That strength was found to be approximately 70-75% ethylacetate.

The first injections for this method were comprised of a series of dilutions of free astaxanthin standard and actual copepod samples. This was an important first step in determining what levels of carotenoid compounds must be present in the injection solvent in order to achieve measurable peaks. After much practice, it was found that a good rule of thumb was to be certain that the actual sample injection solvents had at least a slight orangish/pinkish hue when silhouetted against a bright, white background.

After determining the necessary strength of solvents, the next steps were to determine the times at which the solvent strengths should be increased or remain steady. Initially, a series of stepwise elutions were attempted to both decrease the total sample run time as well as saving on solvents. By ramping up the solvent strengths when no peaks were expected, and holding the

solvent strength steady during times when peaks were expected, it was hoped that an ideal elution profile would be obtained. After much trial and error, it was determined that the cleanest, neatest chromatograms were those obtained with slow, steady increases in non-polar solvent strength over the duration of the program. The following method was that which was found to be the most suitable of all methods attempted for separating and identifying different types of astaxanthin found within alpine copepods.

Carotenoid Analysis

In order to quantify the individual types of astaxanthin present within each zooplankton sample, carotenoids were first extracted from the copepods. After thawing, 10 individual copepods were gently blotted dry and then placed into a 1.5mL microcentrifuge tubule (3 replicates per each population). 1mL of HPLC-grade acetone was then added to each sample, and each sample was bead beaten for 10 seconds to ensure sample homogenization. Extraction tubules were then stored in the dark at 4ºC for a period of 24 hours.

After 24 hours, the sample tubules were centrifuged at 10,000rpm for 5 minutes. The supernatant was transferred to a 15mL glass test tube. Another 1mL aliquot of acetone was added to the microcentrifuge tube, vortexed, and centrifuged again, followed by the supernatant being removed and placed into the same test tube. This process was repeated one more time, ensuring that neither the pellet nor supernatant were visibly pigmented. The test tubes containing the extracted carotenoid compounds were then dried under nitrogen to remove all acetone. They were then resuspended in 200µL of a 75:25 solution of methanol:ethylacetate. These samples were now ready for HPLC analysis.

All samples were analyzed using the following reverse phase HPLC method. This method consists of a gradient elution profile using two different solvents. Solvents were as

follows: Solvent A was a 75:25 v:v mixture of HPLC grade MeOH:H_2O, while solvent B was 100% HPLC grade ethylacetate The column used was a Phenomenex Jupiter 4µ Proteo 90A, size 150mmx4.6mm C18 column. The solvents had a continuous 1 mL/min flow rate throughout the analysis, with an initial 20µL injection volume.

The solvent profile used was as follows: 5 minutes of 25% solvent B, 45 minutes of a steady increase from 25-75% solvent B, 5 minutes of 75% solvent B, 5 minutes of a steady decrease from 75-25% solvent B, and finally 5 minutes 25% solvent B. The detector was set to measure absorbance at 474 nm, which is the experimentally determined absorbance maxima for free astaxanthin (Sigma-Aldrich Product No. 41659). An example of a chromatogram acquired using this method to detect the different carotenoids present in a biological sample are shown in Figure 8.

Quantification of the individual types of astaxanthin was conducted using a mass-balance approach. Free astaxanthin levels were determined by comparison to a standard curve generated by serial dilutions of a concentrated stock solution. As astaxanthin can also exist in an esterified form, bound to either a single fatty acid (mono-esterified) or to two fatty acids (di-esterified), its quantification requires a further, esterase enzyme hydrolysis step, similar to methods used by multiple researchers including Wade et al., (2005), Jacobs et al., (1982), Fuji Chemical Industry, Naturose Technical Bulletin No. AXBUL15.

