

**ULTRAVIOLET RADIATION TOLERANCE IN HIGH ELEVATION COPEPODS FROM THE ROCKY
MOUNTAINS OF COLORADO, USA**

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TABLE OF CONTENTS

CHAPTER 1: LITERATURE REVIEW	1
CHAPTER 2: UVR TOLERANCE OF HIGH ELEVATION COPEPODS	13
CHAPTER 3: ASTAXANTHIN AND CAROTENOPROTEIN QUANTIFICATION IN HIGH ELEVATION COPEPODS	27
CHAPTER 4: CONCLUSIONS	45
REFERENCES	47

LIST OF TABLES

Table 2.1: Characteristics of Study Sites.	16
Table 2.2 Correlation of Habitat Variables to UVA, UVB, and Visible Radiation Tolerance.	24
Table 3.1: Sequences and Predicted Product Sizes of Primers used to Amplify Copepod Carotenoprotein mRNA.	33
Table 3.2: BLASTx analysis of CRNA6 Primer Pair PCR Product Sequenced with CRNA6R Primer.	38
Table 3.3: Spearman's rank correlation of copepod Astaxanthin Concentrations to UVR Tolerance for All Study Sites.	39
Table 3.4: Spearman's Rank Correlation of Habitat Radiation Measures and Elevation with Astaxanthin Concentration.	40

LIST OF ILLUSTRATIONS

Figure 1.1: Generalized calanoid copepod body plan.	2
Figure 1.2: Adult blue <i>Diaptomus shoshone</i> .	4
Figure 1.3: Copepod density versus UVR intensity in surface waters of an alpine lake.	7
Figure 1.4: Crustacyanin interaction with astaxanthin.	10
Figure 2.1: Calanoid copepods collected from various high elevation lakes and ponds in Colorado, USA.	15
Figure 2.2: Ponds 5A, 5B, and 5C.	17
Figure 2.3: Example of UVA and UVB received during UVR tolerance test by a copepod in the full spectrum treatment.	20
Figure 2.4: Mean UVA and UVB dose for copepods from each sample site.	22
Figure 2.5: Comparison of full spectrum and visible treatment survivorship by site.	23
Figure 2.6 Results of the UVR tolerance tests of high elevation copepods.	23
Figure 3.1: Chemical structure of astaxanthin.	27
Figure 3.2: Interaction of astaxanthin with crustacyanin and resultant color morph in high elevation copepods.	29
Figure 3.3: Chromatogram of carotenoids extracted from Crater Lake copepods.	36
Figure 3.4: Relative amounts of astaxanthin in copepods from each study site.	37
Figure 3.5: Schematic diagram of the evolution of crustacyanin genes.	43

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Copepods in high elevation lakes and ponds in Colorado are exposed to significant levels of ultraviolet radiation (UV), necessitating development of UV avoidance behavior and photoprotective physiological adaptations. The copepods are brightly pigmented due to accumulation of astaxanthin, a carotenoid which has photoprotective and antioxidant properties. Astaxanthin interacts with a crustacyanin-like protein, shifting its absorbance from 473 nm (hydrophobic free form, appears red) to 632 nm (protein-bound complex, appears blue). In six sites in Colorado, habitat specific coloration patterns related to carotenoprotein complex have been observed. The objective of this study was to determine whether pigment accumulation or carotenoprotein expression has a greater effect on resistance to UV exposure. For each site, copepod tolerance to UV was assessed by survivorship during UV exposure trials. Control organisms were exposed only to the visible spectrum (shielded from UV). Average UV exposure was determined for each habitat. Astaxanthin profiles were generated for copepods in each site. Ability to withstand UV exposure during exposure trials was significantly different between color morphs ($p < 0.0001$). Red copepods were found to tolerate 2-fold greater levels of UVB than blue or mixed copepods. Additionally, red copepods have much higher levels of total astaxanthin than blue or mixed copepods ($p < 0.0001$) and receive a higher daily UV dose ($p < 0.0003$). Carotenoprotein mRNA sequence varies greatly among crustaceans. Diaptomid carotenoprotein sequence is not homologous with that of other crustaceans in which crustacyanin has been characterized. Carotenoprotein expression differences were not quantified because sequence variation prevented effective amplification. Overall, diaptomid color morph may be an important indicator of UV conditions in high elevation lentic ecosystems.

CHAPTER 1

LITERATURE REVIEW

Introduction to Freshwater Copepods

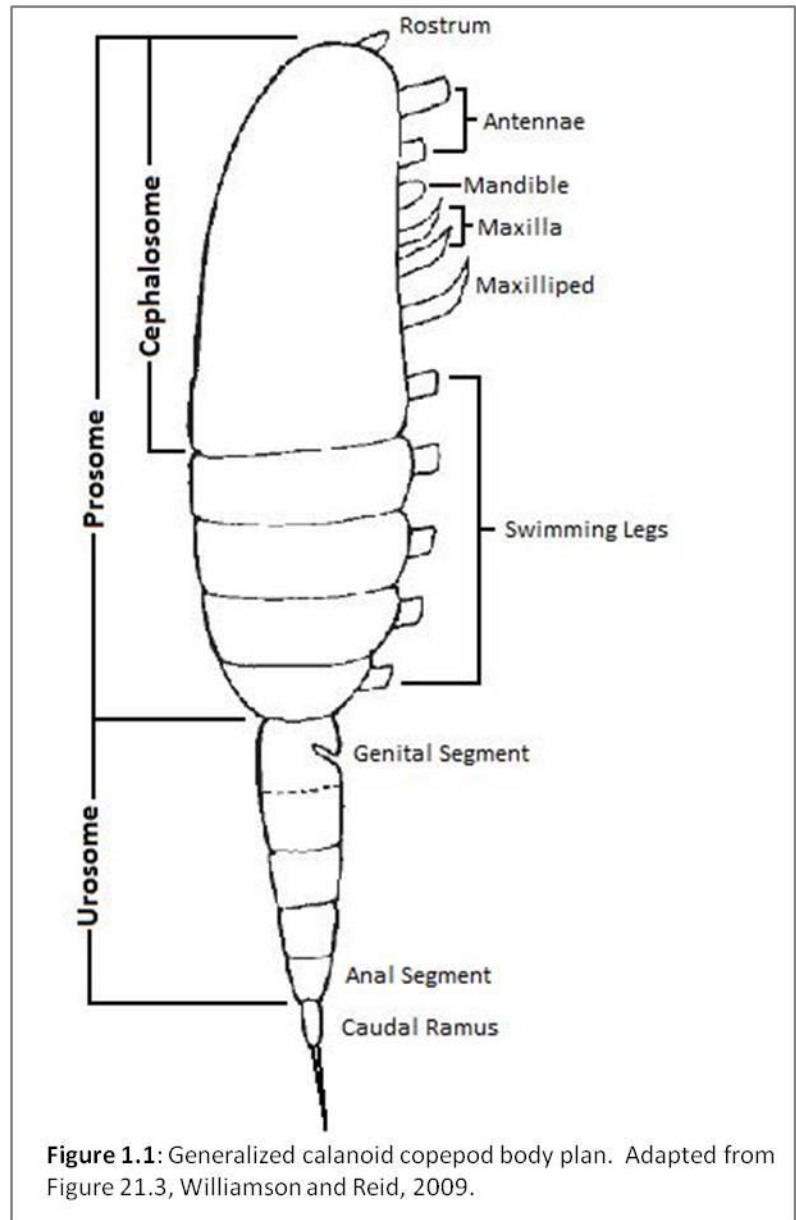
The subclass Copepoda in class Crustacea has 14,000 known species. Of these, 2814 inhabit freshwater ecosystems (Williamson and Reid 2009). In terms of phylogenetic age, abundance, geographic range, and breadth of adaptive radiation, copepod success is on par with that of insects, making copepods one of the most successful groups of metazoans on Earth (Mauchline 1998, Schminke 2007). Copepods originated in the oceans, but often dominate the fauna of freshwater systems they inhabit (Williamson and Reid 2009). Densities of up to 1000 individuals per liter of water have been recorded (Pennak 1978). Typical freshwater copepod habitats range from moist soils and groundwater to lakes, rivers, wetlands, seasonal ponds and even small puddles. Copepods are also commonly found in interstitial and subterranean systems, as well as phytotelmata, the small volumes of water that collect in various parts of plants (Williamson and Reid 2009).

Copepods are important in freshwater food webs as primary and secondary consumers and as a major food source for larger invertebrates and vertebrates including fish (Mauchline 1998). They are predominantly filter feeders, consuming phytoplankton, bacterioplankton, and microzooplankton, but also act as detritovores (Pennak 1978, Williamson and Reid 2009). As grazers, copepods concentrate several types of plankton in their bodies actually increasing food chain efficiency despite adding a trophic level (Williamson and Reid 2009). Copepods frequently make up a large portion of the consumer biomass and thus have a large impact on the energy flow and nutrient cycling in freshwater ecosystems (Williamson and Reid 2009).

Superorder Gymnoplea

includes order Calanoida copepods.

There are 2300 known calanoids, 550 of which live in freshwater. Calanoids are predominantly pelagic and have planktonic or free swimming life histories, as opposed to benthic or parasitic lifestyles. North American freshwater copepod community structure is typically dominated by a single cyclopoid and a single calanoid species (Pennak 1978). Communities in harsh habitats such as the Arctic, Antarctic, and high elevation ecosystems, are typically dominated by one or two calanoid species (Williamson and Reid 2009). Within the order Calanoida, the family

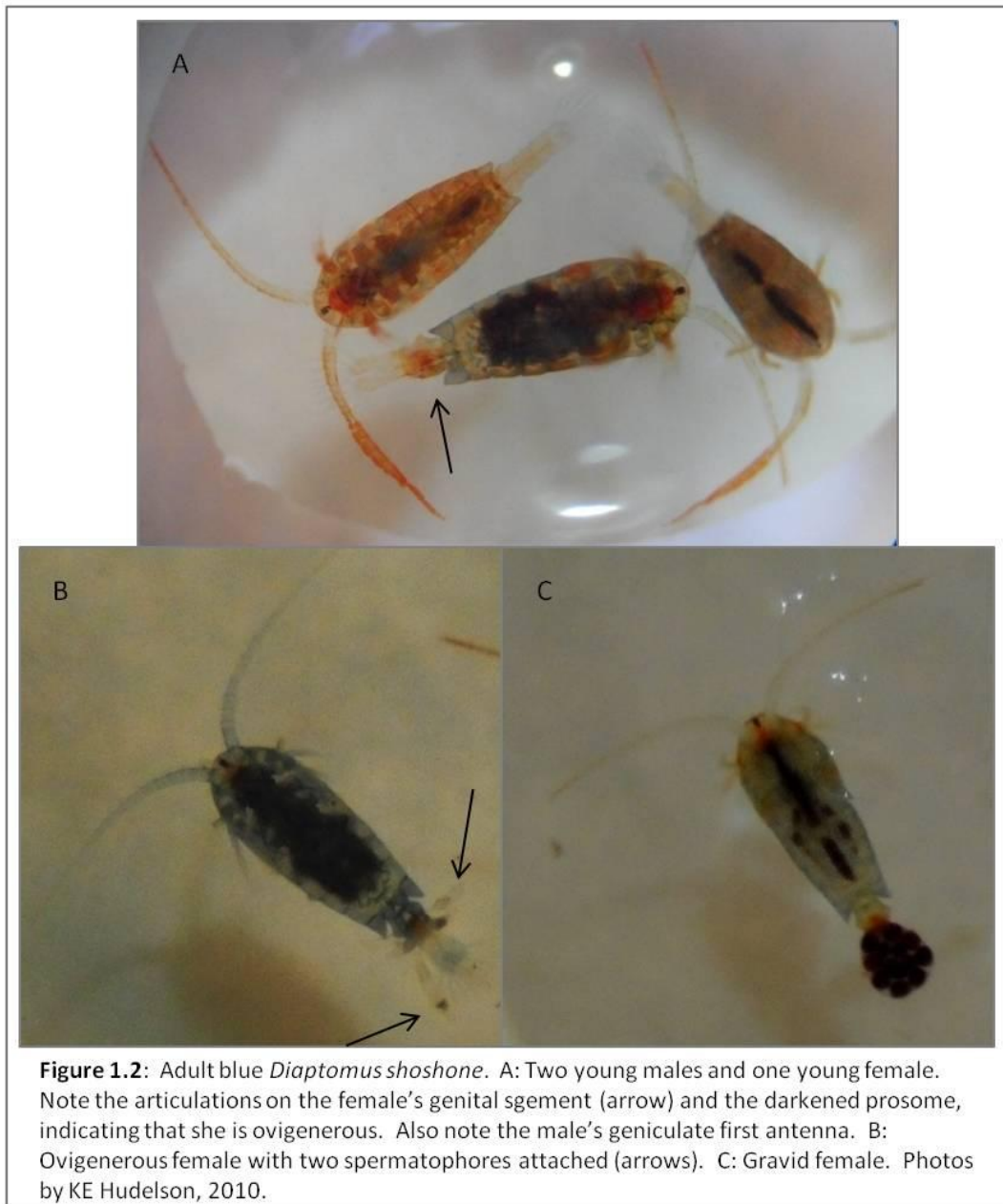


Diaptomidae includes 59 genera, 13 of which are found in North America. This study focuses on Diaptomids, specifically *Diaptomus shoshone*, found in high elevation habitats of the Front Range of the Colorado Rocky Mountains and the Sangre de Cristo subrange in southern Colorado.

Physical Characteristics of Copepods

Copepods have roughly cylindrical, segmented bodies typically 0.5 – 2 mm in length. They have a hard exoskeleton and segmented appendages on their head and thorax. The name copepod is derived from the Greek words *kope podos* which means “oar foot” and refers to the structure of their five pairs jointed, flat, laminar swimming legs (Mauchline 1998, Williamson and Reid 2009). Their prominent first antennae and bristly appendages at the posterior end of their abdomen, known as setose caudal rami, help to distinguish the copepods from other zooplankton groups (Mauchline 1998, Williamson and Reid 2009). The first antennae can consist of up to 25 uniramous (unbranched) segments used in sensory perception and locomotion (Pennak 1978). As shown in Figure 1, the copepod body segments are divided into the prosome (cephalothorax and abdomen) and urosome (genital segment and posterior segments) (Williamson and Reid 2009). The prosome and urosome are clearly divided by a single major articulation. Copepods possess a single anterior simple eye, and their bodies are covered with minute chemoreceptors and mechanoreceptors. Their digestive system consists of a mouth, foregut, midgut, hindgut, and anus. Calanoid copepods possess a dorsal heart consisting of a pair of lateral ostia and a single ventral ostium. A short aorta carries blood anteriorly and is the only blood vessel present (Williamson and Reid 2009).

