EFFECTS OF SEASON, SIZE AND PARASITISM BY THE
ACANTHOCEPHALAN, *PROFILICOLLIS ALTMANI*, ON THE
CAROTENOID CONCENTRATION AND COMPOSITION
OF THE PACIFIC MOLE CRAB, *EMERITA ANALOGA*

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of the Pacific Mole crab, *Emerita analoga*.

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ABSTRACT

The Pacific Mole crab *Emerita analoga* is a filter-feeding crustacean inhabiting sandy beaches along the western coasts of North and South America. The tissues of *E. analoga* contain carotenoids, dietary pigments that are involved in crustacean cryptic coloration and egg development. Despite extensive study of its life history, little is known about how *E. analoga* is affected by parasitism, even though previous groups noted the presence of larval helminths in its body cavity. We examined parasite prevalence and intensity of the acanthocephalan *Profilicollis altmani* infecting *E. analoga* from Pismo Beach, California. We investigated the effects of parasite infection and body size on egg production as well as the effects of parasite infection, body size, season and reproductive condition on tissue carotenoids. We also analyzed *P. altmani* cystacanths for carotenoids to determine if parasite acquisition of host carotenoids was possible. Infection by *P. altmani* did not affect the carotenoid concentration or content of any *E. analoga* tissues. Egg mass production was also unaffected by infection. Crab body size was the only significant predictor of carapace and egg carotenoid concentration, while reproductive season significantly affected ovarian carotenoid concentration. *P. altmani* cystacanths contained carotenoids similar to those found in *E. analoga* tissues, but we could not determine if acquisition was purposeful or from a specific tissue. *E. analoga* esophagus tissue was analyzed for the first time during this study and contained predominantly astaxanthin.

INTRODUCTION

***Emerita analoga* Life History**

The Pacific Mole crab, *Emerita analoga* (Anomura: Hippidae), is a filter-feeding crustacean that inhabits the swash zone of wave swept, sandy beaches in temperate climates along the western coasts of North and South America. Its geographic range spans from Kodiak Island, Alaska to Magdalena Bay in Baja California and from Salaverry, Peru to False Bay in Argentina. There are also a few populations in the northern part of the Gulf of California (Efford 1976; See Figure 1).

Close relatives of *E. analoga* include *E. benedicti*, which inhabits the coastline from South Carolina to Texas and the Gulf of Mexico (Schmitt, 1935), *E. brasiliensis*, which inhabits the Eastern Atlantic shores of South America (Schmitt, 1935), *E. portoricensis*, which is commonly found in the Caribbean (Schmitt, 1935) and *E. talpoida*, which dominates the eastern U.S. coast (Say 1817). *E. portoricensis* has also been found along the northern coast of South America and in parts of Trinidad. (Efford 1976). Common predators of *E. analoga* include gulls, curlews, sandpipers, plovers, ducks, surf scoters and other local seabirds that scavenge the shoreline during daylight hours (Reeder 1951, Schmidt and Kuntz 1967, Ryan et al. 1987, Latham and Poulin 2003). Surf zone fish such as yellowfin and spotfin croakers, the barred surfperch and the California corbina also feed on *E. analoga* when the fish visit nearshore waters

(Dept. Fish and Game 2001). Some predation by sea otters has also been documented (Hennessey and Morejohn 1977, Mayer 2003). *E. analoga* is the dominant macrofauna species of exposed beaches on the California coast, and its geographic variation, growth and reproductive biology have been studied extensively at several California and Chilean beaches (Eickstaedt 1969, Dugan et al. 1994, Jaramillo 2000).

***E. analoga* Reproductive Biology**

Along the Pacific coast, *E. analoga* reproduction begins in spring and continues through the summer, however a second, brief reproductive period has been observed in late fall. As crabs crawl onshore at night to mate, several males may simultaneously mount the same female at a time and attach sperm packets to her ventral side. Once eggs have left the oviducts and are fertilized, they are immediately deposited underneath the telson and held in place by pleopods (Wenner et al. 1987). The time between egg deposition under the telson and hatching, known as the “egg incubation period” has been reported as 29-32 days in the laboratory by Boolotian et al. (1959) and Dudley and Cox (1967). Females produce multiple broods during their lifetimes and may mate more than once during each reproductive season (Wenner et al. 1987).