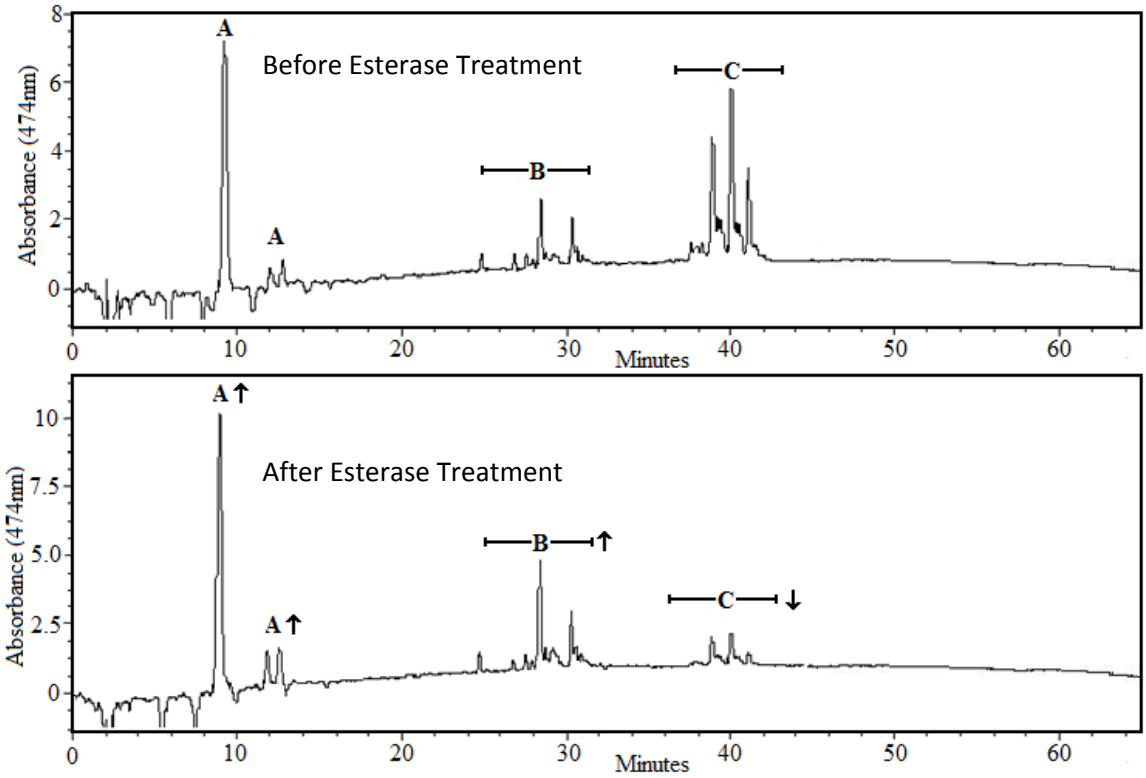

Figure 8. Sample chromatograms of extract from a copepod from Crater Lake before and after esterase treatment. Peaks A represent isomers of free astaxanthin, peaks B represent mono-esterified astaxanthin, and peaks C represent di-esterified astaxanthin.

To accurately quantify the amount of esterified astaxanthin present within a sample, a sample's extract (in acetone) was split into two fractions. 150µl of the first fraction was added to 150µL of a solution of cholesterol esterase (Sigma # C9281) in 50mM Tris buffer (pH adjusted to 7 using HCl) to a concentration of 5 units of esterase per mL buffer in a 1.5mL microcentrifuge tubule. The second fraction was exposed identically to the same buffer, without the addition of the cholesterol esterase. The different fractions were incubated at 37°C for 3 hours and mixed by inversion every 15 minutes. Contents were then transferred into a glass test tube, and the microcentrifuge tubule was rinsed with acetone 3x to remove any residual carotenoid. A 1mL aliquot of hexane was added to the test tube, which was vortexed. Upon this

addition, a hexane/acetone layer was present at the top of the solution, which appeared orange indicating the carotenoid had all been transferred to this layer. The hexane layer was removed and placed into a fresh test tube. This hexane extraction was completed two more times to ensure all carotenoid was transferred. This hexane layer was then dried under nitrogen and resuspended in 75:25 v:v MeOH:ethylacetate and run via the above HPLC procedure.

This esterase reaction resulted in the cleaving of the individual ester bonds, releasing the fatty acid groups and leaving free, unesterified astaxanthin. If a complete hydrolysis occurs, peaks in Group A increase dramatically, while all peaks in Groups B and C are reduced to little more than small bumps on the chromatogram. If a partial hydrolysis occurs however, peaks in Groups A and B increase, while peaks in Group C decrease (seen in Figure 8). An astaxanthin molecule esterified to a single fatty acid is more non-polar than the parent compound, it elutes later in reverse phase chromatography. An astaxanthin molecule esterified to two fatty acids is more polar than a mono-ester or free form and thus elutes last. In the process of cleaving ester bonds, a di-ester must become a mono-ester before it can become a free astaxanthin molecule. In a partial hydrolysis, peaks in Group C (likely di-esters) correspond with an increase in peaks in Group B (likely mono-esters).

While Wade et al., (2005) identifies peaks in Group C as astaxanthin esters, those in Group B are explained by the authors to be "unidentified carotenoids". When looking at their chromatograms, however, the same mono-ester and di-ester trend (under partial esterase hydrolysis) is evident. Fuji Chemical identifies the peaks in Group A as Astaxanthin optical isomers, Group B as astaxanthin mono-esters, and Group C as astaxanthin di-esters. Though these esters have not been compared to a standard, results of my esterase treatments and the results of others suggest these to be the correct ester identifications. In lieu of this information,

peaks in Group A are identified in this thesis as free astaxanthin, peaks in Group B are identified as astaxanthin mono-esters, and peaks in Group C are identified as astaxanthin di-esters.