Like many crustaceans, copepods increase their body size by moulting their exoskeleton. The first nauplius hatches from the egg, proceeds through five moults to the sixth nauplius, which moults to the first copepodid. The copepodid then proceeds through five moults to the adult form. The adult form does not moult, but retains the same exoskeleton for the rest of its life (Mauchline 1998). Maturation to adulthood is typically 1-3 weeks, and adult lifespan is 1-3 months (Williamson and Reid 2009). However, time necessary to complete an entire life cycle from egg to egg is dependent on species and environmental conditions, and thus is highly variable. Some diaptomids have a year-long life cycle (Pennak 1978). Copepods are strict sexual reproducers. Female copepods are typically larger than the males, and female diaptomids have lateral protrusions on genital segment. Calanoid males can



also be distinguished by their right first antennae, which is geniculate and modified for grasping the female during copulation. Copulation consists of the male attaching spermatophores to the female's paired seminal receptacles on her genital segment. Eggs are fertilized as they are extruded into the egg sac, which remains attached to the female's genital segment until it drops off just prior to hatching. The eggs can be subitaneous, in which case they will hatch 1-5 days after detachment of the egg sac, or diapausing, in which case they can remain dormant for a few weeks, a year, or as long as a century.

Both forms of eggs can pass through fish or bird digestive tracts intact (Williamson and Reid 2009). See Figure 2.1 for photographs of copepods in various stages of reproduction. Water temperature, salinity, and oxygen concentrations are the main factors determining dormancy of diapause eggs, but salinity and oxygen concentration are often negligible relative to water temperature (Mauchline 1998).

Because copepods act as a direct link between phytoplankton and higher trophic positions and are often the predominant fauna, they are of vital importance to freshwater ecosystem functioning (Mauchline 1998, Williamson and Reid 2009). In high elevation lakes and ponds of the Colorado Rockies, species diversity is low and *D. shoshone* typically dominate the zooplankton communities. The low diversity amplifies the role of *D. shoshone* as a link between primary producers and higher trophic positions in these systems.

Ultraviolet Radiation in High Elevation Lakes and Climate Change

High elevation lentic ecosystems are harsh due to the low nutrient availability, low temperatures, short productive period, and highly variable ultraviolet radiation (UVR) conditions (Sommaruga 2001). Primary productivity is limited by the available nutrients and cold temperatures, and thus these ecosystems tend to be ultra-oligotrophic, contributing to the transparency that is the hallmark of high elevation lakes (Sommaruga 2001, Rose et al. 2009b). Cold water temperatures also allow for very high dissolved oxygen concentrations; up to 50% saturation has been reported in alpine Rocky Mountain Lakes (Rawson 1942). The open (ice-free) season in the alpine areas of the Rocky Mountains is typically June – August (Bowman and Seastedt 2001). Because these ecosystems are covered with snow and ice for most of the year, and exposed during the time of year when UVR is greatest, UVR exposure varies drastically during the year.

UVR is an important driver in aquatic ecosystems, particularly in high elevation systems due to their naturally higher exposure to UVR. The shortest wavelength and highest energy form of solar

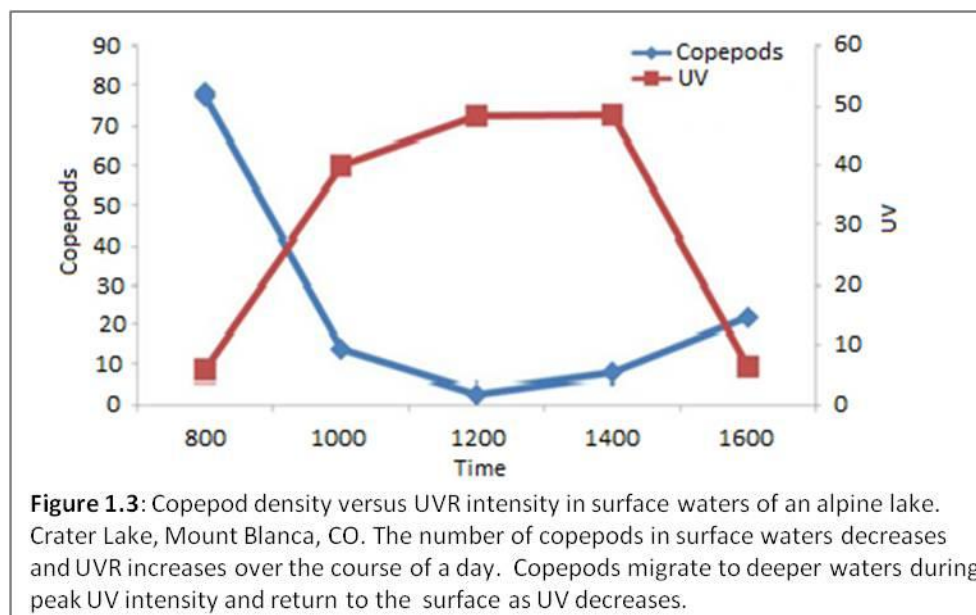
radiation that passes into earth's atmosphere is called ultraviolet radiation (Williamson and Rose 2010). UV-B (290 – 320 nm), the most biologically active type of UVR, has been reported to increase about 18% with every 1000 m increase in elevation (Ambach et al. 1993, Blumthaler et al. 1997). The wavelengths that reach earth's surface are between 290 and 400 nm, and are capable of compromising the physiology and performance of organisms they reach (Lesser et al. 2001, Williamson et al. 2001). UV damage can occur through direct interaction of UV photons with biomolecules or through the generation of reactive oxygen species (ROS). ROS generated as a result of oxidative conditions can lead to damage of biological tissues including lipids, proteins, and nucleic acids. ROS interaction with membranes resulting in peroxidation of lipids is one of the most prevalent mechanisms of cellular injury by ROS (Lesser 2006). The accumulation of ROS damaged protein constituents is hypothesized to be a component of the aging process (Lesser 2006). ROS generated lesions in DNA can result in deletions, mutations, strand breakage, and cross-linking to proteins (Lesser 2006). Direct damage by UV photons can generate pyrimidine dimers in DNA strands, preventing normal transcription (Peak and Peak 1992). DNA absorbs strongly in the UV range, and DNA damage is the most prevalent form of direct UV damage (Diffey 1991, Peak and Peak 1992). DNA-protein crosslinking can also occur (Diffey 1991). Chromophoric dissolved organic matter (CDOM), the main attenuator of UVR in aquatic environments (Cooke et al. 2006), decreases with increases in elevation (Sommaruga 2001). Because primary productivity and CDOM concentrations are both very low in high elevation lakes, these systems are some of the most UV-transparent habitats in the world (Sommaruga 2001).

The ozone layer absorbs, reflects, and scatters solar UVR thereby shielding the earth's surface. Ozone depletion, a component of global climate change, has increased UVR reaching the Earth's surface at temperate latitudes (Williamson et al. 2002). UV-B has been reported to increase 10-20% each decade in northern and southern temperate zones (Stolarski et al. 1992, Kerr and McElroy 1993).

Because high elevation lakes are naturally UV-transparent, they may be particularly vulnerable to increased UVR associated with climate change (Hader et al. 2007).

Overall, inhabitants of high elevation lakes experience very intense UVR because the water in these systems is very UV transparent and UVR is naturally higher at high elevations (Vinebrooke and Leavitt 1998). The UVR conditions necessitate the development of specialized adaptations by the zooplankton community, as UVR has been shown to enhance mortality and impair reproduction in zooplankton (Stutzman 1999). Adaptations are characterized either as behavioral (seeking UVR refuge) or physiological (accumulation of photoprotective compounds or repair of photodamage).

Zooplankton Adaptations to UVR



Zooplankton deal with UVR stress in three ways: photoavoidance behavior (Leech et al. 2005), photoenzymatic repair of damaged DNA (Zagarese and Williamson 1994), or accumulation of photoprotective compounds (Moeller et al. 2005). Some zooplankton exhibit light- and temperature-dependent photoenzymatic repair to DNA (Connelly et al. 2009), but photorepair is not well studied in copepods. Zooplankton exhibit a predictable pattern of migration in the water column known as diel

migration, which consists of swarming in surface waters during low light conditions, and moving down the water column in intense light conditions, depicted in Figure 1.3 (Pennak 1978, Hader et al. 2007, Hansson et al. 2007). Avoiding intense UVR allows zooplankton to avoid photodamage as well as predation by visual predators (Hairston 1979b, Williamson 1995, Gal et al. 1999, Hansson 2000). Diel migration has been observed in many groups of copepods (Williamson et al. 2001, Leech et al. 2005), including diaptomids (Hairston 1976) but is not consistent among copepods, although it is very consistent in *Daphnia* (Leech et al. 2005, Hansson et al. 2007). Rather, copepods rely more heavily on accumulation of dietary pigments that act as photoscreens and as antioxidants to mitigate UVR damage (Hansson et al. 2007, Williamson and Reid 2009), although pigmentation increases predation pressure (Luecke and O'Brien 1981).

Advantages and Disadvantages of Pigmentation

The most common pigment molecule isolated from copepods is the carotenoid astaxanthin (AX), a potent antioxidant associated with enhanced UVR survivorship in many studies and gives copepods a vibrant red or orange color (Hairston 1976, Byron 1981, 1982, Hansson 2004, Hylander et al. 2009). Astaxanthin can be bound by a carotenoprotein, resulting in pale green or blue pigmented copepods (see next section for detailed description of carotenoprotein) (Luecke and O'Brien 1981, Byron 1982). Calanoid copepod pigmentation typically increases with elevation (Sommaruga 2001); a trend that has been reported in the Front Range (Byron 1982). Most researchers agree that carotenoids act as a photoprotectant, but alternative roles of carotenoids in zooplankton have been suggested as well. Byron reported increased metabolic rate in pigmented versus nonpigmented copepods and hypothesized that carotenoid accumulation allowed for localized warming of important metabolic processes, imparting an advantage to pigmented copepods in cold waters (Byron 1981). Other researchers have implicated carotenoids in reproductive processes of copepods because the pigments

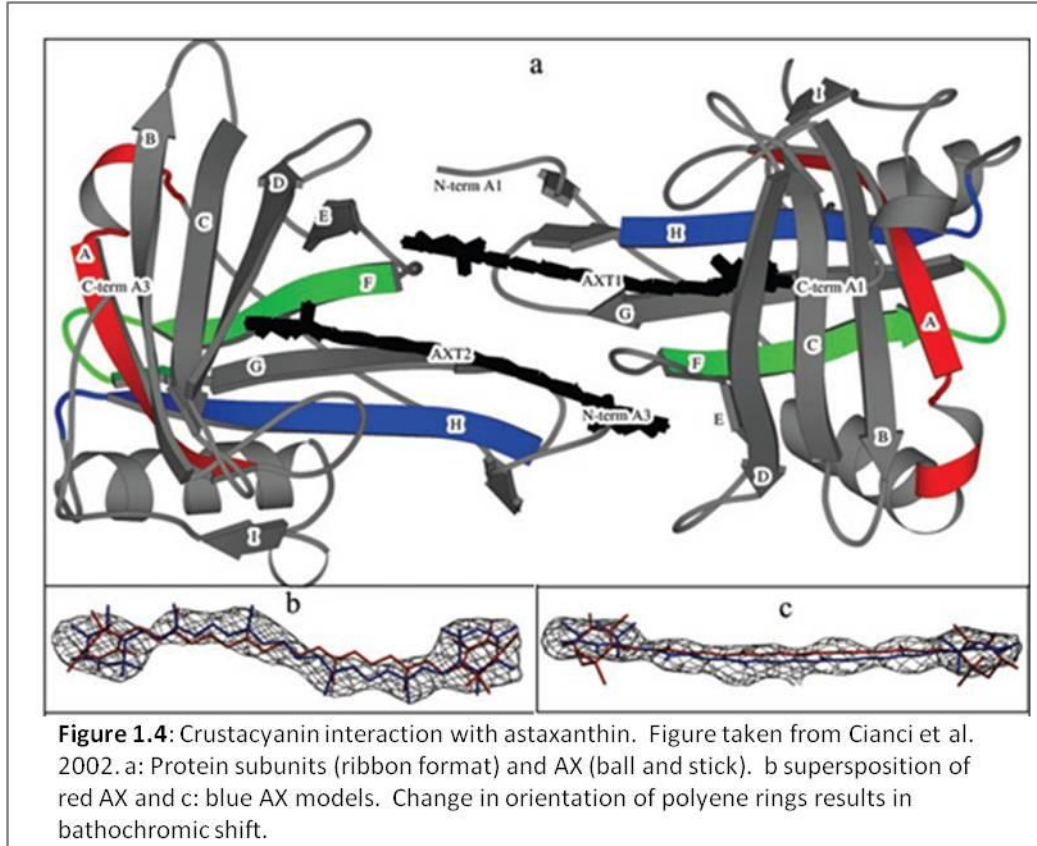
are often localized in the gonads and are concentrated in the eggs and nauplii (Pennak 1978, Hairston 1979a). Others simply suggest that carotenoids are accumulated as food reserves (Pennak 1978). While AX is an important component of copepod diet and may be essential in several biological processes, increased accumulation of AX when exposed to UVR (Luecke and O'Brien 1981, Stutzman 1999) and enhanced survivability of copepods exposed to UVR strongly implicates that AX plays an important role as a photoprotectant.

Copepods also accumulate mycosporin-like amino acids (MAAs) which assist in mitigation of UVR stress (Hansson et al. 2007). It is unlikely that MAAs also enhance predation in the same way that carotenoids do by increasing visibility (Hansson et al. 2007) and MAAs are thus accumulated preferentially in the presence of fish (Hylander et al. 2009).

Predation by sight hunting predators often counter-balances pigment accumulation in zooplankton (Hansson 2000, Boeing et al. 2004). In a study of *Heterocope septentrionalis* in 37 lakes in the Toolik Lake region of Alaska, pale green copepods were found in lakes with fish, while fishless lakes contained bright red copepods. The red copepods had greater UV survivorship but were preferentially preyed upon by arctic grayling fish (Luecke and O'Brien 1981). Likewise, red copepods were preferentially preyed on, and periods of pigmentation maxima were constricted in the presence of fish in central Washington (Hairston 1979a).

In general, accumulation of carotenoids leads to increased UV survivorship, and increased predation rate. Zooplankton must respond to both threats to be successful in their habitats. Carotenoprotein-carotenoid interactions may also play an important role in photoprotection while reducing predation risk.