After eggs from each brood fully develop, the larvae hatch into the ocean water and begin the first zoeal stage, characterized by crabs having a short spine, large, stalked eyes and a clear carapace. The larvae pass through four other

zoeal stages, each marked by morphological changes in the telson, antennae, mandibles, and maxillae along with increasing size. During all zoeal stages, larvae remain pelagic and disperse widely as they develop. (Johnson and Lewis 1942, Wenner et al. 1987). A study of *E. analoga* populations from Santa Barbara, California by MacGinitie (1938) suggested that this life cycle produces two major reservoirs: an immature, pelagic population supplying nearby beaches with new megalopae each fall, winter, and spring, and an intertidal population consisting of sexually reproductive adults supplying pelagic larvae during summer and fall. Developing *E. analoga* larvae belonging to the pelagic reservoir will return to the swash zone as sexually immature megalopae, or “recruits.” Once here, they repopulate the beach by joining existing aggregations under the sand and becoming new members of the intertidal reservoir (MacGinitie 1938, Eickstaedt 1969). During mid-winter, the intertidal population may be scarce, and the same is true of the pelagic population during the summer months (Barnes and Wenner 1968).

The reservoirs of on-shore, sexually mature *E. analoga* adults are re-established each reproductive period with new individuals. The constituents of these aggregations vary in age and sex. For example, analysis of *E. analoga* populations inhabiting the north and south ends of a large beach in Chile during February 2001 revealed that males outnumbered females by 1.6:1 and 2.4:1, respectively (Lastra et al. 2004). Similar analysis of *E. analoga* populations inhabiting Santa Barbara (Barnes and Wenner 1968) and Monterey beaches in

California (MacGintie 1938) has shown that large females typically predominate the swash zone in summer and fall, comprising anywhere from 75-100% of the population. Smaller crabs, most of which were assumed to be male, predominate during the winter months (Barnes and Wenner 1968, MacGintie 1938), similar to the demographic pattern of the Chilean population. Sex ratios have not been previously documented for *E. analoga* populations from central California beaches.

Two separate studies have suggested that the preponderance of large females in the summer, coupled with large numbers of small males early in the year, is due to protandric hermaphroditism (Barnes and Wenner 1968, Wenner 1972).

However, a later study by one of the same groups concluded that this was an unlikely occurrence. Laboratory rearing of *E. analoga* males and females revealed that the molt increment of males is much smaller than that of females, and once the maximum size of a male was reached, subsequent molts do not result in increased size. Furthermore, during the entire evaluation, none of the captive males transformed into females. This was evident from final analysis of reproductive organs and sexual characteristics, including the presence or absence of pleopods, an oviduct, and ovarian tissue. (Wenner and Haley 1981).

Another important aspect of the reproductive biology of *E. analoga* that deserves attention is the conspicuous orange pigmentation of egg and gonad tissues.

Eickstadt (1969) studied this extensively as part of a larger project during the late

1960s. He found that during periods of reproduction, gonad indices for ovigerous females increased substantially compared to the non-reproductive season, when ovarian tissue mass decreased (Eickstaedt 1969). Other studies have documented distinct color changes in eggs as a function of their developmental stage, changing from bright orange to dark brown as they mature. In *Emerita asiatica*, varying concentrations of two or three predominant egg carotenoids were reported for different developmental stages (Tirumalai and Subramoniam 2001), however in *E. analoga* the concentrations of egg carotenoids within each developmental stage were not significantly different from one another (Gilchrist and Lee 1972). Developmental stages have not been formally established or documented for *E. analoga*, making it difficult to compare the results of these studies. Although the two species are similar, It is unknown if changes in *E. analoga* egg color can be attributed to the same developmental factors found in *E. asiatica*.

***E. analoga* Behavior**

E. analoga is known for forming dense aggregations in the sand and migrating along the beach in response to tidal patterns and changing beach morphodynamics (Eickstaedt 1969). As inhabitants of the swash zone where water is often turbid, crabs filter feed to collect phytoplankton and other small particles from passing waves using feathery antennae that protrude out of the sand for several minutes at a time (Eickstaedt 1969). When not feeding, they burrow completely underneath the sand or swim in short spurts to new locations.