Individual copepod size differed between populations. To account for this, all carotenoid data were converted from astaxanthin/individual to astaxanthin/unit dry weight. To obtain dry weights, small (~5mm x 5mm) aluminum foil squares were folded into small packets. All packets were weighed in a microbalance and placed into a specific well on a 96 well plate (for easier reference). One individual copepod was placed into each packet and then the entire plate was placed into a drying oven at 60°C for 24 hours. The foil packets were then re-weighed, and the original mass of the aluminum packet was subtracted to obtain an individual dry weight. 10 individuals from each population were weighed, and the mean dry weight was used as the dry weight per individual from each population. All carotenoid data were then expressed as astaxanthin units per unit dry weight.

Results

In situ Copepod Culturing

After a 2 week incubation, attempts to collect the copepods from the enclosures were made. Though the enclosures themselves were still intact, no copepods were found. As no water quality parameters were measured in the actual enclosures, the cause for the absence of the copepods remains unknown. Instead, copepods were collected from several of the study lakes to obtain populations with a range of astaxanthin content and composition.

Carotenoid Content

Overall carotenoid content was highest in the organisms from Crater Lake, while organisms from Emerald Lake had an intermediate level and the organisms from Ponds 5C, 5A, and 0A had the lowest levels (Figure 9). The highest overall levels of carotenoid compounds

corresponded to the organisms who appeared red in color, while the lowest content was for those organisms displaying blue or a mixed red/blue coloration. The presence of a red coloration in the red and mixed individuals corresponded with the presence of a large amount of di-esterified astaxanthin. The organisms appearing blue and mixed had the largest percentage of free astaxanthin in relation to total astaxanthin. As protein-bound astaxanthin is in its unesterified form, it is logical that the unesterified form would be more prevalent in those organisms with high levels of blue pigment.

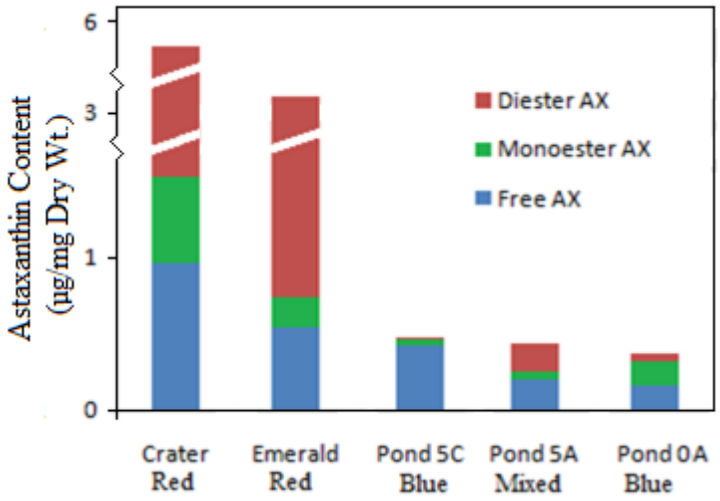

Figure 9. Carotenoid content of each population of copepods.

Phototoxicity Experiments: Dose-to-Death

A median phototoxic dose-to-death (LD50) was calculated for each population using probit regression. The following statistically distinct ($\alpha = 0.05$) populations were found in terms of their median lethal phototoxic dose-to-death: Emerald Lake > Crater Lake > Pond 0A > Pond 5A = Pond 5C (Figure 10). Dose-to-death data were also calculated for each population using the 8 µg/L fluoranthene treatment only. Total astaxanthin content (in ug/mg dry weight) was found to have a significant positive relationship with dose-to-death (Figure 11).

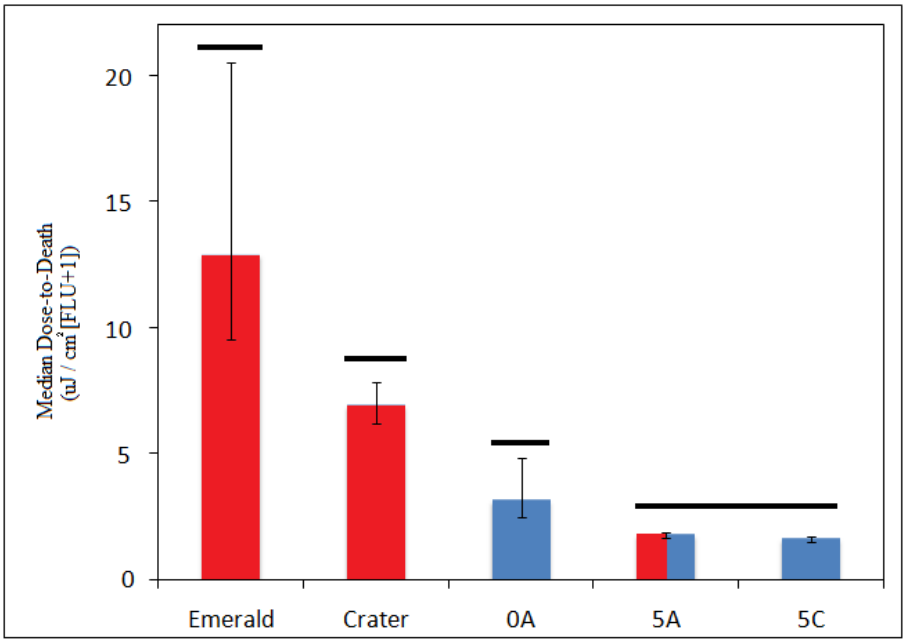

Figure 10. Median phototoxic dose-to-death for each different population of copepods. Statistically distinct populations ($\alpha = 0.05$) denoted by broken bars.