Characteristics of Astaxanthin and Crustacyanin



The carotenoid AX has photoprotectant properties capable of shielding biota from solar radiation (Byron 1982, Wang et al. 2003) and is also a potent antioxidant that quenches reactive oxygen species generated by photooxidation (Beutner et al. 2001, Lesser 2006). The conjugated double bond system allows AX to quench oxygen radicals by radical addition, absorb unstable electrons, or hydrogen abstraction at allylic carbons (Woodall et al. 1997). Astaxanthin is composed of two polyene rings linked by an 18 carbon conjugated chain (Higuera-Ciajara et al. 2006). Each ring bears a hydroxyl group at which esterification can occur. Therefore, AX can be found in free or unesterified form, monoester, or diester form (Hussein et al. 2005, Higuera-Ciajara et al. 2006). (Young and Lowe 2001). Astaxanthin accumulation is positively correlated to increased UV survivorship of copepods in many studies (Zagarese and Williamson 1994, Hansson 2000, Moeller et al. 2005, Hansson et al. 2007) and has been

detected in the free state, monoester, and diester form in copepods (Lotocka and Styczynska-Jurewicz 2001, Kovach 2010). In crustaceans, AX is often bound by the carotenoprotein α -crustacyanin, which changes the orientation of the pigment molecule and results in a shift from reflecting red light (472 nm) to reflecting blue light (632 nm) (Britton et al. 1997). α -crustacyanin isolated from *Homarus gammarus*, the European clawed lobster, is composed of eight subunits, each consisting of two AX molecules and two protein subunits, designated A and C (Figure 1.4) (Cianci et al. 2002). Thus the holoprotein is a 16-mer capable of binding 16 AX molecules. The blue and green color morphs of copepods are probably due to a similar carotenoprotein interaction with AX, but they have not been characterized to the extent of other crustaceans. A carotenoprotein complex was isolated from the hypodermal tissue of *Labidocera acutifrons*, a marine copepod, had a similar molecular weight (720 kD) to crustacyanin, but was found to bind three AX per subunit (Zagalsky and Herring 1972).

Changes in prevalence of carotenoprotein expression in the Front Range

In 1981, Earl Byron included a series of permanent and ephemeral ponds, the Moraine ponds in the University of Colorado Mountain Research Station, in a survey of copepod pigment biogeography in the front range of the Rocky Mountains. Dr. Byron explicitly states that "...blue copepods were relatively rare among the copepod populations I surveyed. No populations from which I estimated pigmentation intensity were predominantly blue," (Byron 1982). During the next decade, UVR increased 10-20% above normal levels (Stolarski et al. 1992, Kerr and McElroy 1993). In 2007, Dr. Aaron Roberts returned to the UC-MRS Moraine ponds and collected bright blue and mixed (blue and red) copepods from the ponds. At similar elevation to the blue copepod habitats, red copepods are observed in deep lake habitats which contain fish. Because the UC-MRS ponds are relatively shallow (2 m or less), the copepods in these systems cannot effectively migrate away from UVR, and must instead rely strongly on carotenoid pigmentation to provide UVR protection.

Experimental Summary

Because the appearance of the novel copepod color morphs coincided with a period of documented increase in UVR on the Front Range, and because the novel color morph appeared in habitats where behavioral UVR avoidance was not possible, it was hypothesized that copepods from shallow pond habitats were 1) experiencing more UVR than copepods from deep lake habitats and that 2) the blue copepods were using carotenoprotein complexes to enhance the photoprotective properties of AX as a way to ameliorate their greater UVR burden. In order to test these hypotheses, two sets of experiments were carried out. First, UVR in copepod habitats, as well as the UVR tolerance of copepods, was measured in six sites of the Colorado Rocky Mountains. These experiments are described in Chapter 2: UVR Exposure and Tolerance in High Elevation Copepods. Additionally, AX content of each population was quantified and carotenoprotein expression quantification was attempted, as detailed in Chapter 3: Photoprotection by Astaxanthin and Characterization of Carotenoprotein in High Elevation Copepods.

CHAPTER 2

UVR TOLERANCE OF HIGH ELEVATION COPEPODS

Introduction

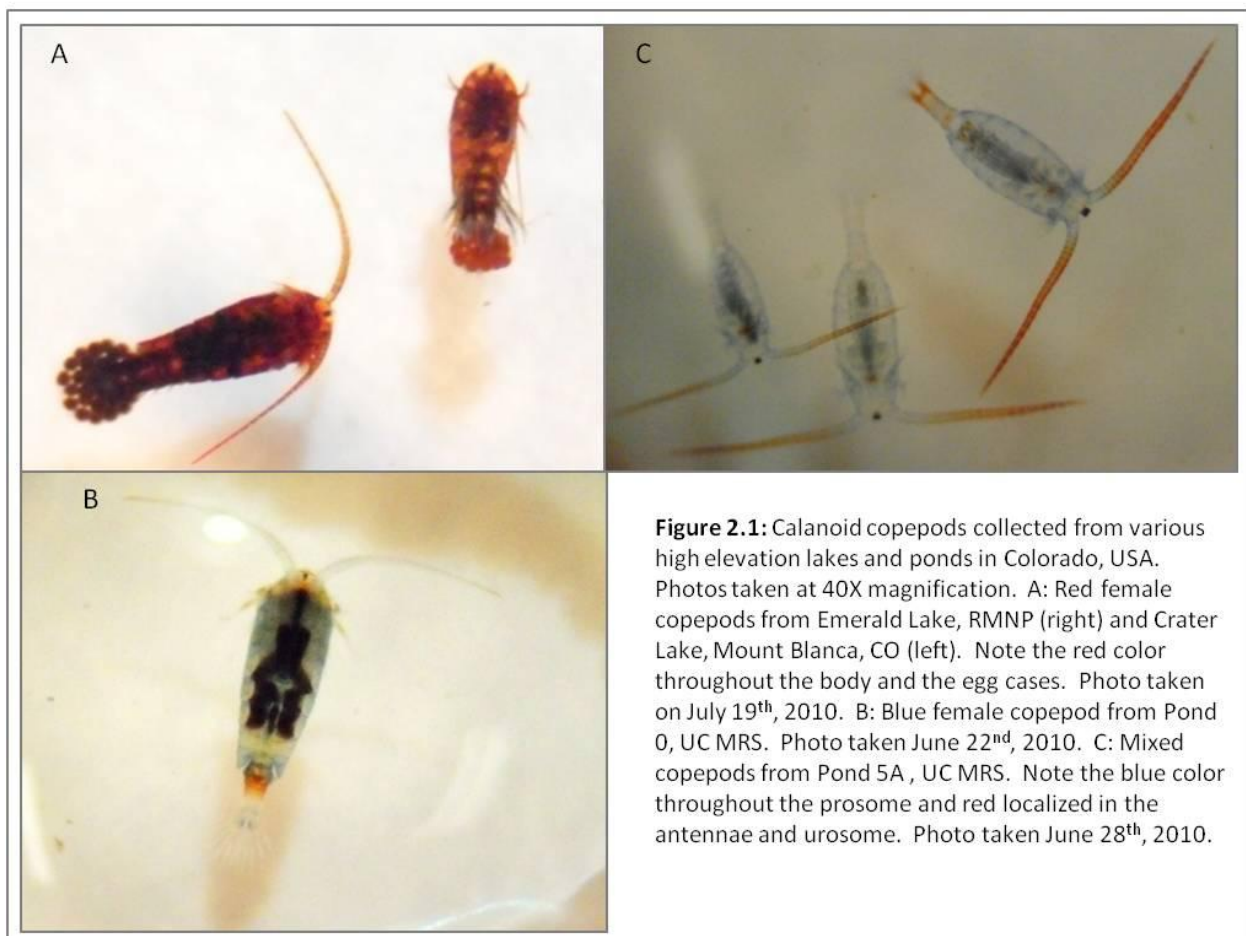
Ultraviolet radiation (UVR) is the shortest wavelength and highest energy form of solar radiation that passes into earth's atmosphere (Williamson and Rose 2010). The wavelengths that reach earth's surface are between 290 and 400 nm, and are capable of degradation of DNA, proteins, and membrane lipids, potentially compromising the physiology and performance of organisms they reach (Lesser et al. 2001, Williamson et al. 2001). High elevation aquatic ecosystems are exposed to high levels of UVR due to their high elevation and high water transparency (Sommaruga 2001). In fact, UV-B (290 – 320 nm), the most biologically damaging type of UVR, was found to increase about 18% with every 1000 m increase in elevation (Ambach et al. 1993, Blumthaler et al. 1997). In aquatic ecosystems, UVR is attenuated by chromophoric dissolved organic matter (DOM) (Cooke et al. 2006), which is present in very low concentrations in alpine lakes making them some of the most UVR transparent ecosystems in the world (Vinebrooke and Leavitt 1999).

Because alpine lentic ecosystems are harsh, species richness and diversity is low in these ecosystems (Rose et al. 2009a). Organisms that inhabit these systems must be well adapted in order to thrive in intense UVR conditions. Calanoid copepods, often the dominant zooplankton taxa in high elevation lentic systems, exhibit two forms of adaptation to UVR: diel migration and accumulation of carotenoid pigments. Diel migration is a behavior consisting of migrating deeper in the water column to increase attenuation of solar radiation, but rising back to surface waters to graze or mate when solar radiation is less intense. This behavior has been observed in many zooplankton (Hairston 1976, Gal et al. 1999, Williamson et al. 2001, Cooke et al. 2006, Hansson et al. 2007). Additionally, high elevation calanoid copepods develop striking reddish pigmentation, due to accumulation of dietary carotenoids,

which increases with altitude (Sommaruga 2001). Byron confirmed the correlation of increasing pigmentation with altitude in the front range of the Rocky Mountains in Colorado (1982). The consensus among researchers is that accumulation of carotenoid pigments by copepods is a photoprotective mechanism, though carotenoids may also play other important roles in copepod biology (Hairston 1976, 1979a, Hairston 1979b, Byron 1981, 1982, Hansson et al. 2007). Previous studies by Hairston have shown that carotenoids enhance survival of calanoid copepods exposed to short wavelength visible light (450 nm). Byron exposed highly pigmented and colorless copepods to sunlight and reported significantly increased mortality in colorless copepods. Byron did not test the effects of UVR independent of visible light, but he suspected that “carotenoid pigments are probably not protective against UV light,” (1982). However, a calanoid copepod UVR tolerance study conducted in Sweden and Siberia by Hansson and others, in which the UVR tolerance of a carotenoid poor species (*Eudiaptomus gracilis*) and a carotenoid rich species (*Leptodiaptomus angustilobus*), were compared, there was no significant difference in the survivorship of copepods exposed to UVR or shielded from UVR (2007). Interestingly, these researchers report that copepods exposed to UVR accumulated 3-10% more carotenoid during the UVR exposure than did the UVR shielded copepods, in which carotenoid pigment decreased 14-22% (Hansson et al. 2007). Additionally, the study did not detect significant diel migration in copepods, but found that the behavior was very predictable for daphnids. The researchers concluded that accumulation of pigmentation was the primary mode of UVR defense for copepods in the study.

Depletion of the ozone layer, which shields the earth from solar UVR, has increased UVR at temperate latitudes (Williamson et al. 2002). UV-B was reported to increase 10-20% each decade in northern and southern temperate zones (Stolarski et al. 1992, Kerr and McElroy 1993). In 1981, Earl Byron included the Moraine ponds in the University of Colorado Mountain Research Station in a survey of copepod pigment biogeography in the front range of the Rocky Mountains. In 2007, Dr. Aaron

Roberts returned to the UC-MRS Moraine ponds and collected bright blue and blue and red copepods from the ponds. Dr. Roberts confirmed with Dr. Byron that this was a novel color morph not found during Byron's extensive studies of the ponds (Byron 1982) (Byron-Roberts personal communication). Because the UC-MRS ponds are relatively shallow (2 m or less), the copepods in these systems cannot effectively migrate away from UVR, and must instead rely heavily on carotenoid pigmentation to provide UVR protection. It was hypothesized that the novel color morphs arose as a response to the recent increase of UVR in the habitats of these copepods. To test this hypothesis, UVR measurements and copepods were collected from six sites of varying depth in the Colorado Rockies and the daily dose of UVR and visible radiation copepods receive was calculated for each site. Additionally, UVR tolerance of copepods from each site was assessed. Results of these studies demonstrate that the novel color morphs are likely not an adaptive response to increased environmental UVR, as the shallow waters of the Moraine ponds attenuate ambient UVR very efficiently.



Materials and Methods

Study Sites

Copepods were collected from a range of high-elevation, habitats in Colorado both above and below treeline (Table 2.1). Each study site contained calanoid copepods that were brightly pigmented entirely red, entirely blue, or blue with red antennae and genital segments as adults. The color morphs of the adult copepods are shown in Figure 2.1 and will be described as blue, red, or mixed throughout this work. In each study site only one color morph can be found.

Table 2.1: Summary of Characteristics of Study Sites

Site	Color Morph	Temp. °C	Cond. mS/cm	Hardness mg/L CaCO ₃	Alkalinity	pH	Lat.	Long.	Elev. (m)
Pond 0	Blue	16.21	0.023	12	20	6.15	40°01' 48.68"	105°33' 06.66"	3108
Pond 5A	Mixed	16.27	0.019	8	20	6.39	40°01' 49.83"	105°33' 41.53"	3116
Pond 5B	Blue	13.78	0.018	8	20	6.08	40°01' 47.55"	105°33' 41.00"	3112
Pond 5C	Mixed	17.26	0.021	8	20	6.01	40°01' 48.20"	105°33' 38.71"	3114
Crater Lake	Red	4.26	---	24	---	4.13	37°49' 19.82"	105°30' 1.28"	3703
Emerald Lake	Red	8.63	0.011	20	---	5.71	40°18' 36.49"	105°40' 5.30"	3081
Como Creek	---	8.0	0.015	28	20	5.49	40°18' 36.49"	105°40' 5.30"	2958

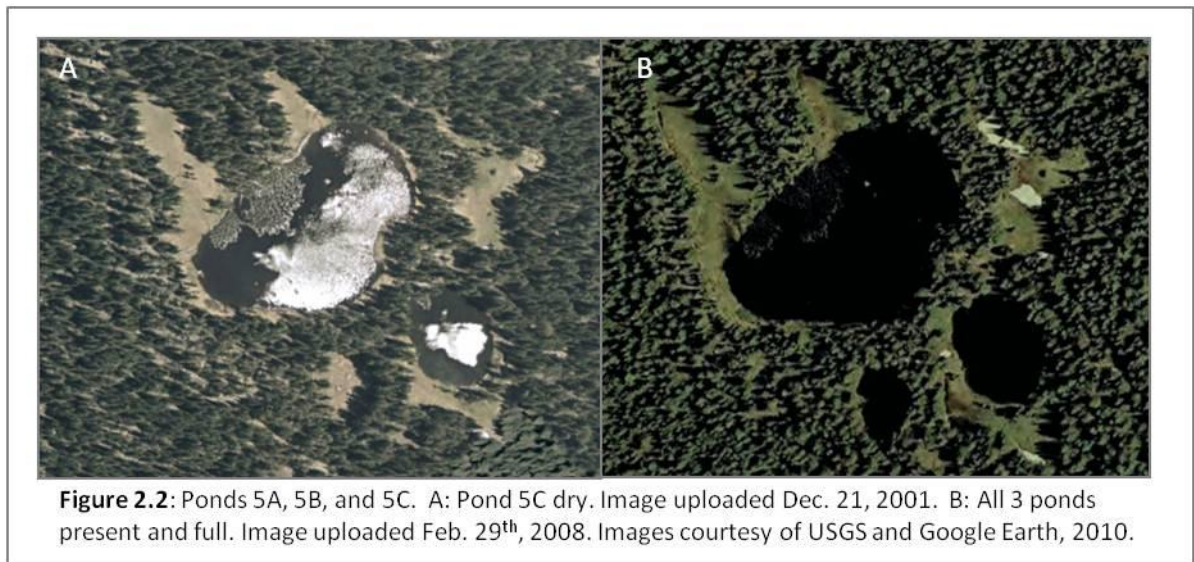
Moraine Ponds; University of Colorado Mountain Research Station (UC MRS)

The University of Colorado Mountain Research Station is located within Roosevelt National Forest and overlaps the Niwot Ridge Long Term Ecological Research Site (LTER). Below treeline within the LTER there are several moraine ponds between elevations 10,200 and 10,224 ft that are home to blue or mixed copepods. Dr. Earl Byron surveyed calanoid copepods in these ponds in the late 1970's

but did not note the presence of blue copepods at that time (Byron 1981, 1982). All of the ponds are fishless.