Some studies focusing on the burrowing behavior of *E. analoga* have reported that burrowing rates are affected by both sand type and beach morphodynamics (Braziero 2005 and Kolluru, Green, Vredevoe, Kuzma, Ramadan, and Zosky unpublished data); However, other studies have reported that *E. analoga* is a “sediment generalist,” capable of burrowing at equal speeds in either coarse or fine sands (Dugan et al. 2000 and Jaramillo et al. 2000). In any case, burrowing behavior is one of the few mechanisms used by *E. analoga* to avoid predation by scavenging fish and seabirds. Nocturnal activity may serve as another predator avoidance mechanism (Fusaro 1980).

***E. analoga* Growth and Morphology**

E. analoga is a sexually dimorphic species in which males are significantly smaller than females in terms of carapace length; females can be distinguished by tiny appendages underneath the telson known as pleopods that hold a developing egg mass in place. In general, size is a function of age, with the largest crabs in the population being the oldest. Population studies in Monterey by Eickstaedt (1969) indicated that ovigerous females were larger on average than non-ovigerous females. Furthermore, he found a positive correlation between egg production and carapace length. It was also described that female age and size at maturity is affected by external environmental factors such as water temperature and food availability (chlorophyll *a*). For example, when compared to females from San Clemente beach and Goleta beach in California, *E. analoga* females from Pismo Beach (located in a colder climate) produced

eggs much later in life, usually not until their second year of maturation. In general, female size at maturity and largest and smallest crab size increase significantly from southern to northern beaches, specifically from La Jolla in San Diego County to Clam Beach in Humboldt County. It has been suggested that this is a result of slower ovarian development due to colder waters but a faster overall growth rate due to greater food availability (Wenner et al. 1987, Dugan et al. 1994). Observations of *E. analoga* populations from California central coast beaches including Morro Bay, Cayucos, Montaña de Oro, Avila Beach, and Pismo Beach indicate that these populations are also sexually dimorphic, have large ovigerous females and share egg production patterns similar to previously studied populations from other California beaches (Vredevoe and Kolluru, unpublished data).

Previous studies have shed light on the life history, distribution, and behavior of *E. analoga*. However, none have not addressed the effects parasitism has on these populations. In most ecosystems, parasitism often plays a hidden yet fundamental role in species competition, predation, survivorship and fecundity (Barnard and Behnke 1990, Maitland 1994, Vance 1996, Lafferty 1999). Taking this into consideration, it is unlikely that *E. analoga* populations are completely free from the effects of parasitism.

Parasites of *E. analoga*

Crustacean endoparasites often have complex life cycles that involve trophic transmission from intermediate to definitive hosts. In these cycles, crustaceans are generally intermediate hosts in which the larval stages of the parasite develop before transmission to a final host. Many host-parasite systems involving aquatic crustaceans have been identified, and a large body of research exists specifically on the effects of larval helminths in these systems. This includes the larvae of nematodes, trematodes and acanthocephalans, all of which have been shown to infect various species of amphipods, isopods, copepods, cirripeds, and decapods (Eickstaedt 1969, Schlechte 1969, Haye and Ojeda 1998, Gaillard et al. 2004, Fredensborg and Poulin 2005, Dezfuli et al. 2007).

Previous groups have documented the presence of larval helminths inside the body cavity of *Emerita analoga* (Eickstaedt 1969, Schlechte 1969). Although the effects that the larvae had on crab hosts were not investigated in these studies, an attempt was made to identify each parasite to species level. At least one unknown species of nematode, a gregarine and two larval stages of a digenetic trematode thought to be either *Levinseniella cruzi* or *Spelotrema nicolli* have been found encysted in *E. analoga* tissues (Eickstaedt 1969, Schlechte 1969). Infection of *E. analoga* by the acanthocephalan *Polymorphus kenti* was initially recognized in sand crab populations from Coos Bay, Oregon in 1949, shortly after a description of acanthocephalan morphology was published by H.J. Van Cleave in 1947 (Reish 1950). In 1967, results from a study on the life history of acanthocephalan species *Profilicollis altmani* revealed that it infected 90% of

5,900 *E. analoga* and an unknown number of shore birds from the coast of California (Karl 1967). *P. altmani* cystacanths have also been found to infect *E. analoga* populations in Peru & Chile (Tantalean et al. 2002).