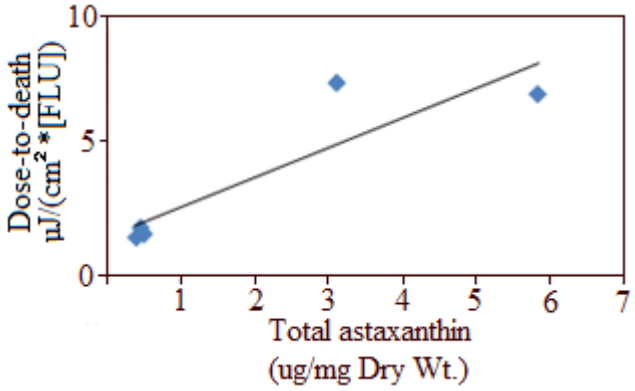

Figure 11. Regression of dose-to-death and total astaxanthin content ($p = 0.04$, $r^2 = 0.80$).

To determine if size was a confounding factor in survival against phototoxic dose-to-death, a regression of dry weight vs. dose-to-death was analyzed. When regressed against

phototoxic dose for all organisms exposed to 8µg/L fluoranthene, the individual copepod size had no significant relationship with phototoxic resistance (Figure 12).

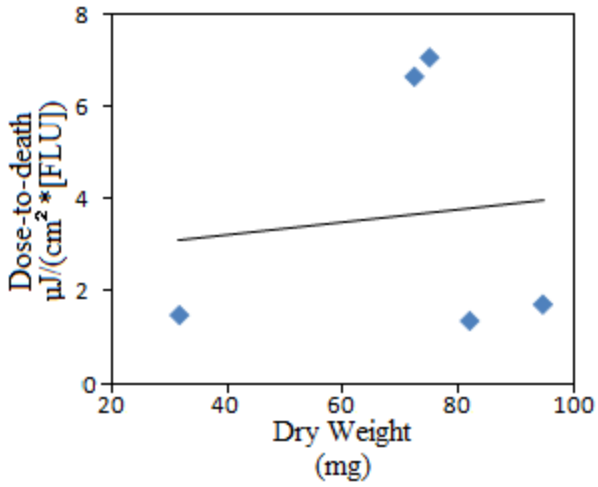

Figure 12. Dry weight regressed against phototoxic dose-to-death. No significant relationship was found (SLR, p = 0.85).

Lipid Peroxidation Analysis

No significant relationship was found between lipid peroxidation levels (in terms of malondialdehyde content) and total astaxanthin in either UVR or non-UVR exposed copepods (Figure 13) as well as between malondialdehyde content and esterified astaxanthin in either UVR or non-UVR exposed copepods (Figure 14).

When compared by color, no significant differences in malondialdehyde levels were detected between either UVR exposed copepods of differing colors or non-UVR exposed copepods of different colors (Figure 15). There was also found no significant differences between UVR and non-UVR exposed blue, mixed, and red copepods (Figure 15).