Pond 0 contains blue copepods and is an ephemeral pond. Pond 0 did not dry completely during the 2010 field season, but has dried completely in previous years.

Ponds 5A, 5B, and 5C are a series of ponds in very close proximity (Figure 2.2). Pond 5A is the largest pond (approximately 7440 m²) that feeds into Pond 5C via an 18 m stream. Both ponds 5A and 5C contain mixed copepods and do not appear to be ephemeral. Pond 5B is an ephemeral pond and is home to blue copepods. Despite the short distance between these ponds (less than 20 m) the copepod color morph in 5B is distinct from Ponds 5A and 5C.



Como Creek is a small creek near the UC-MRS laboratory where UV tolerance testing was carried out. As shown in 1.1, the water from this creek has similar properties as that in the study sites and therefore was a suitable source of water used to maintain copepods during testing.

Emerald Lake; Rocky Mountain National Park

Emerald Lake is located within Rocky Mountain National Park in northern Colorado. This lake sits between Hallett's Peak and Flattop Mountain. The water is very clear; UVA and UVB penetrate to the bottom. This lake is inhabited by fish as well as red copepods. It is below treeline.

Crater Lake; Mount Blanca

Crater Lake is on Mount Blanca in the Sangre de Cristo mountain range in southern Colorado. It is above treeline. The water is very cold and clear, with UVR penetration to the bottom. It is inhabited by fish and red copepods.

Measurement of Radiation in Each Study Site

In order to approximate the daily dose of UVR copepods receive in each study site, a PUV multi-wavelength radiometer (Biospherical Instruments Inc., San Diego, CA) was used to measure UVA ($\lambda = 380 \text{ nm}$), UVB ($\lambda = 318 \text{ nm}$), and photosynthetically active radiation (PAR, $\lambda = 400\text{-}700 \text{ nm}$, visible light). Continuous light and depth measurement were taken along the entire water column. Peak UVR occurs between 10 AM and 2 PM in Colorado during summer, and so measurements were taken at approximately 10:00 AM, 12:00 PM, and 2:00PM for each site. Measurements were taken on two representative days for the moraine ponds, but only one profile was completed for Emerald and Crater Lakes. The solar radiation profile of Emerald Lake was taken during the summer of 2008 and was not repeated during the summer of 2010 due to limitations on research permits. For measurement of solar radiation in the Moraine ponds, the radiometer sensor was placed, two minutes were allowed for sediments to settle, and the sensor was allowed to collect data for three minutes. For measurement of solar radiation in Crater Lake and Emerald Lake, the sensor was lowered to the bottom of the lake and then raised back to the surface to take a complete measurement. An 80 μm closing Wisconsin

zooplankton tow net (Wildco Inc., Yulee, FL) was used to collect copepods from two meter increments of the water column. Copepods from each tow were counted. A multiparameter sensor (556 MPS, YSI Inc., Yellow Springs, OH) was used to measure water temperature, pH and conductivity.

Habitat Radiation Data Analysis

For each site, three seconds of data was averaged from each solar radiation reading to generate doses in ($\mu\text{W}/\text{m}^2$) for UVA and UVB and ($\mu\text{E}/(\text{cm}^2\text{nm})$) for PAR. For the shallow pond habitats, the daily dose of each type of radiation was calculated separately. The dose received by the copepods in deep lake habitats was determined by multiplying the percentage of sampled copepods at each depth and the amount of radiation at that depth, then summing the values. UVA, UVB, and PAR doses between sites were compared using a general linear model (GLM) ANOVA (SAS v. 9.1, SAS Institute Inc., Cary, NC) with $\alpha = 0.05$, and Scheffe's post hoc means separation. Scheffe's pairwise comparisons were used because the number of measurements was not equal for all sites and this test minimizes type I experimentwise error. Additionally, sites were grouped according to color morph and compared using GLM, $\alpha = 0.05$, and Scheffe's test.

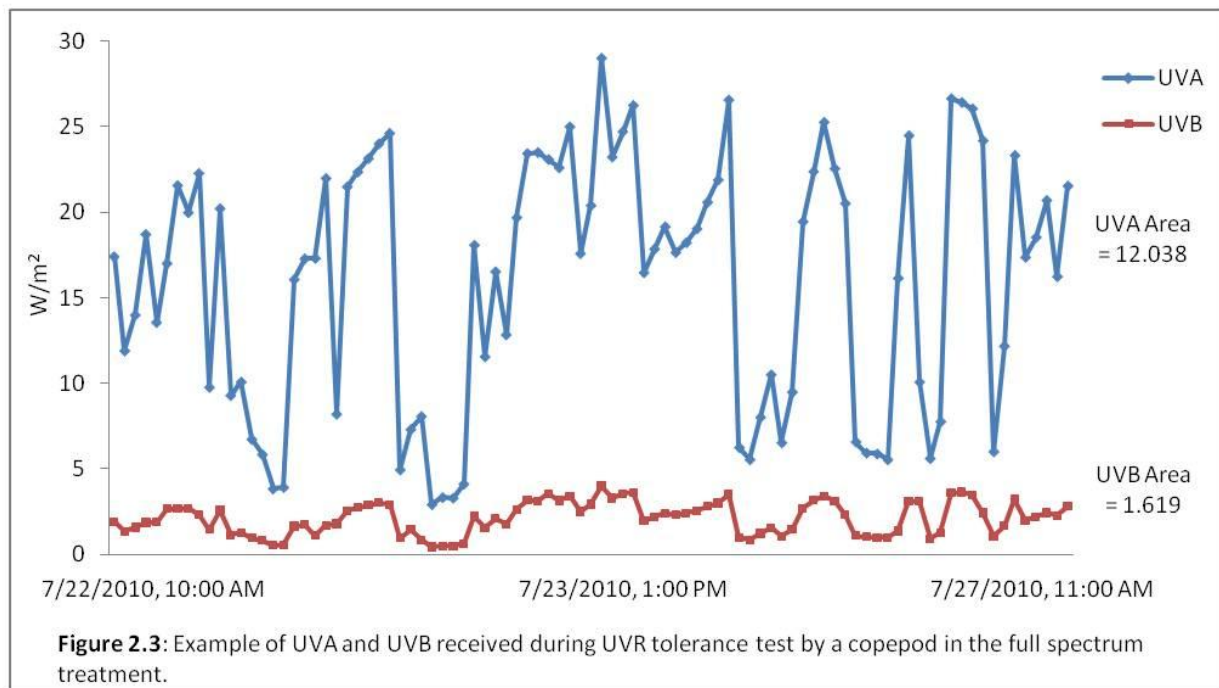
UVR Tolerance Testing

Adult copepods were collected from each sampling site using a 12" diameter 250 μm toss net 24 hours prior to UVR tolerance testing. Copepods were transported in water from their habitats to the UC-MRS laboratory, where they were transferred to water from Como Creek which had been filtered using a 45 μm filter to remove large particulates. The animals were then stored at 4-6 °C in the dark. The UVR tolerance tests began July 21st 2010, and were completed on August 6th 2010. Prior to commencement of testing, individual copepods were placed in cups with 20 mL of fresh filtered Como Creek water. Each cup was then placed in a water table. The water table allowed water to flow around the cups without mixing with the water inside the cups in order to maintain the same temperature in all

the cups. Temperature was maintained at $\sim 15^{\circ}\text{C}$ during tests by addition of chilled water to the water table. Half of the water table was covered in UV transparent Aclar plastic sheeting (Honeywell Int. Inc. Morristown, NJ; 96% UVA transparent, 93% UVB transparent, 92% visible light transparent), and the other half was covered in UV opaque Cortgard plastic sheeting (CPFilms Inc., St. Louis, MO; 99% UVA and UVB opaque, 18% opaque to visible light); thus the treatment groups were designated full spectrum and visible. Copepods from each sampling site were randomly placed in each treatment so that there were 10 copepods from each sampling site in each treatment. Cup location within each treatment was also randomized. UVR tolerance test exposures began at 10 AM and stopped at 2 PM each day during the test period (weather permitting). UVA (λ peak 365 ± 2 nm), UVB (λ peak 311 ± 2 nm), and visible light (W/m^2) as well as water temperature ($^{\circ}\text{C}$) was measured every 15 minutes during exposures using a UV-203 (Macam Photometrics Ltd., Livingston, Scotland) radiometer and thermometer. Copepod mortality was monitored every hour during the exposures and every 4 hours post-exposure during the test period. Mortality was determined by copepod movement after gentle contact with transfer pipette. 100% water replacement in the cups was carried out every 12 hours.

UVR Tolerance Testing Data Analysis

The UVA, UVB, and visible light received by each copepod during the UVR tolerance test was determined by plotting the recorded radiation values during the test and integrating the interval corresponding to each copepod's test period (PSI-Plot v. 9.03). An example of the UVR collected during the UVR tolerance test is shown in Figure 2.3. For all statistical analyses, α was set at 0.05. Because visible light was constant between treatments, visible light dose received by each treatment was compared to test the effects of UV shielding. In this case, because visible light varied over time but was constant between treatment groups, visible light was a more appropriate metric for determination of efficacy of the test apparatus than simple test lifespan of each organism. Therefore, for each site, the



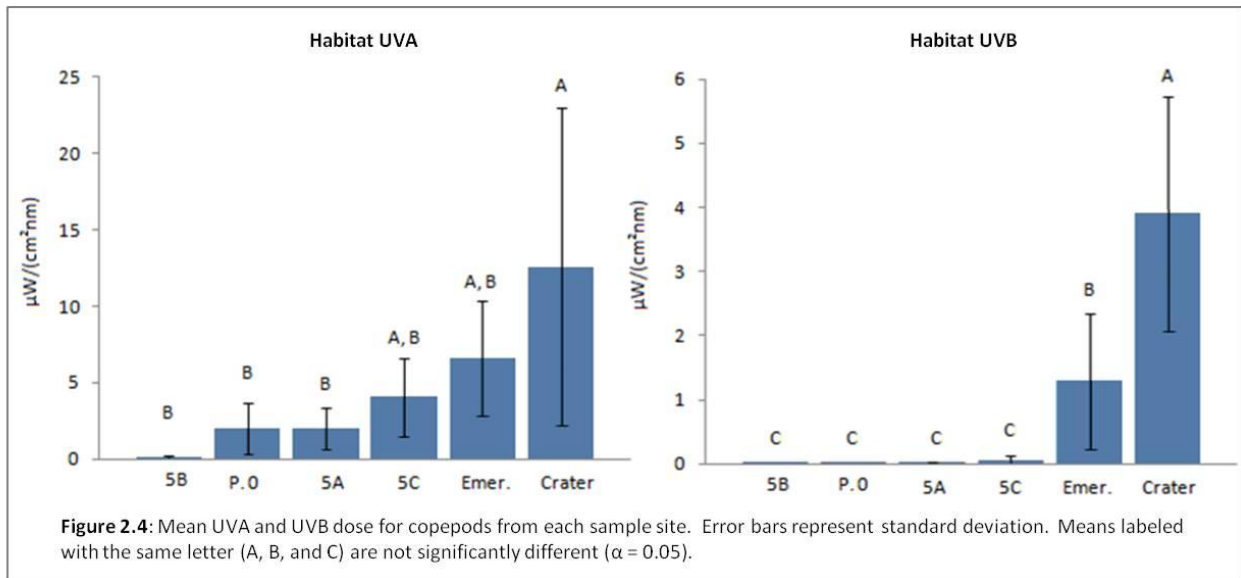
mean visible light dose for the FS and V treatments were compared by T-test. UVA dose and UVB dose for the FS treatment were compared between sites using a GLM procedure (SAS v. 9.1) with least squares means contrast as post hoc mean separation. Additionally, UVA and UVB doses were grouped by color morph (blue, mixed, and red) and compared using GLM with orthogonal contrasts.

Also, habitat variables best able to predict UVR tolerance were examined. The elevation, habitat UVA dose, habitat UVB dose, and habitat PAR dose were correlated to UVA tolerance, UVB tolerance, and Visible light tolerance of copepods in the FS treatment using the CORR procedure (SAS v. 9.1, SAS Institute Inc., Cary, NC).