Three senior projects completed by undergraduate biology students at Cal Poly San Luis Obispo served as preliminary resources for cystacanth identification, where hundreds of acanthocephalans recovered from *E. analoga* along central coast beaches were keyed out using the key by Amin (Amin 1987). Results from these projects indicated that local sand crab populations were infected with *P. altmani* based on the number of spines on the trunk and proboscis morphology. (Barber 2007, Zopfi 2007, Stingely and Martini 2008). Based on these studies, we identified any cystacanths infecting *E. analoga* along the central coast of California as *P. altmani*.

Acanthocephalan Life History

Acanthocephalans or “thorny-headed worms” are endoparasitic helminths which belong to the phylum Acanthocephala. Acanthocephalans use a two-host life cycle consisting of larval development in an intermediate host and transmission to a vertebrate definitive host for final maturation into adults and subsequent reproduction (Near 2002). They are cylindrical, un-segmented worms equipped with a long neck and spheroid proboscis covered in spiny projections that allow

them to burrow into varied intermediate host tissues and into the digestive tract of the final host [Figure 2] (Schmidt 1973, Nickol et al. 1999). They lack an alimentary tract and nutrient absorption occurs entirely through the body wall (Schmidt 1973).

Acanthocephalans may infect both terrestrial and aquatic organisms and use a variety of arthropods such as copepods, amphipods, isopods and decapods as intermediate hosts (Crompton and Nickol 1985, Benesh and Valtonen 2007). Extensively studied systems include freshwater amphipod species *Gammarus pulex*, *G. roeseli* and *Echinogammarus tibaldii* that are infected by juvenile *Polymorphus minutus* (Gaillard et al. 2004, Dezfuli et al. 2007). Other well-studied systems include the marine shore crabs *Hemigrapsus edwardsi* and *H. crenulatus* (Haye and Ojeda 1998), the mud crab *Macrophthalmus hirtipes* (Latham and Poulin 2001, 2002a) and the marine rock crab *Cancer irroratus* (Schmidt and MacLean 1978). Additionally, acanthocephalan infection of lobster and crayfish has also been documented (Nickol et al. 1999).

Definitive hosts of acanthocephalans include mammals, fish and birds that normally prey upon the afore-mentioned arthropods. In aquatic systems, this includes a variety of shorebirds, gulls and ducks (Ching 1989, Brokerhoff and Smales 2002) as well as local predatory fish such as trout and perch (Fielding et al. 2003, Benesh and Valtonen 2007). In marine habitats, adult acanthocephalans have been found inside the black-backed gull (*Larus dominicanus*) in both Chile and New Zealand (Zdzitowiecki 1985, Latham and Poulin 2002b) as well as the pale-faced sheathbill (*Chioniz alba*) in Chile (Torres et al. 1991). In the U.S., gulls

such as *Larus occidentalis* and *L. glaucescens* commonly harbor adult acanthocephalans (Reish 1950).

Acanthocephalan Life Cycle in Sand Crabs

Profilicollis altmani is an acanthocephalan that has adapted to complete part of its life cycle in sand crabs (See Figure 3 for a summary of the life cycle). In this host-parasite system, the sand crabs serve as an intermediate host and local birds that prey upon them serve as definitive hosts. As they feed, sand crabs ingest acanthocephalan eggs from the water (Reish 1950). Once ingested, eggs hatch and microscopic larvae or acanthors are carried through to the digestive tract where they hatch and increase in size. Eventually, the acanthors exit through the gut wall of the digestive tract and freely infiltrate the hemocoel where they develop into cystacanths, encysted juveniles capable of infecting a definitive host but unable to reproduce inside the intermediate host. During this dormant life stage, the proboscis is inverted inside a receptacle and remains unexposed. Infection with multiple cystacanths is common due to the crab continually ingesting eggs as it filter feeds over the course of its life (Reish 1950, Crompton and Nickol 1985).