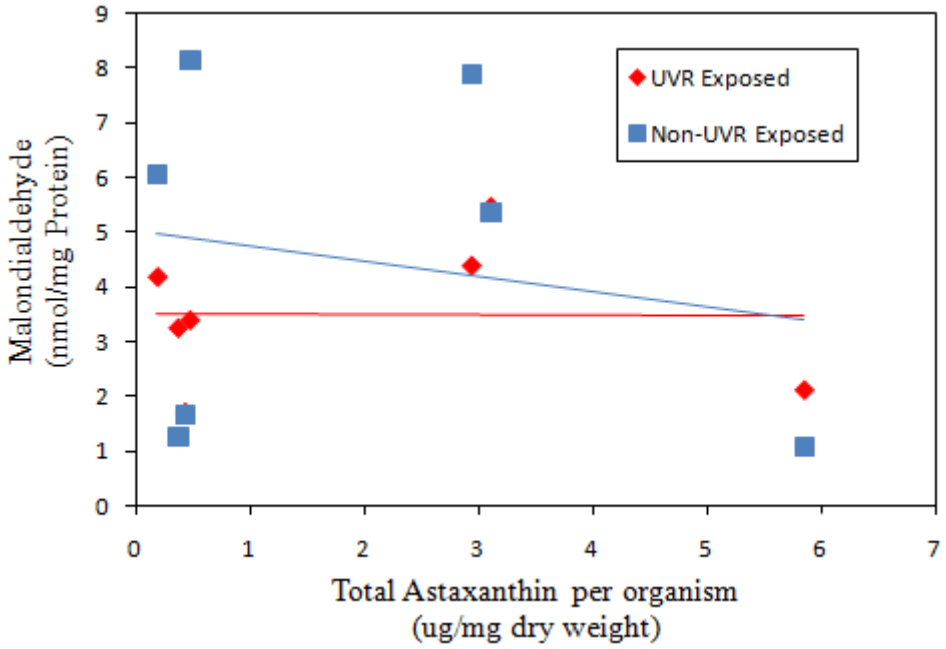

Figure 13. Malondialdehyde content regressed against total astaxanthin. No significant relationship was seen for UVR exposed (SLR, $r^2 < 0.01$, $p = 0.98$) or non-UVR exposed individuals (SLR, $r^2 = 0.04$, $p = 0.68$).

Upon finding no significant results in malondialdehyde content in copepods, a similar test was conducted with *Daphnia magna*. When exposed to 0, 0.5, and 8µg/L fluoranthene and placed into direct sunlight for 30 minutes (as compared to the copepods' 2 hour exposure), significant differences were seen between *D. magna* treatments in terms of MDA content (Figure 16).

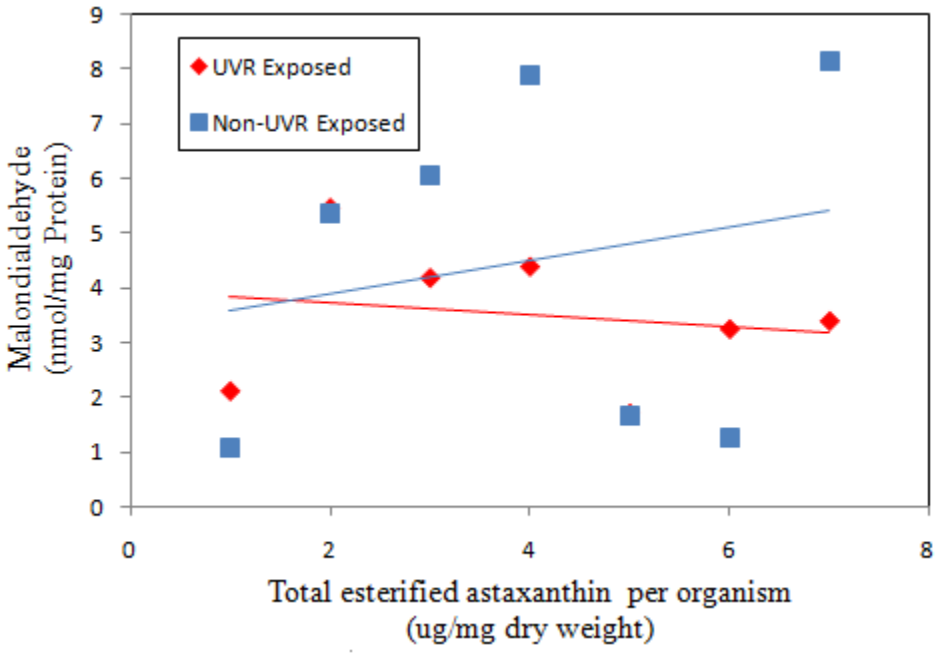

Figure 14. Malondialdehyde content regressed against total esterified astaxanthin. No significant relationship was seen for UVR exposed (SLR, $r^2 = 0.03$, $p = 0.97$) or non-UVR exposed individuals (SLR, $r^2 = 0.05$, $p = 0.71$).

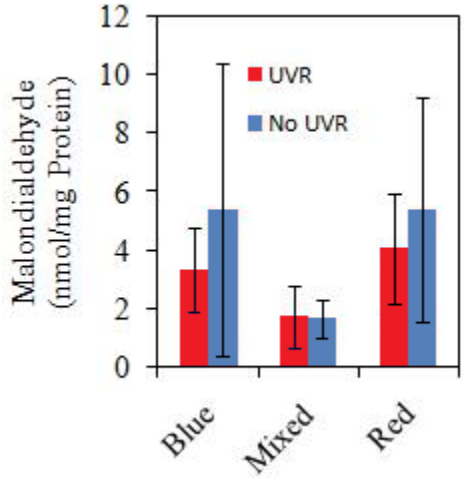

Figure 15. Malondialdehyde content across different copepod colors. No significant differences were seen between UVR exposed (ANOVA, $p = 0.12$) and non-UVR exposed (ANOVA, $p = 0.40$). No significant differences were seen between copepods of the same color in terms of UVR or non-UVR exposure.