Results

Habitat Radiation

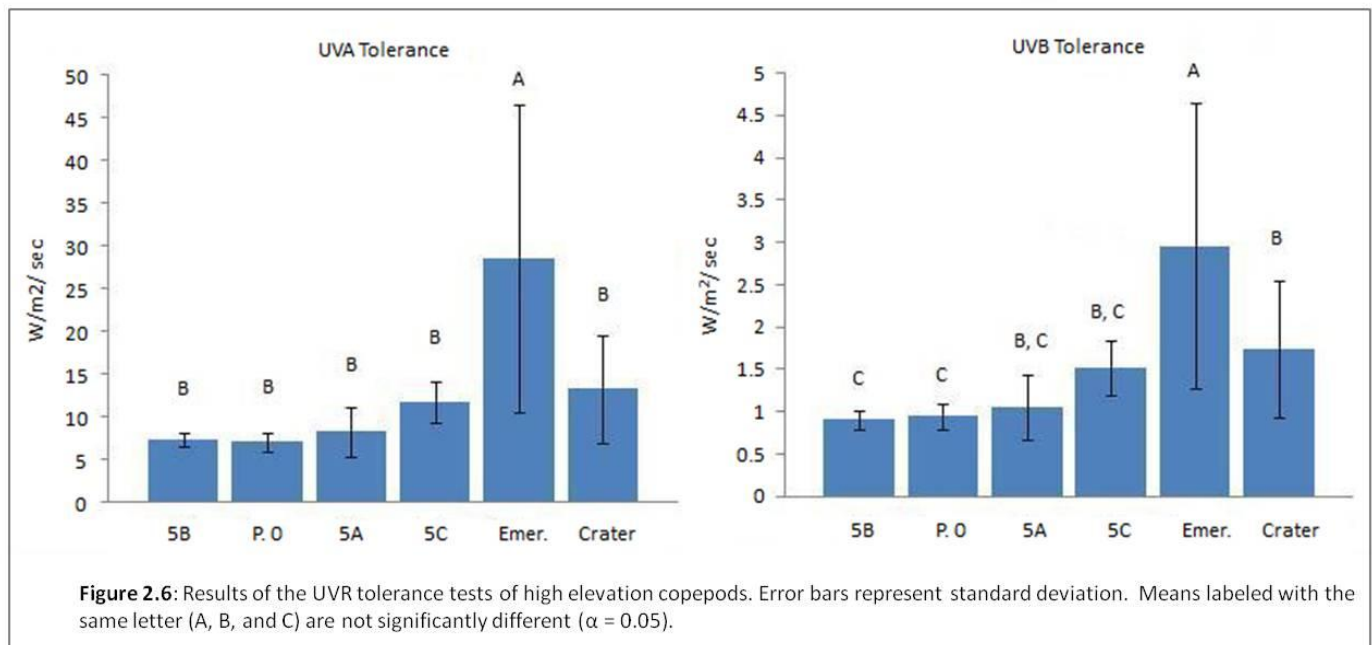
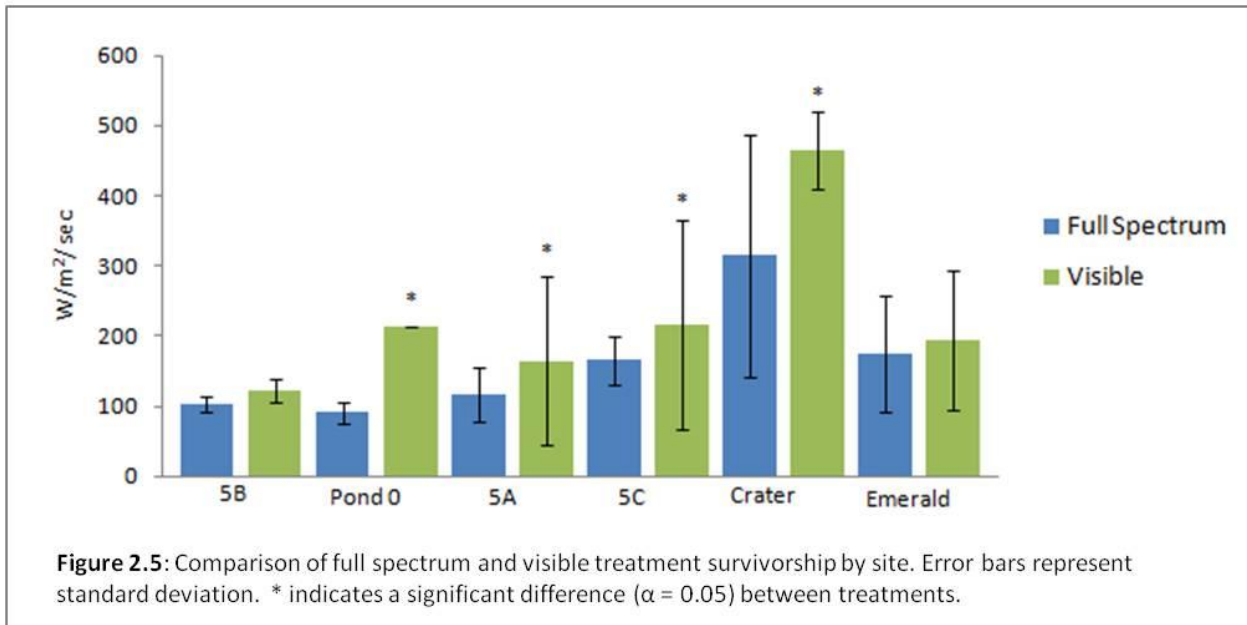
As shown in Figure 2.4, the amount of UVA and UVB differed between sites ($p = 0.001$ and $p < 0.0001$, respectively). Red copepods received significantly more UVB in their habitats than blue or mixed copepods. Red copepods from Crater and Emerald lakes also receive significantly more UVA than



the blue copepods found in 5B and Pond 0 and the mixed copepod habitat 5A, but were not significantly different from the mixed copepod habitat 5C. For the PAR (visible light) measurements, the only detected significant difference was between Pond 0 and 5B ($p = 0.0154$), the two blue copepod habitats. 5B had a mean PAR exposure of $0.0199 (\mu\text{E}/(\text{cm}^2\text{nm})/\text{sec})$, while Pond 0 had a mean PAR of $0.0804 (\mu\text{W}/\text{cm}^2\text{nm})/\text{sec}$; a four-fold difference. Despite a large disparity between means of the PAR measurements of 5B and Emerald Lake (mean for 5B = $0.0199 (\mu\text{W}/\text{cm}^2\text{nm})/\text{sec}$, mean for Emerald Lake = $0.0851 (\mu\text{W}/\text{cm}^2\text{nm})/\text{sec}$) significant differences were not detected between means due to the large standard deviation in the Emerald Lake measurements. Interestingly, the PAR measurements did not follow the same trend of significantly higher measures in the red sites and lower measures in the blue sites. In fact, 5B was the only site with a mean lower than the mean PAR of Crater Lake (mean = $0.0215 (\mu\text{W}/\text{cm}^2\text{nm})/\text{sec}$).

When the habitats were grouped by color morph and compared, the red copepod habitats were found to receive significantly more UVA ($p = 0.0007$) and UVB ($p < 0.0001$) than the blue and mixed copepod habitats, between which significant differences were not detected. Differences in PAR were not detected between any of the color morphs ($p = 0.8255$).

UVR Tolerance



The UVR tolerance test revealed differences in the UVR tolerances of the copepod populations.

Firstly, the visible treatment received more visible light than the full spectrum treatment for each

population, and significantly more for four of the six study populations (Figure 2.5). When the mean UVA tolerance of each population was compared, it was found that Crater Lake copepods are significantly more UVA tolerant than all the other populations ($p = 0.0012$, Figure 2.6). The same trend was apparent for UVB; Crater copepods tolerated a higher dose than all the other tested populations ($p < 0.0001$). Additionally, Emerald Lake copepod UVB tolerance was not different from the 5A and 5C copepods ($p = 0.1664$ and $p = 0.1041$, respectively), but they were significantly more UVB tolerant than the 5B and Pond 0 copepods ($p = 0.0224$ and $p = 0.0012$, respectively). When populations were grouped by color morph and contrasted, the trend of higher UVR tolerance in red copepod populations persisted ($p = 0.0003$ for UVA, $p = 0.0003$ for UVB). Differences were not detected between the mixed and blue copepod populations for UVA ($p = 0.3348$) or UVB ($p = 0.1932$).

Table 2.2: Correlation of Habitat Variables to UVA, UVB, and Visible Radiation Tolerance							
		habitat UVB $\mu\text{W}/(\text{cm}^2\text{nm})$	habitat UVA $\mu\text{W}/(\text{cm}^2\text{nm})$	habitat PAR $\mu\text{E}/(\text{cm}^2\text{sec})$	Elevation m	Temp. $^{\circ}\text{C}$	pH
UVA tolerance W/m^2	R^2	0.3295	0.2213	0.2736	0.0297	0.4098	0.1799
	P	0.2336	0.3465	0.2870	0.7442	0.1709	0.4020
UVB tolerance W/m^2	R^2	0.1973	0.1235	0.1542	0.0060	0.4281	0.1164
	P	0.3776	0.4944	0.4413	0.8841	0.1586	0.5081
Visible tolerance W/m^2	R^2	0.0528	0.1052	0.0968	0.2392	0.0189	0.09875
	P	0.6614	0.5305	0.5485	0.3249	0.7952	0.5442

Table 2.2 contains the results of the correlations of the habitat variables UVB, UVA, PAR, elevation, temperature, and pH to the UVR and visible light tolerance of the copepods from each site. None of the variables were significantly correlated to UVR tolerance.

Discussion

The UVR dose of copepods in deep lake habitats was significantly greater than that of copepods in shallow pond habitats, despite their migration down the UVR gradient in the water column. This

could be due in part to frequent rain and cloud cover on Niwot Ridge (Hudelson personal obs., 2010). On average, Niwot ridge receives 172.1 mm of precipitation during the months of June, July, and August (Greenland 1989). Cloud cover could be shielding the ponds from much of the solar UVR.

In freshwater, UVR is attenuated mainly by DOM and also by phytoplankton (Arrigo and Brown 1996, Cooke et al. 2006, Rose et al. 2009a). While the amount of DOM and density of phytoplankton was not measured in this study, it was observed that the shallow habitats were more productive and had higher amounts of DOM than did the deep lake habitats. Increased productivity is most likely related to higher amounts of DOM, and also to the higher temperature of the ponds (Table 2.1). The findings of Hansson indicate that UVR tolerance is highly plastic and that pigments are accumulated as UVR exposure increases (Hansson 2000). Hansson also found that the presence of fish scent lead to decreases in zooplankton pigmentation in as little as four days. Hansson and Luecke found that copepod pigmentation was much less marked in lakes inhabited by fish; the opposite of the trend in this study's sample sites (Luecke and O'Brien 1981, Hansson 2000). The absence of fish, however, does not mean that zooplankton predators are absent. Chaoborus larvae, predators of diaptomid copepods (Hairston 1979b, Byron 1982), also inhabit the shallow pond habitats, perhaps putting predation pressure on zooplankton that counter-balances the UVR pressure to accumulate pigmentation.

The role of additional naturally occurring stressors in determination of UVR tolerance for a population of copepods deserves further exploration. *Leptodiaptomus ashlandi* from subalpine Beartooth Lake exhibited negative effects when exposed to UVR at colder temperatures and low levels of ambient dissolved organic matter, but no significant effects when exposed to UVR at warmer temperatures or when dissolved organic matter was added (Cooke et al. 2006). These results demonstrate the importance of water quality parameters in determination of zooplankton community UVR sensitivities.

The effects of climate changes on zooplankton in regards to increasing UVR may not be positively related to their UVR tolerance. Rather, as UVR increases, lentic productivity may increase as well (Prowse et al. 2006). Further, as global warming increases productive periods in lentic habitats (Magnuson et al. 2000), and changes in precipitation patterns result in greater amounts of DOM (Williamson et al. 2009), UVR penetration may decrease in these systems, reducing the need for accumulation of dietary carotenoids in zooplankton. Whether the ponds included in this study have experienced increased primary productivity, or whether increased productivity has influenced UVR tolerance of copepods is unclear, but long-term observations of these interesting sites could prove valuable as global climate change progresses.

In addition to these findings, this study seeks to determine the importance of the interaction of the carotenoid pigment with the crustacyanin homolog expressed by crustaceans and the value of the carotenoprotein complex as a photoprotectant. The following chapter describes the astaxanthin content and expression of the crustacyanin homolog in copepods from the described study sites.

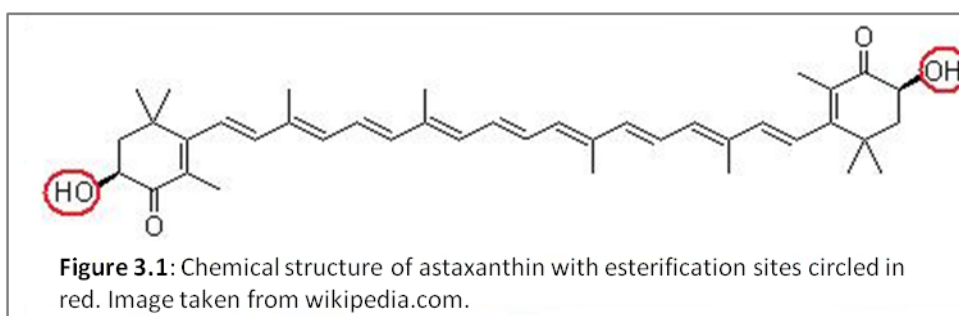
CHAPTER 3

ASTAXANTHIN AND CAROTENOPROTEIN QUANTIFICATION IN HIGH ELEVATION CALANOID COPEPODS

Introduction

High elevation zooplankton are excellent models for the study of ultraviolet radiation (UVR) tolerance because of the intense ambient UVR and UV transparency that characterizes their habitats (Sommaruga 2001, Cooke et al. 2006). Zooplankton deal with the UVR threat in three ways: photoavoidance behavior, photoenzymatic repair of damaged DNA, or accumulation of photoprotective compounds (Hairston 1979b, Zagarese and Williamson 1994, Leech et al. 2005, Moeller et al. 2005). Copepods rely more heavily on accumulation of photoprotectants than behavioral UVR avoidance (Hansson et al. 2007) in the absence of an environmental refuge from UVR.

Calanoid copepods commonly accumulate carotenoid pigments, predominantly astaxanthin (Figure 3.1) (Hairston 1976) and/or mycosporin-like amino acids (Hader et al. 2007, Hansson et al. 2007) in response to environmental UVR. Astaxanthin is a photoprotectant capable of shielding biota from solar radiation (Byron 1982, Wang et al. 2003) and is also a potent antioxidant capable of quenching reactive oxygen species generated by photooxidation (Beutner et al. 2001, Lesser 2006). The antioxidant properties are imparted by the polyene chain linking the two β -ionone rings, creating a conjugated double bond system, as shown in Figure 3.1 (Woodall et al. 1997, Young and Lowe 2001).