A vertebrate host such as a gull, surf scoter or sandpiper becomes infected when it preys upon *E. analoga* and ingests the cystacanths (Brockerhoff and Smales 2002). Once inside the intestinal tract of the bird host, cystacanths excyst, embed in the intestinal lining and sexually reproduce. Eggs are shed in definitive host

feces and re-enter the aquatic environment to be consumed by filter feeding sand crabs (Reish 1950, Crompton and Nickol 1985). Generally, only birds can serve as definitive hosts and infection may cause adverse effects in some species. For example, the sudden increase in mortality of eider ducks in Denmark were attributed to dense *P. minutus* infections (Christiansen 1948) . In another instance, heavy infections of *P. minutus* were found to be the cause of mortalities in two flocks of the Canadian swan, *Cyngus olor*. The birds gradually lost weight and may have died as a result of vast intestinal perforation (Sanford 1978). *Profilicollis altmani* was found to have the same effect on female surf scoters in Monterey, California (Perry 1942).

Other vertebrates may also be deleteriously affected as a consequence of ingestion of the parasites. Accidental infection of acanthocephalan larvae by the sea otter *Enhydra lutris nereis* may be fatal for the otter due to peritonitis or necrotic ulceration from parasite attachment to the intestinal lining, followed by intestinal perforation (Mayer et al. 2003). This may occur when sea otters ingest large numbers of infected sand crabs rather than foraging for sea urchins and other preferred prey items (Kreuder et al. 2003). The use of sea otters as hosts is not advantageous to acanthocephalans in this system. Although juveniles may survive transiently in the mammalian intestinal tract, maturation to adulthood is often suspended and reproduction does not occur (Crompton and Nickol 1985; Mayer et al. 2003). This was shown to be true in rats that had been experimentally infected with *P. kenti* cystacanths; when dissected, the rats only

harboured female juvenile acanthocephalans (Reish 1950). This is one of the only laboratory studies conducted that has revealed the fate of polymorphid cystacanths in the mammalian intestine.

Acanthocephalan Taxonomy

Acanthocephalans may serve as useful model systems to test the effects of parasitism on the physiology, behavior, and reproductive potential of many species (Near 2002). Early ancestral acanthocephalan species dominated aquatic habitats and later evolved to inhabit hosts from terrestrial habitats. However, the use of an arthropod as an intermediate host has remained constant (Near 2002). There are three classes in the phylum Acanthocephala: Archiacanthocephala, Palaeacanthocephala and Eoacanthocephala. The latter class primarily uses fish as definitive hosts, while the other two use mammals and birds. Intermediate hosts include myriapods and insects for Archiacanthocephala, malacostracans for Palaeacanthocephala and maxillopods for Eoacanthocephala (Bullock 1969, Crompton and Nickol 1985, Near 2002). The class Palaeacanthocephala includes the families Echinorhynchida and Polymorphida. (See Figure 4 for a breakdown of the host characteristics of each acanthocephalan phylum.) Interestingly, some phylogenetic studies using 18S rRNA have suggested that acanthocephalans are related to Rotifera (Garey et. al 1998, Near 2002) and they were even proposed as a monophyletic group in one study (Gary et. al 1998). Figure 5 illustrates the possible phylogenetic relationship between rotifers and acanthocephalans using 18S rRNA analysis

and also illustrates the relatedness of various acanthocephalan species to one another.

The members of Polymorphida have successfully utilized birds and mammals as definitive hosts (Near 2002), and this order includes ten genera with approximately 127 species, all of which use malacostracan intermediate hosts such as decapods and amphipods (Near 2002, Garcia-Varela 2005). Adults and juveniles of different polymorphid species are generally distinguished by proboscis morphology, neck length, the number of spines on the trunk of the body and the presence of polar swellings on the egg coat (Nickol et al. 1999 and 2002, Garcia-Varela 2008) Our host-parasite system of interest, *E. analoga* infected by *P. altmani*, provides one example of the polymorphid life cycle in a malacostracan crustacean.