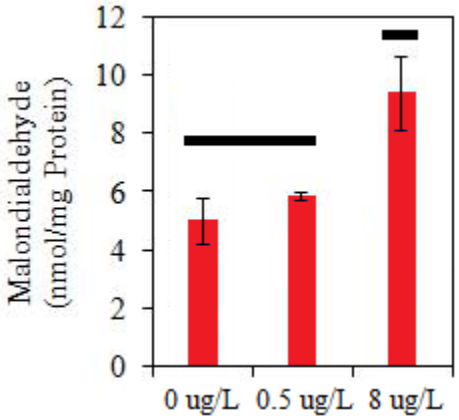

Figure 16. Malondialdehyde content across different *D. magna* fluoranthene doses (in µg/L). Significant differences in denoted by a broken bar (ANOVA, p = 0.03, SNK Multiple Range Comparison Test, α = 0.05).

Discussion

Dose-to-Death

A significant positive relationship was found between total astaxanthin content and the dose of phototoxicity needed to kill the organisms. As hypothesized, as astaxanthin content increases, copepods become less susceptible to PAH-UVR damage. This is similar to the trends reported by Byron (1982) and others in which copepods containing higher carotenoid content are less susceptible to UVR stress alone. While this trend exists, it sheds little light on a potential mechanism of protection. One potential explanation for this observation is due to differences in organism size. The larger an organism is, the less likely harmful UVR will penetrate throughout the entire organism.

To study this hypothesis, a regression between dry weight of the different copepod populations and the phototoxic dose to death at 8µg/L fluoranthene was constructed. This regression showed no significant relationship ($p = 0.85$, $r^2 = 0.01$). This suggests that size plays no major role in susceptibility of alpine copepods to PAH-UVR phototoxicity.

As size plays no significant factor, four hypothetical explanations exist:

Direct p*hotoprotection* – This method of protection would be akin to the carotenoid compounds acting as a type of sunscreen. These compounds would reflect or absorb damaging wavelengths of light before they have a chance to react with the fluoranthene molecule and damage biological tissues. If the harmful light is removed before ever having a chance to have a deleterious effect, the potential for damage is removed altogether.

In its red form (which includes the esterified forms), astaxanthin appears red due to its absorbance of light in the shorter, blue wavelengths of the visible spectrum, reflecting light in the longer reddish/orangish wavelengths thus appearing as a reddish/orangish compound. While it may be possible that this red compound does, in fact, reflect light in the ultra-violet range, it seems somewhat unlikely.

In its visibly blue protein bound form (crustacyanin, Cianci et al., 2002), astaxanthin absorbs light in the longer, reddish wavelengths of light, reflecting the shorter wavelength blue and violet light. This causes the compound to take on a bluish/violet color. This shift in absorbance from short wavelength (blue) light to the longer wavelength (red) light could provide an important key to identifying a potential protective mechanism. If the compound reflects blue and violet (just within the UV-A range) wavelength light, it is possible that it may also reflect the shorter ultraviolet wavelengths outside of the visible spectrum. It seems more likely that this blue or violet appearing caroteno-protein complex would act as a photoprotectant by reflecting damaging UVR more than would the red-appearing esterified (or free, unbound) forms of astaxanthin. As reflectance was not measured, investigation into this topic is incomplete and only speculations can be made.

The distribution of the various carotenoid compounds may further elucidate their potential role as photoprotectants. In the blue copepods, the caroteno-protein pigment appeared most numerous in the carapace of the organism, with little blue coloration within the interior of the organism. The red forms including esterified forms, are present within the organism, noticeably within lipid globules (Ringelberg, 1980). While lipids are highly susceptible to damage by phototoxicity, the placement of a UVR reflecting compound would more logically be exterior to the sensitive locations. While there may be other causes for the placement of blue caroteno-proteins on the exterior of the organism (such as camouflage), its presence in the carapace supports the idea that the blue coloration acts as a reflecting photoprotectant.

The most effective location to put a reflecting photoprotective compound would be the most exterior locations of the organism. While the red compounds may function this way, their absorbance spectrum and location within the organism suggests an alternative mechanism. Under this mechanism, it seems likely that the blue-appearing copepods would be less sensitive to phototoxicity. However, it should also be noted that while the organisms from Emerald and Crater Lakes were characterized as being "red" in color, upon examination under a dissecting microscope many of them did reveal blue carotenoid compounds in their carapaces. This blue coloration, however, was essentially "drowned out" by the prevalence of large amounts of red pigment on the interior of the organism, causing the organisms to appear dark red and, in some cases, almost black. If the free, unesterified astaxanthin found within the organisms is predominantly in the protein-bound form, the organisms labeled as "red" may actually contain more of the blue pigment than the copepods appearing blue as the red organisms contained more free astaxanthin per unit body mass than did the blue copepods.