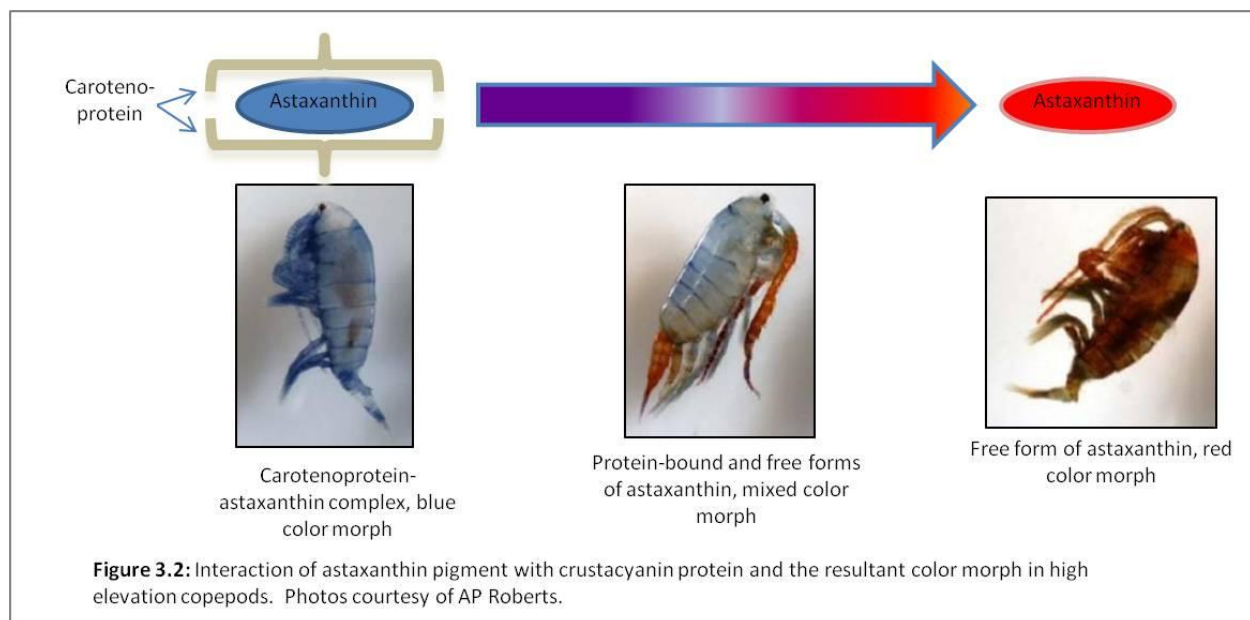


Astaxanthin exists in an unesterified or free state, and also in monoester and diester (see Figure 3.1) form in copepods (Lotocka and Styczynska-Jurewicz 2001, Kovach 2010). In crustaceans, astaxanthin is often bound by a carotenoprotein complex, α -crustacyanin, which changes the orientation of the pigment molecule and results in a shift from reflecting red light (472 nm) to reflecting blue light (632 nm) (Britton et al. 1997). α -crustacyanin isolated from the hypodermis of *Homarus gammarus*, the European clawed lobster, was composed of 8 apoprotein subunits, each consisting of two astaxanthin molecules and 2 protein subunits, designated A and C (Cianci et al. 2002). While the astaxanthin molecule remains intact and the conjugation of the molecule is not altered by protein binding (Zagalsky and Herring 1972, Cianci et al. 2002), its availability for interaction with ROS is likely lessened by protein binding. However, carotenoproteins stabilize and enhance the solubility of carotenoids in aqueous media (Britton et al. 1997), potentially enhancing their photoprotective ability.

An extensive study of the evolution of crustacyanin in crustacea by Wade et al. detected crustacyanin mRNA sequences in nine malacostracans (eight decapods and one stomatopod), but not in branchiopoda (*Daphnia* and *Ligula*) or in maxillopoda (*Lepas*, a barnacle) (Wade et al. 2009). Copepods were not included in the study. Degenerate primer PCR, bioinformatic database searches, and immunoassays were used to detect crustacyanin in 9 crustaceans in the study. Primer sequences were derived from the protein sequence of the A and C protein subunits of crustacyanin isolated from *H. gammarus* (Keen et al. 1991b, a). Copepods are also in the class maxillopoda, and have been observed to have blue pigmentation, attributed to carotenoprotein complex (Hairston 1976, Byron 1982) but carotenoproteins have not previously been studied in any freshwater copepods. Carotenoprotein was isolated from the marine copepod *Labidocera acutifrons*, and was found to have a similar molecular weight, pI, and wavelength of maximal absorption to that of crustacyanin-bound astaxanthin isolated from decapods (Zagalsky and Herring 1972). The carotenoid associated with the protein isolated from *L.*

acutifrons was AX, but the complex was found to be capable of binding three astaxanthin molecules per subunit, whereas decapod isolates are capable of binding only two (Figure 1.3, (Cianci et al. 2002).

Astaxanthin content has been positively correlated to increased UV survivorship of copepods (Zagarese and Williamson 1994, Hansson 2000, Moeller et al. 2005, Hansson et al. 2007) but the effectiveness of the carotenoprotein complex as a photoprotectant has not been directly assessed. The Colorado Rocky mountains present interesting sites for the study of calanoid copepods, their accumulation of astaxanthin, and carotenoprotein expression. A series of permanent and ephemeral ponds in the University of Colorado Mountain Research station is inhabited by copepods that are predominantly blue, and copepods that have blue prosome and metasome with red genital segments and antennae. At similar elevation (See Chapter 2, Table 2.1) to the blue copepod habitats, red copepods are observed in deep lake habitats. Figure 3.2 displays the color morphs of high elevation copepods from three of six study sites in the Colorado Rocky Mountains identified as *D. shoshone* (Kovach 2010) and a summary of the interaction of astaxanthin and carotenoproteins.



Because of the functional similarities of copepod carotenoprotein to decapod crustacyanin, it was hypothesized that the primers used by Wade et al. to amplify crustacyanin could also be used to amplify the carotenoprotein visibly expressed by copepods. Additionally, it was hypothesized that astaxanthin content and carotenoprotein expression would differ significantly between habitat types. Therefore, the concentration of astaxanthin in six populations of copepods was measured from both habitat types. Additionally, carotenoprotein expression differences were investigated. Astaxanthin concentrations were significantly higher in copepods from deep lakes, and copepod carotenoprotein mRNA sequence differs from that of marine crustaceans studied by Wade.

Methods

Study Sites and Sample Collection

Sampling sites previously described (Table 1.1) were revisited for this study. Briefly, the Moraine Ponds are a series of shallow ponds at elevation 3108 – 3116 m, inhabited by blue and mixed color morph *D. shoshone*. Emerald Lake (3081 m) and Crater Lake (3703 m) are deep, cold lakes inhabited by red *D. shoshone*.

Samples of adult male and female *D. shoshone* were collected from July 16th – August 2nd, 2010 using a 12" diameter 250 µm toss net. Samples were transported to the research station and snap frozen in liquid nitrogen and stored in a -80 °C freezer prior to analysis.

Astaxanthin Quantification

Two replicates of total carotenoid was extracted from 20 copepods from each population using a modified Folch method (Folch et al. 1957). Copepods were homogenized in 500 µL 6 M urea using a rotary micropestle. 100 µL of the homogenate was removed to quantify total protein using the Bradford assay. For the Bradford assay, three 50% serial dilutions were prepared from each homogenate and a

five-point BSA standard curve was used to determine the protein concentration. The Bradford reactions were carried out in optical 96 well plates according to the manufacturer's standard procedure for microtiter plates (Bio-Rad Inc., Hercules, CA). Absorbance at 595 nm was measured for each sample dilution and standard using a Synergy 2 (BioTek Inc., Winooski, VT) plate reader. The remaining 400 μ L of whole copepod homogenate was transferred to a glass centrifuge tube. The microcentrifuge tube and micropestle were rinsed with an additional 500 μ L of 6 M urea, which was added to the glass centrifuge tube, along with 3 mL of 2:1 chloroform: methanol. The tubes were vortexed, incubated on ice for 10 min, then centrifuged at 2500 rpm for 5 minutes to separate the mixture into two phases. The chloroform phase was removed, dried down under nitrogen, and reconstituted in 100 μ L 9:1 acetone: water in preparation for chromatographic separation. The chromatographic methods were adapted from Miao et al. (2006). Reverse phase chromatography was performed on an HP1100 (Agilent Technologies) HPLC, using a DAD monitoring at 474 nm with a reference wavelength of 800 nm and a Jupiter Proteo 90A 150 x 4.6 mm column (Phenomenex). The run time was 55 min, during which the percentage of acetone was ramped from 83% to 98% and the flow rate was held constant at 0.8 mL/min. The injection volume was 100 μ L. Detected peaks were consistent with those in previous studies of astaxanthin (Wade et al. 2005, Kovach 2010). Concentration was determined by standard curve produced from commercial astaxanthin (Sigma-Aldrich Co. St. Louis, MO). The concentration of astaxanthin for each population is expressed as mg AX/mg protein.

Data Analysis

When astaxanthin was very concentrated in the sample extract, the extracts were divided into two vials and a single extract was analyzed twice by HPLC. This was the case for both of the Crater Lake and Emerald Lake extracts and one of the extracts from Pond 0. There was not significant variation between technical replicates as determined by t-test ($\alpha = 0.05$). Both replicates were retained for

subsequent statistical analyses. The concentration of each form of astaxanthin (non-esterified, monoesterified, and diesterified), as well as the total amount of astaxanthin, was compared between sites and between color morphs by ANOVA using the GLM procedure and Scheffe's post hoc test (SAS v. 9.1). For all analyses, $\alpha = 0.05$. Means are reported as the value $\pm$ one standard deviation.

Spearman's rank correlation coefficients were calculated for the concentration of total AX, non-esterified astaxanthin, monoesterified astaxanthin, and diesterified astaxanthin relative to the results of the radiation tolerance tests detailed in Chapter 2, as well as to the habitat variables of elevation (ft), temperature ($^{\circ}\text{C}$), pH, UVA dose ($\mu\text{W}/(\text{cm}^2\text{nm})$), UVB dose ($\mu\text{W}/(\text{cm}^2\text{nm})$), and visible light dose ($\mu\text{E}/(\text{cm}^2\text{sec})$). For all correlations, $\alpha = 0.05$.

Carotenoprotein expression quantification

Total RNA was extracted from 10-30 mg of copepod wet tissue with the RNEasy Mini Kit (Qiagen Inc., Valencia, CA) and quantified by spectrophotometer (Nanodrop Inc., Wilmington, DE) prior to removal of contaminating genomic DNA and reverse transcription reaction (Quantitect Reverse Transcription Reaction, Qiagen Inc., Valencia, CA).

Three methods of primer design were employed in attempts to amplify the carotenoprotein mRNA. Primer sequences are reported in Table 3.1. All primers were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). Conventional PCR reagents were purchased from Promega Inc. (Madison, WI). Universal 18S primers were used as a positive control for all PCR reactions (Ambion, Inc., Austin, TX). PCR reaction volumes were 10 μL , consisting of 3 mM MgCl_2 , 0.2 mM dNTPs, 1 μM primer, 0.025 U Taq polymerase, double distilled water, and 1 μL of template (20 ± 2 ng). Gradient PCR was employed to optimize reactions on an Eppendorf Mastercycler pro thermal cycler (Eppendorf, Inc., Hamburg, Germany). Firstly, the degenerate primers designed by Wade et al. were tested. Annealing temperature gradients were varied by ± 5 $^{\circ}\text{C}$ about the average melting temperature of the primer set.

Table 3.1: Sequences and predicted product sizes of primers used to amplify copepod carotenoprotein mRNA				
Primer	Sequence	Expected product size (bp)	Designed by	
CRCN-A2.1	GTN GCN AAY CAR GAY AYY TTY TTY GA	not reported	Wade 2009	
CRCN-A2.2	AAY TCN CAN CCR TTY TTR TTR AA			
CRCN-C1.1	TAY CAR YTN ATH GAR AAR TGY GT	not reported		
CRCN-C1.2	CCR AAR TTR TAR TCD ATR CA			
CRCN C1A2.1	TTY GTN CAN GCN GGN AAR TGY GC	not reported		
CRCN C1A2.2	GTY TCN AND AYN WCN WRN GGN GC			
CRN1F	TAY GCN GGN CGN TGG TAY	unpaired	Clustal W alignment of crustacyanin A mRNA sequences (Wade 2009)	
CRN2F	TAY GGC TTC AAB GTS ACY	unpaired		
CRN2R	RGT WAC VTT GAA GCC RTA	unpaired		
CRN3F	CCM GCN GCN CAY ATG CTY	unpaired		
CRN3R	RAG CAT RTG AGC NGC KGG	unpaired		
CRN4R	AGG AGT AGA YGC AGG AGT	unpaired		
CRN4dR	NGG NGT NGA YGC NGG NGT	unpaired		
CRNB1F	CGT TAT GCT GGT CGT TGG TAT	233		Clustal W alignment of crustacyanin A amino acid sequences (Wade 2009)
CRNB1R	GGA GCA GCA AAA ACA GAA GG			
CRNB1Fd	CGN TAY GCN GGN MGN	233		
CRNB1Rd	CCN WSN GTN TTY GCN GCN CC			
CRNB2F	TTT CCT GCT GCT CAT ATG CTT	173		
CRNB2R	GCA GGA CCA GAA GTT TGA GG			
CRNB3F	CGT TAT GCT GGT CGT TGG TA	229		
CRNB3R	CAG CAA AAA CAG AAG GAG CA			
CRNB4F	CGT CGT TAT GCT GGT CGT T	236		
CRNB4R	GGA GCA GCA AAA ACA GAA GG			
CRNA1F	CTT TGG GCT GAA CAA ACT CC	215		
CRNA1R	GGT TCA CCA AAA GGA TTA GGA			
CRNA1Fd	CTN TGG GCN GAR CAR ACN CC	215		
CRNA1Rd	CNA AYC CNT TYG GNG RCC NC			
CRNA2F	CTT TGG GCT GAA CAA ACT CC	216		
CRNA2R	AGG TTC ACC AAA AGG ATT AGG A			
CRNA3F	CTT TGG GCT GAA CAA ACT CC	217		
CRNA3R	GAG GTT CAC CAA AAG GAT TAG G			
CRNA4F	TTT GGG CTG AAC AAA CTC CT	216		
CRNA4R	GAG GTT CAC CAA AAG GAT TAG G			
CRNA5F	CGC CCC CGA ATA CGC GAC TAG	264	Clustal alignment of CRNA1d CRNA1dF sequence product alignment	
CRNA5R	TCT TTG GGC GGA GCA GAC CC			
CRNA6F	TTC GCC CCC GAA TAC GCG ACT	266		
CRNA6R	TCT TTG GGC GGA GCA GAC CCC			
CRNA7F	ATG CGC CTG GGG TTT TCG C	260		
CRNA7R	CCG GGG CAG GTG TAC TTC GAA			
CRNA8F	TGC GCC TGG GGT TTT CGC CC	260		
CRNA8R	CCC GGG GCA GGT GTA CTT CGA A			
CRNA1dcR2	ACT TCG AAT TGG CGG ATT T	211		Reverse primers to complement CRNA1dF
CRNA1dcR3	GTA CTT CGA ATT GGC GGA TTT	214		
CRNA1R2	ATT GGC GGA TTT GGA G	211		
CRNA1R3	GGC GGA TTT GGA GGT A	214		

Primer naming conventions: A: crustacyanin A chain; C crustacyanin C chain; F: forward primer; R: reverse primer; d: degenerate.