The taxonomy of Polymorphidae has been controversial. For example, the genus *Polymorphus* was once classified into two subgenera, *Polymorphus* and *Profilicollis* (Nickol et al. 1999). *Profilicollis* was originally referred to as *Filicollis* and included species that belonged to both *Polymorphus* and *Profilicollis*. As additional species were discovered, new morphological distinctions were made and re-classifications occurred (Nickol et al. 1999). Later, there was debate as to whether *Profilicollis* should be a separate genus due to its distinct, fully ovoid proboscis, compared to the proboscis of *Polymorphus*, which is thought to simply be a bulbous expansion of an otherwise cylindrical organ. Initially, some researchers concluded that the original subgenus ranking for *Profilicollis* was still

appropriate based on the fact that a distinct ovoid proboscis did also occur in certain *Polymorphus* species (Nickol et al. 1999). However, subsequent research revealed that infection by species with characteristic *Profilicollis* attributes always involved decapod crustaceans. Once this intermediate host information was combined with the existing morphological characteristics, the genus status of *Profilicollis* was reinstated within Polymorphidae due to its distinct life history (Nickol et al. 1999, Garcia-Varela 2008). Some studies have made these re-classifications based on gene sequence analyses of 18S rRNA, cytochrome C oxidase, and nuclear small and large subunit (SSU and LSU) rDNA (Near 2002, Garcia-Valera and Nalder 2005, Garcia-Varela 2008). Additional sequence data will further elucidate these phylogenetic relationships.

With respect to acanthocephalans that infect *Emerita analoga*, three species have been described, currently identified as *Profilicollis altmani*, *Polymorphus kenti* and *Polymorphus bullocki* (Crompton and Nickol 1985, Balboa 2009). Another acanthocephalan, *Polymorphus texensis*, was once distinguished as a species separate from *P. altmani* when two specimens were found in a sanderling at Galveston Island, Texas (Webster 1948). However, it was concluded that *P. altmani* and *P. texensis* are synonymous after 2,000 adult acanthocephalans classified under each species were analyzed (Karl 1967). *Polymorphus* and *Profilicollis* species were once distinguished from one another based on the larger proboscis of *P. kenti* that lacks trunk spines (Crompton and Nickol 1985). However, two studies suggest that *P. altmani*, *P. kenti* and *P.*

texensis are a single species, and *P. altmani* has been given nomenclatural priority (Karl 1967, Nichol et al. 2002).

Effects of Parasitism on Behavior

Many parasites have evolved mechanisms by which they alter or manipulate intermediate host physiology in order to enhance their chances of being transmitted to a final host. This may include modifying behavior to increase or decrease mating and feeding habits as well as general movement and activity. Often, there appears to be some degree of correlation between increased parasite infectivity and changed host behavior (Combs 1991, Poulin 1995, Poulin and Thomas 1999, Moore 2002). However, it is hard to say for certain if forces exerted by the parasite truly facilitate transmission or if host pathology is simply a product of parasitism. One striking example is the effect the liver fluke *Dicrocoelium dendriticum* has on the behavior of its second intermediate host, the ant species *Formica fusca*. Migration of a single metacercaria into the sub-esophageal ganglion of the ant causes it to leave the colony at night and crawl to the top of a blade of grass, where it remains until the sun rises or it is ingested by a grazing ruminant, which is the final host of the liver fluke (Esch 2007). Other well known cases involve parasitoid wasp species inducing their spider hosts to weave intricate cocoon structures to protect the wasp's pupae from falling rain (Thomas et al. 2005). Another example is the effect the digenean trematode *Microphallus papillorobustus* has when it encysts within the nervous system tissue of its amphipod intermediate host and induces risky, hyperactive behavior

that makes the host more vulnerable to predation by aquatic birds, the definitive hosts for the parasite (Brown et al. 2003).