Another direct protection mechanism would function similar in fashion to the previously mentioned method, except that the photons of light in the ultra-violet wavelengths would be absorbed by the carotenoid pigments rather than reflected. The end product may be the same – less damaging UVR striking fluoranthene-containing, susceptible tissues, but the mechanism differs. The different colorations of the carotenoid compounds may play a significant role in the type of photoprotection they offer to the organism. This type of photoprotection could be important for compounds found not only in the carapace of the organism, but also within the most susceptible tissues.

As mentioned before, red carotenoid compounds absorb light in the shorter blue wavelengths of light, reflecting red wavelength light. These compounds absorb light near the ultra-violet range, suggesting the potential for the absorbance of UVR. However, an absorbance spectrum for astaxanthin shows that very little absorbance occurs in the ultraviolet range. If these compounds act as photoprotectants via absorbance of ultraviolet wavelength light, it seems merely coincidental as their absorbance spectrum barely overlaps with the UV-A range.

For the protein-bound forms of astaxanthin, the blue and violet forms seem even less likely to exhibit photoprotection via absorbance of UVR than did the red compounds. These bluish compounds absorb light within the longer wavelengths of the visible spectrum and exhibit no absorbance in the ultraviolet range (Cianci et al., 2002), discounting the notion that these compounds may play a role as UVR protectants via absorbance.

Another type of astaxanthin caroteno-protein exists which, rather than resulting in a bathochromic shift in absorbance from ~474nm (free form) to ~650nm (crustacyanin), results in a hypsochromic shift in absorbance from ~474nm to ~410nm (Buchwald and Jencks, 1968). This caroteno-protein - crustochrin, has been seen in crayfish and other crustaceans (Buchwald and

Jencks, 1968; Cianci et al., 2002) but has not been reported in alpine copepods. This shift in absorbance would likely put the compound's absorbance well into the range of UV-A radiation. While this seems a possible mechanism for photoprotection, the absence of knowledge about its presence in copepods makes this hypothesis speculative at best.

Indirect photoprotection – On its own, UVR causes little damage. However, UVR damage is primarily manifested by generation of ROS. If the ROS can be removed from the system before damage is produced, little harm is done. By acting as an antioxidant, carotenoid compounds are likely to scavenge ROS. While it's been speculated that carotenoid compounds may act by direct photoprotection, no causative data exists. There is, however, data to support astaxanthin's antioxidant function (Edge et al., 1997). Data collected in this study showed that *D. magna* with no measurable levels of astaxanthin were much more sensitive to PAH phototoxicity than are the copepods that contain high levels of astaxanthin. It's highly likely that the astaxanthin within these copepods acts as an antioxidant.

No photoprotection –Because I was unable to manipulate carotenoid content in a single population, much of the data produced here is correlative across populations and not causative. The presence of high amounts of carotenoid compounds in those organisms that are less susceptible to PAH-induced phototoxicity may be simply an artifact of the organism's diet or other factors. These compounds may play little to no role in the actual photoprotection of the organism. It is possible that other factors such as the UVR absorbing mycosporine-like amino acids are present at levels reflecting those of the carotenoid compounds. This could potentially account for astaxanthin's supposed photoprotective function.

While the possibility exists that no causal relationship exists between carotenoid compounds and susceptibility to UVR, it seems highly unlikely. In reality, it may be a

combination of both direct and indirect photoprotection, as well as direct photoprotection via absorbance and reflectance. Each type of astaxanthin (different types of caroteno-proteins, and esterified forms) may play its own unique role in the photoprotection of an organism.

Carotenoid Analysis

Differential susceptibilities to PAH-induced phototoxicity was found among different color phenotypes (as seen in Figure 12), with the red coloration being significantly less susceptible to PAH-induced phototoxicity than either blue or a mixed (blue and red) phenotype. While this data may suggest that the blue pigmentation is less effective in reducing UVR, care must be taken in making major assumptions. First, these data are essentially qualitative only in terms of "color" measurement. As evidenced by the carotenoid analysis, each population contains free form, monoesterified and diesterified astaxanthin. In reality, though each population may "look" mostly blue or red, each contains varying amounts of both pigments, resulting in each organism being technically classified into the "mixed" category.

Secondly, the type of astaxanthin contained in the blue, protein-bound form is free, unesterified astaxanthin. However, in terms of total astaxanthin, both red populations have more free-form astaxanthin (in µg astaxanthin/mg dry weight) than do the blue or mixed populations (Figure 8). As mentioned previously, it should also be noted that though appearing dark red, many of the individuals from both Emerald Lake and Crater Lake actually have carapaces that appear blue.

Lipid Peroxidation Analysis

Certain PAHs (including fluoranthene) and UVR have been shown by Choi and Oris (2000) and Newsted and Giesy (1987) to generate large amounts of reactive oxygen species. The dose-response curves obtained show that increased doses of PAH-UVR result in increased rates

of mortality in all populations studied. As fluoranthene coupled with UVR has been shown to induce ROS, its presence in the organisms is extremely likely.