Additionally, MgCl_2 concentrations of 1 mM, 1.5 mM, and 2 mM were assayed. Also, primer concentration was doubled (2 μM), and template concentration was increased (40 ± 2 ng) while MgCl_2 concentration was held constant at 3 mM in attempts to obtain a PCR product. PCR products were separated by agarose electrophoresis, stained with ethidium bromide, and imaged under UV.

As suggested by Wade (Wade-Hudelson personal communication, 2010) annealing temperature was decreased to 45 °C and increased by 1°C in a gradient PCR reaction, with 10 μL , consisting of 3 mM MgCl_2 , 0.2 mM dNTPs, 1 μM primer, 0.025 U Taq polymerase, double distilled water, and 1 μL of template (20 ± 2 ng).

Clustal W 2.0.12 (European Molecular Biology Laboratory, European Bioinformatics Institute, Cambridge, UK) multiple alignment tool was used to align translated crustacyanin A sequences first sequenced by Wade et al. during their study of crustacyanin (GenBank Accession numbers FJ498899, FJ498900, FJ 49894, FJ 49895, FJ 49896, and FJ 49897). Regions of homology among the mRNA sequences generated by Wade et al. were chosen and degenerate and non-degenerate primers were designed. The primers were designed to anneal to homologous regions, not necessarily to be paired. Therefore, forward primers (designated F) were paired with reverse primers (R) of the closest annealing temperature and thus product sizes for the reactions were variable. See Table 3.1 for the sequences of these primers. The primer optimization procedures described above were repeated for these primers and PCR products were visualized by electrophoresis.

Next, primers were designed based on translated mRNAs generated by Wade et al. Amino acid sequences were obtained from GenBank and two common amino acid sequences were detected: YAGWRY and EVIETDY. mRNA sequences of FJ49889, FJ498894, FJ49885 and FJ498898 were searched

for the nucleic acid sequence encoding the common sequences. The nucleic acid sequences were then used to design primers CRNB1F – CRNA4R. The optimization process described above was repeated for these paired primer sets.

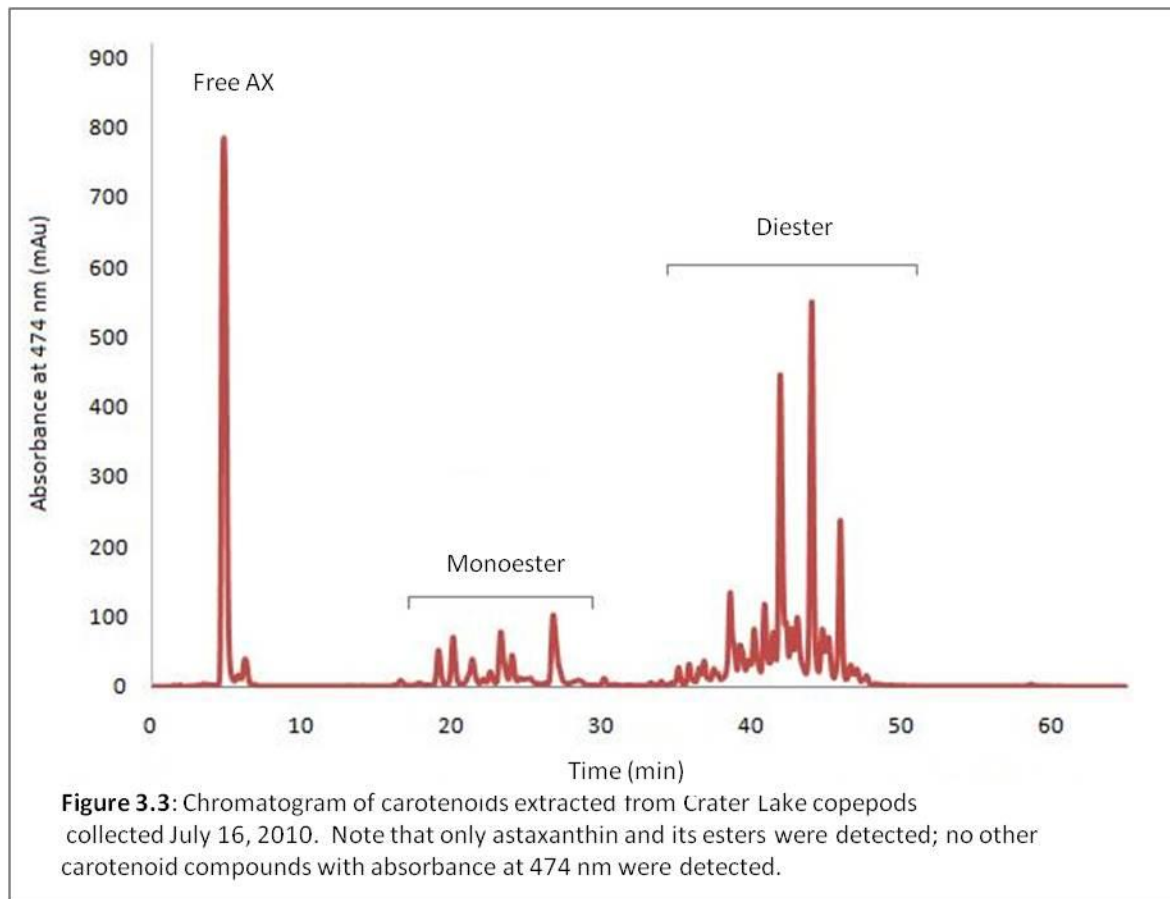
The primer set CRNA1Fd and CRNA1Rd at 64 °C annealing temperature, 3 mM MgCl₂, and 1 μM primer, generated bands of sizes 350 and 200 bp. Resulting bands were excised, cleaned up (Wizard SV Gel and PCR Cleanup System, Promega Inc., Madison, WI), and sequenced using an capillary electrophoresis (3130 Genetic Analyzer, Applied Biosystems Inc., Carlsbad, CA). CRNA1Fd PCR product sequences were aligned using Clustal W and the resulting consensus sequence was used to design primer pairs and reverse primers to complement CRNA1Fd. Primers were designed using Primer-BLAST (National Center for Biotechnology Information, U.S. National Library of Medicine, Bethesda, MD). PCR reactions carried out using CRNA6F and CRNA6R at annealing temperature 57.6 °C generated a faint band at 270 bp, which were submitted for sequencing. CRNA6R sequences were processed with Geneious Basic 5.3.6 (BioMatters Ltd., Auckland, New Zealand). Next, optimization of the CRNA6 primer set was attempted for use in quantitative PCR. The QuantiTect SYBR Green PCR kit (Qiagen Inc., Valencia, CA) was used in a Rotor-Gene thermal cycler (Corbett Research, Mortlake, Australia). Each run included an 18S positive control and a no template negative control.

Results

Astaxanthin Quantification

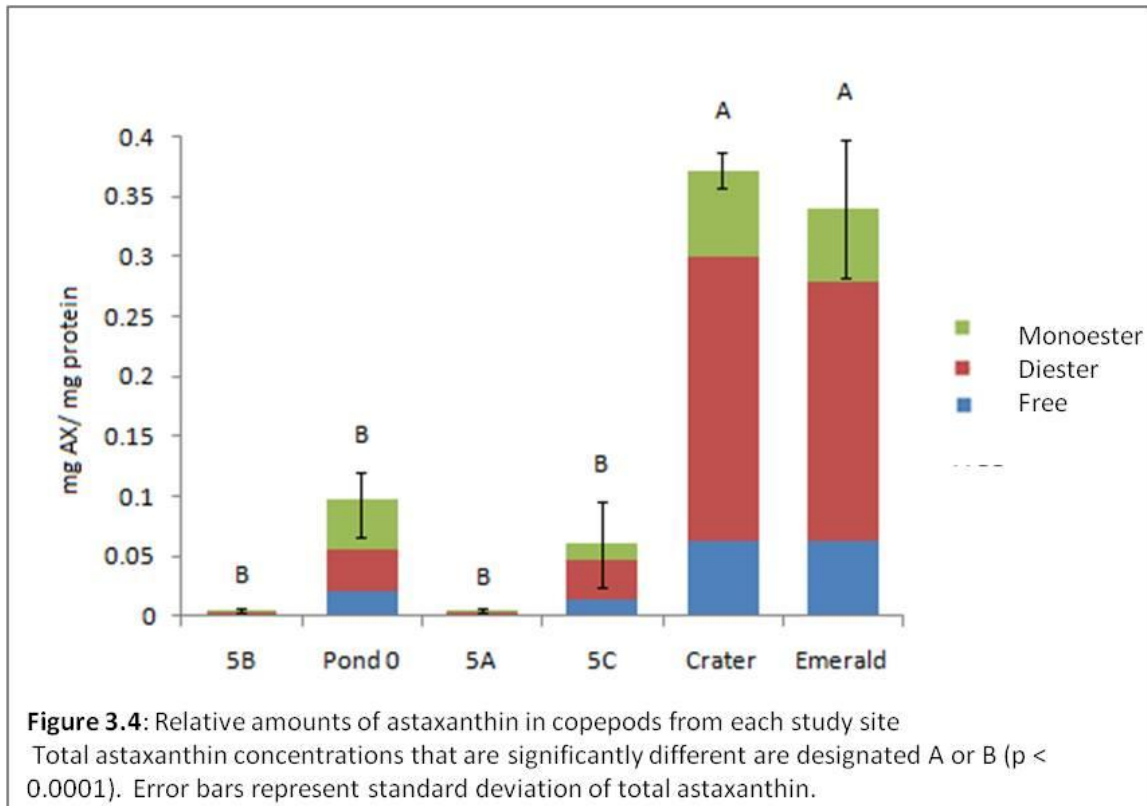
Figure 3.3 is a chromatogram from which concentrations of astaxanthin and its esters were calculated. As shown in Figure 3.4, the concentration of total astaxanthin varied greatly between sites ($p < 0.0001$). The mean concentration of total astaxanthin found in Crater Lake copepods was 0.371 ± 0.013 std. dev. mg AX/ mg protein, meaning that these copepods were composed of about 37% AX per

unit protein. The Emerald copepods were found to have a total astaxanthin concentration of 0.341 ± 0.057 mg AX/ mg protein.



The concentration of each form (diester, monoester, or free) also varied between sites ($p < 0.0001$ for each form). Crater and Emerald lake copepods contained significantly more free and diesterified astaxanthin than did copepods from the blue or mixed sites. However, differences were not detected between the amount of monoesterified astaxanthin from Pond 0 copepods and Crater or Emerald copepods (difference between means 0.0286 and 0.0187 mg AX/mg protein, respectively).

When astaxanthin concentrations were compared by color, the red copepods were found to contain significantly higher concentrations of each form of astaxanthin (diester, monoester, and free, $p < 0.0001$ for each) than blue and mixed copepods. No significant differences in concentration of astaxanthin were detected between the blue and mixed copepods (Fig. 3.4).



Carotenoprotein mRNA expression quantification

No PCR products were obtained during the optimization process for the Wade primers (CRCN-A2.1 – CRCN1A2.2). No PCR products were obtained from the primers designed from alignment of mRNAs generated in the Wade study (CRN1F – CRN4dR). A PCR product was obtained from the primer set CRNA1Fd, which was sequenced and the resulting sequence was used to design four additional sets of primers.

The resulting sequences were of poor quality; only the CRNA1Fd primer with the 200 bp PCR product yielded results from the sequencing reaction. Additional reverse primers were designed to complement CRNA1Fd, however these did not improve the PCR reaction.

In order to determine the identity of the sequenced CRNA1d PCR products, BLASTn and BLASTx (National Center for Biotechnology Information, U.S. National Library of Medicine, Bethesda, MD) searches of nucleotide and protein data bases were performed. No significant homology was detected (bitscores < 40) but one of the sequences aligned with a crustacyanin-like lipocalin protein (bitscore 24.6) isolated from subepidermal adipose tissue of *Macrobrachium rosenbergii*, a giant freshwater prawn (GenBank accession number ABC88388.1). Additionally, crustacyanin mRNA sequences were compared to the PCR products using BLASTn and BLASTx. Bitscores of these comparisons were < 40.

CRNA6R sequence quality was poor; chromatograms were difficult to read. Despite this, several PCR product sequences aligned with crustacyanin isolated from *H. gammarus*, but bitscores remained low: 22.3 at the highest (Table 3.2).

Table 3.2: BLASTx analysis of CRNA6 primer pair PCR product sequenced with CRNA6R primer				
Database	Accession number	Source information	Bitscore	Citation
PDB protein database	1S2P A	Crustacyanin A	20.4	(Habash et al. 2004)
PDB protein database	1H91 A	Crustacyanin A	20.4	(Habash et al. 2003a)
PDB protein database	10BQ_A	Crustacyanin C1	20.4	(Habash et al. 2003b)
Refseq protein	P80029.1	Crustacyanin-C1	20.8	(Cianci et al. 2002)
Swissprot	P58989.1	Crustacyanin-A1	20.8	(Cianci et al. 2002)
Swissprot	P80029.1	Crustacyanin-C1	20.8	(Cianci et al. 2002)

The quantitative PCR reactions with CRNA6 primer did not yield a detectable amplification signal above the level of the no template control. Positive control reactions (18S primer) appeared normal; melt curves indicated a single PCR product and no template 18S reactions did not generate a similar

amplification signal, indicating that the quality of the template cDNA was acceptable for quantitative PCR.

Correlation of ultraviolet radiation experiments and astaxanthin content

During the UVR tolerance test described in Chapter 2, UVA (λ peak 365 ± 2 nm), UVB (λ peak 311 ± 2 nm) and visible light varied at approximately the same rate because the source of radiation was solar, but since UVB are the most biologically damaging wavelengths (Madronich et al. 1998) it is likely that UVB is also the most influential in determining lifespan during UVR tolerance testing.

Table 3.3: Spearman's Rank Correlation of Copepod Astaxanthin Concentrations to UVR Tolerance for All Study Sites				
		UVA tolerance W/m^2	UVB tolerance W/m^2	Visible exposure W/m^2
Total AX mg/mg protein	r_s	0.5318	0.6027	0.5081
	p	0.0340	0.0135	0.0445
Free AX mg/mg protein	r_s	0.6470	0.7002	0.6233
	p	0.0068	0.0025	0.0099
Diester mg/mg protein	r_s	0.4461	0.5229	0.4254
	p	0.0833	0.0377	0.1004
Monoester mg/mg protein	r_s	0.6248	0.6987	0.5864
	p	0.0097	0.0026	0.0170

As shown in Table 3.3, the total concentration of astaxanthin is positively correlated to UVA ($r_s = 0.5318$, $p = 0.0340$) and UVB ($r_s = 0.6027$, $p = 0.0135$) tolerance. Comparison of Spearman's rank correlation coefficients indicates that free astaxanthin concentration is the most predictive variable for determining UVB ($r_s = 0.7002$) and UVA ($r_s = 0.6470$) tolerance. In addition to total astaxanthin content, concentration of each etherification state of astaxanthin (free, monoester, and diester) was correlated to UVR tolerance in order to determine if one form of astaxanthin is accumulated preferentially by UVR tolerant copepods (Table 3.3). Free astaxanthin was found to have the highest r_s values and the most

significant p-values when compared to UVB tolerance ($r_s = 0.7441$, $p = 0.0009$) and UVA tolerance ($r_s = 0.5500$, $p = 0.0273$). Neither PAR (visible light) nor elevation were correlated to the concentration of any form of astaxanthin.

Table 3.4 displays Spearman's rank correlation values and p values for correlations of the concentration of astaxanthin to the habitat variables UVB dose, UVA dose, PAR dose, and elevation. This analysis was carried out in order to determine which habitat variables were most predictive of astaxanthin concentrations. UVB dose ($\mu\text{W}/(\text{cm}^2\text{nm})$) was found to have the strongest correlation to each measure of astaxanthin concentration. Significant correlation was not detected between elevation and astaxanthin concentration.

Table 3.4: Spearman's Rank Correlation of Habitat Radiation Measures and Elevation with Astaxanthin Concentration					
		Total AX mg/mg protein	Free AX mg/mg protein	Diester mg/mg protein	Monoester mg/mg protein
habitat UVB $\mu\text{W}/(\text{cm}^2\text{nm})$	r_s	0.8235	0.7441	0.8324	0.6647
	P	<.0001	0.0009	<.0001	0.0050
habitat UVA $\mu\text{W}/(\text{cm}^2\text{nm})$	r_s	0.6471	0.5500	0.6824	0.4353
	P	0.0067	0.0273	0.0036	0.0920
habitat PAR $\mu\text{E}/(\text{cm}^2\text{sec})$	r_s	-0.0618	-0.1500	-0.1353	-0.1735
	P	0.8202	0.5792	0.6174	0.5204
Elevation meters	r_s	0.0286	-0.0857	0.0286	0.0286
	P	0.9572	0.8717	0.9572	0.9572

Discussion

Astaxanthin Quantification

The concentrations of astaxanthin were high (0.371 ± 0.013 and 0.341 ± 0.057 std. dev. mg AX/mg protein) in red *D. shoshone* from lake habitats, consistent with the findings of Kovach (2010). The high concentration of astaxanthin in these populations of *D. shoshone* points to the importance of this

compound for the survival of high elevation copepods. Moeller and others found that *Leptodiaptomus minutus* exposed to UVR and fed a diet limited in mycosporin-like amino acids accumulated carotenoids in concentrations up to 1% dry weight (Moeller et al. 2005). Although mycosporin-like amino acids were not quantified in this study, the concentrations of astaxanthin accumulated by copepods studied here indicate that it is likely that carotenoids are the primary photoprotectant employed by copepods. Hylander et al. found that predation pressure and lack of dietary mycosporin-like amino acids promote carotenoid accumulation by zooplankton (Hylander et al. 2009). Red copepod habitats included in this study are known to contain fish, and so carotenoid accumulation could be disadvantageous. However, UVB exposure is also greater in these habitats (Figure 2.4, Chapter 2), therefore driving copepods to accumulate photoprotectants.

The correlations between UVR tolerance test results and carotenoid content revealed that free astaxanthin concentration was the most powerful predictor of UVA and UVB tolerance, even though significant differences were detected between the concentration of total AX, monester, and diester. Interestingly, the free form of carotenoid is the only form that can be bound by the carotenoprotein crustacyanin; esterification prevents the protein from binding tightly to the astaxanthin molecule according to the models developed by Cianci et al. (2002). The free form is also preferable in terms of use by animals and more readily incorporated into membranes than the esterified forms (Pintea et al. 2005). Esterification also increases hydrophobicity of the astaxanthin molecule and enhance its ability to quench ROS (Lotocka and Styczynska-Jurewicz 2001). Byron observed that red pigments are distributed throughout the body interior and that the exoskeleton can have a blue color, most likely due to the carotenoprotein complex (1981). The carotenoprotein complex is typically found in the hypodermis of crustaceans (Wade et al. 2009), including copepods (Zagalsky and Herring 1972). Analysis of the localization of each form of astaxanthin in *D. shoshone* could provide greater insight into its role as an antioxidant and photoprotectant. If the free form is predominantly complexed with

carotenoprotein in the hypodermis, while the esterified forms are stored in fat droplets or concentrated in the ovaries, it could indicate that the esterified forms function as antioxidants while the carotenoprotein complexed with free astaxanthin acts as a photoscreen.

As summarized in Table 2.1, Chapter 2, the deep lake habitats were colder than the shallow pond habitats. Byron's hypothesis that copepods accumulate carotenoid in order to increase light absorbance and thereby raise their body temperature in order to increase metabolic rate seems to be supported by this data (Byron 1981, 1982). However, carotenoid content was not significantly correlated to water temperature in this study ($p = 0.2080$ for total astaxanthin concentration).

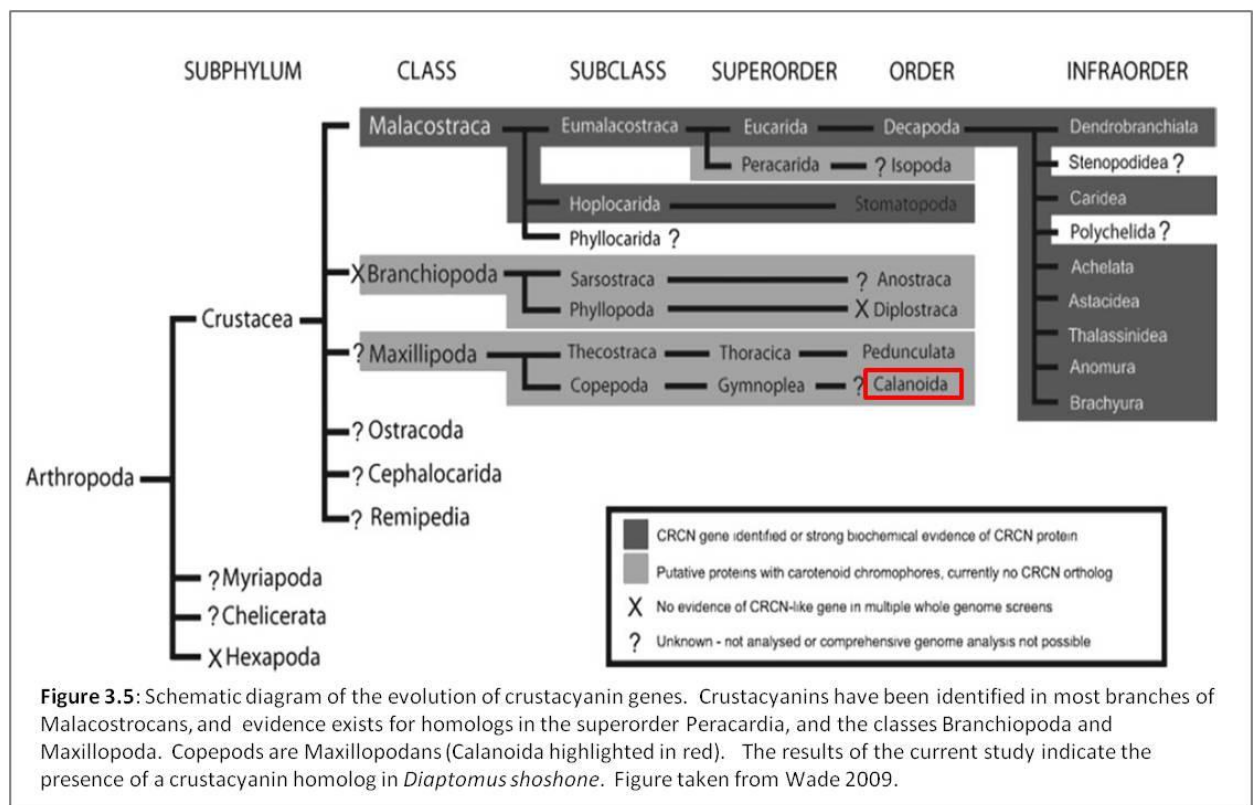
Correlations between the UVB, UVA and PAR dose copepods receive in their habitats and the concentration of astaxanthin revealed that habitat UVB dose is the strongest predictor of astaxanthin concentration in the study sites. UVB is the most biologically active form of UVR; it has the greatest capability for degrading biomolecules and negatively impacting physiology of organisms (Madronich et al. 1998). The high correlation of UVB to carotenoid concentration ($r_s = 0.8235$ for total AX) suggests that astaxanthin is utilized as a photoprotectant by high elevation copepods.

While significant increases in copepod pigmentation along elevation gradients have been observed in other studies (Byron 1982), this study did not detect such a trend. It is likely that if a larger range of elevations was included this trend might have been detected.

Carotenoprotein Expression

Carotenoprotein mRNA expressed by copepods, despite having a similar function, body localization, and cofactor, does not have a great degree of sequence homology to crustacyanin. The lack of homology prevented effective PCR amplification of copepod carotenoprotein. Crustacyanin is a crustacean specific-innovation (Wade et al. 2009) prevalent in the class Malacostraca, which arose

during the middle Cambrian (Schminke 2007). Copepods belong to the class Maxillopoda, which arose during the upper Cambrian (Schminke 2007). Figure 3.5 summarizes Wade's findings for crustacyanin genes among the Malacostraca and other crustaceans. Interestingly, querying nucleotide databases with PCR generated copepod carotenoprotein sequences did not generate any alignments with crustacyanin sequences, but queries of protein databases resulted in alignments with crustacyanin A and C subunits, although they were of low homology. Homology of the CRNA6 PCR product with decapod crustacyanin proteins, but not with crustacyanin mRNAs could indicate differential post transcriptional modification patterns between the two groups. Alternatively, the protein-coding regions of the genes could be significantly different sequences encoding similar protein sequences due to the degeneracy of nucleic acids relative to proteins. Sequencing of a calanoid copepod genome or transcriptome could reveal crustacyanin homologs, but it is likely that analysis of a calanoid copepod carotenoprotein sequence would be a more straightforward approach to determination of the mRNA



sequence. Further research into this topic could include characterization of carotenoprotein isolates as well as investigations of the mRNA sequence of the crustacyanin homolog expressed by diaptomids.

Overall Conclusions

In addition to its photoprotective properties (Hairston 1976, Hansson et al. 2007), astaxanthin plays a role in the diaptomid reproductive cycle (Hairston 1979a), has been correlated to increased metabolic rate (Byron 1981), and is a potent antioxidant (Young and Lowe 2001). This study indicates that astaxanthin plays a significant role in UVR tolerance, but whether astaxanthin functions as a sunscreen, antioxidant, or both was not examined. Further, the results of this study indicate that UVB dose is the most powerful predictor of carotenoid content for high elevation copepods, and that nonesterified astaxanthin is the best predictor of UVR tolerance. Additionally, this study has made significant progress into discerning the sequence of a copepod crustacyanin homolog.

CHAPTER 4

CONCLUSIONS

This study found that in six sites of the Colorado Rocky Mountains, *Diaptomus shoshone* in deep lakes receive higher amounts of UVB on average than do *D. shoshone* in shallow pond habitats and that UVR tolerance was greater in red copepods from deep lake habitats. The high correlation of UVB exposure to carotenoid concentration ($r_s = 0.8235$ for total AX) suggests that astaxanthin is utilized as a photoprotectant by high elevation populations of *D. shoshone*, consistent with previous reports (Hairston 1976, Byron 1982, Kovach 2010) in pigmented diaptomid populations.

UVB promotes oxidation reactions; astaxanthin is a potent antioxidant. Based on these findings it is likely that the role of the astaxanthin molecule in photoprotection is tied to its antioxidant properties. The correlations between UVR tolerance test results and carotenoid content reveal that free astaxanthin concentration was the most powerful predictor of UVA and UVB tolerance. While the esterified forms of astaxanthin are more effective at quenching ROS (Madronich et al. 1998), the free form is more readily assimilated into animal membranes (Lotocka and Styczynska-Jurewicz 2001), where it may play an important role of stabilizing and preventing radical degradation of the membrane.

Carotenoprotein mRNA expressed by copepods, despite having a similar function and localization of protein product, does not have a great degree of sequence homology to crustacyanin genes. The protein-coding regions of the genes could be quite different and still encode similar protein sequences due to the degeneracy of nucleic acids relative to proteins. Degenerate primers were designed with this scenario in mind, yet successful amplification did not occur. It is possible that there are differences in post transcriptional modifications to mRNAs between the two groups. Sequencing of a calanoid copepod genome or transcriptome could reveal crustacyanin homologs, but targeted analysis

of the crustacyanin protein homologs expressed by Diaptomids is likely to be the most direct method of elucidating the protein and mRNA sequence in this taxa.

UVR is attenuated by DOM and phytoplankton in freshwater systems. Changes in rainfall patterns, resulting in greater amounts of DOM in the water, increased length of productive period, and increased water temperature have all been linked to global climate change effects in freshwater ecosystems, and can lead to increased attenuation of environmental UVR (Williamson et al. 2009). Additionally, these changes generally lead to increased primary productivity, which can also increase attenuation of UVR. Increased UVR attenuation results in lessened UVR dose for zooplankton and thereby reduced UVR tolerance. It is possible that the difference in color morph observed in the Moraine Ponds in the late 1970s (red) and 2007 (blue) was caused by the changing UVR conditions for zooplankton linked to global climate change. As their environment attenuated increasing amounts of UVR, the UVR stress placed on copepods decreased and accumulation of carotenoids decreased as well.

Continual monitoring of Diaptomid carotenoid content in settings like the Moraine ponds could reveal important trends for similar ecosystems impacted by global climate change. Because changes in zooplankton color can be easily and quickly detected, unlike changes in phytoplankton community or other environmental variables affecting UVR, copepod color morph may be a useful indicator of changes in UVR conditions. Therefore, diaptomid copepod color morph could be a simple and valuable metric used to assess changes in aquatic ecosystem UVR attenuation.

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