No effect of astaxanthin on lipid peroxidation as measured by malondialdehyde formation was observed. However, phototoxicity in the *D. magna* positive control did result in the formation of measurable levels of malondialdehyde. It is unlikely that no lipid peroxidation occurred in the copepods, but rather that the copepod samples were below the detection limit of the TBARS assay. The kit that was used, supplied by Cayman Chemical, recommended at least 25mg of wet tissue be used per sample, which would require approximately 25 individuals per population. While it was impossible to increase the size of the samples, it was possible to decrease the total volume of the assays. The initial incubations of the TBARS assay called for a total volume incubation of 4ml, of which, only 200µL were actually measured on a spectrophotometer. To increase the amount of sample relative to the amount of total volume, all assay solution volumes were halved, which effectively doubled the total amount of tissue used in the test. Even with this increase in sample size, the malondialdehyde concentrations were at the lower level of detection of the kit used.

Though it was hypothesized that carotenoid compounds act as photoprotectants by scavenging reactive oxygen species, no conclusive results on mechanism were obtained. As these organisms are inherently small in relation to the required materials for the TBARS assay, a much larger sample size may be required to obtain significant results. Also, though an ample size of tissue was used in terms of wet weight, it's likely that the relative abundance of water within a copepod is likely much greater than that of, say, liver tissue used by Roberts and Oris (2004). While increasing the sample size will help by increasing the amount of malondialdehyde present,

increasing the phototoxic dose by a longer UVR treatment and/or increased fluoranthene concentration would also serve to increase levels of lipid peroxidation.

Future Directions for Study

In-situ culturing – Though the survival was significantly small, data from Hansson (2004) seems promising that organisms are able to be cultured to reduce actual carotenoid content. This could provide very suggestive data towards a causal link between the carotenoid compounds and a mechanism of protection to phototoxicity. Using different water bodies to collect organisms with varying degrees of carotenoid compounds has worked, but the potential for a change in carotenoid compounds due to a single factor (alteration of UVR, for instance) would allow for more controlled experiments.

Analysis of other photoprotective pigments – The amount and exact identity of the blue caroteno-protein (likely α- or β-crustacyanin) is not known. It is also unknown if carotenoprotein, which may have a more direct photoprotective function as previously described, was present in the organisms. Another common type of photoprotective compound, mycosporine-like amino acids, have been shown by Moeller et al. (2005) to play a significant role in reducing UVR photodamage but these were not measured in the current study.

The source of carotenoid compounds – Though their presence is conspicuous in the copepods, their actual source is unknown. Copepods are traditionally thought of as pelagic feeders, along with the majority of other zooplankton, so it is likely that these compounds are obtained through their phytoplankton food source. A type of bacteria isolated from the study lakes in Rocky Mountain National Park by a fellow student, Jenny Paul, seems to contain high concentrations of carotenoid pigment, though the actual type does not match up to any type of

astaxanthin when analyzed on HPLC. Analyses such as stable isotope mass spectrometry on both whole copepod as well as carotenoid compounds may suggest a potential source location.

Further malondialdehyde analysis – The TBARS assay used in this study did not appear to be sensitive enough for the task of measuring malondialdehyde in copepods. Two options exist to continue studying this trend. As mentioned previously, increasing the sample size could allow the use of the same assay kit with a better resolution. Another potential method exists, which would be by use of chromatographic techniques to isolate and quantify the actual amounts of malondialdehyde. This could allow a similar (or potentially smaller) sample size, potentially yielding more precise results.

Conclusions

Dose-response relationships of phototoxic dose and mortality were obtained for five different alpine copepod populations exhibiting a range of carotenoid compounds as well as color phenotypes. As expected, an increase in astaxanthin compounds correlated with lower susceptibility to fluoranthene phototoxicity in the populations studied. Different color phenotypes (red, blue, and a red/blue mixed phenotype) exhibited significantly different sensititivities to PAH induced phototoxicity, though the coloration of the organisms may play less of a role in the survival of the copepods in relation to phototoxicity than does overall carotenoid content. No significant differences were observed between treatments in lipid peroxidation in copepods so the exact mechanism of protection cannot be determined. Regardless, the data supports the notion that carotenoid compounds have a photoprotective role in helping copepods cope with environmental stressors (both anthropogenic and natural).

Data collected show that when challenged with PAH induced phototoxicity, increased levels of carotenoid compounds are correlated with increased survival. As a pigment, astaxanthin

may play a role in the prevention of UVR from reacting with sensitive tissues. As astaxanthin is a potent antioxidant, it likely acts as a photoprotectant by scavenging UVR produced ROS. It is also likely that astaxanthin's antioxidant functioning allows organisms to better cope with not only UVR induced ROS, but also other xenobiotic induced ROS as well. Thus, carotenoid compounds in alpine ecosystems may play a broader ecological function than earlier research has suggested.

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