In crustacean species, particular attention has been paid to infection by acanthocephalans, which are notorious for altering intermediate host behavior (Crompton and Nickol 1985). *Hemigrapsus crenulatus* crabs naturally infected with acanthocephalans have higher metabolic rates and are generally more active than un-parasitized crabs (Haye and Ojeda 1998). In the mud crab, *Macrophthalmus hirtipes*, individuals that were exposed at the surface of mud flats during receding high tide harbored more *Profilicollis antarcticus* and *P. novaezealandensis* cystacanths than their hidden, burrowed counterparts (Latham and Poulin 2001). Another study found that *Gammarus* amphipods infected with *Polymorphus* cystacanths displayed altered geotactic and clinging behaviors (Bethel and Holmes 1973), and later these behavioral changes were linked to altered serotonergic activity in the same species (Bauer et al. 2005, Tain et al. 2006). Like most crustaceans, *Emerita analoga* must also rely on evolved behavioral mechanisms in order to avoid predation and compete with other individuals in the population. Increased shore exposure and/or a change in metabolic activity that might alter energy expenditure (such as increased burrowing time) in response to parasitism could increase the frequency of predation on infected crabs, thereby affecting survivorship.

Effects of Parasitism on Reproduction

Parasitic infection may also alter host fitness by forcing infected individuals to re-allocate reproductive energy towards parasite survival and transmission. Reproductive effects induced by parasites may vary from decreasing egg load to complete castration. A classic example of parasitic castration is observed in European and California green crabs infected by *Sacculina*, rhizocephalan barnacles. *Sacculina* larvae inject a germinal cell mass into the crab that form microscopic root-like structures that tap into the host nervous system. Eventually, the abdominal organs and gonads are completely infiltrated and destroyed as the parasite develops (Goddard et al. 2005). The parasitic isopod *Hemioniscus balani* has a similar effect on sessile adults of many free-living barnacle species, and infection by a single *H. balani* adult causes complete castration of the barnacle (Blower and Roughgarden 1989). Other parasites simply repress reproductive output or sexual development to a lesser extent. This occurs in threespine sticklebacks (*Gasterosteus aculeatus*) infected with the cestode *Schistocephalus solidus*, which causes females to have smaller ovarian masses and reduction of their ability to carry fully mature gametes (Schultz et al. 2006). In *Gammarus pulex* and *Gammarus roeseli* amphipods, infection with the acanthocephalan, *Polymorphus minutus*, prevents sexual maturity, gonadal development, and inhibits copulation in females. Infected females that do mate successfully produce smaller eggs and have significantly smaller egg masses as well. (Gaillard et al. 2004 and Dezfuli et al. 2007).

Very little attention has been given to reproductive changes in parasitized decapods such as *E. analoga*, even though numerous accounts of infection by acanthocephalan species exist and egg production of this species is relatively easy to study. Infection by *P. altmani* may lead to decreased reproductive success in *E. analoga* populations with a high infection rate. This may be especially true for female crabs because they must allocate a large amount of energy towards egg production each reproductive season.

Parasitic Acquisition of Host Nutrients

One vital component of a parasitic relationship is the manipulation of physiological elements necessary for host survival and homeostasis. A variety of parasites do this by depleting host nutrients and other important compounds that they cannot synthesize *de novo*. For example, in *Gammarus pulex*, the lipid and glycogen content of gravid females infected with acanthocephalan *Pomphorhynchus laevis* were significantly decreased compared to the fat content of uninfected females (Plaistow et al. 2001). Another study involving *G. pulex* revealed that parasitism by the same acanthocephalan causes depressed copper and protein content of the midgut (Bentley and Hurd 1995). Profound changes in host physiology may have a more obvious effect on the life history and success of infected organisms. This may be especially true when there are visible alterations in pigmentation or cryptic coloration of the host. In many aquatic crustaceans, coloration patterns are often achieved by carotenoid pigment

deposition into various tissues. These molecules are an important component of both plant and animal physiology. Parasites may alter host pigmentation by impacting the uptake, metabolism, and/or deposition of pigment, leading to detrimental effects such as decreased host nutrition and increased visibility to predators (Czeczuga 1980a, Crompton and Nickol 1985, Fried et al. 1993, Gaillard et al. 2004).

Introduction to Carotenoids

Carotenoids are lipid-soluble, organic pigments that cannot be manufactured by animals and must be obtained through diet (Linan-Caballo 2002). For example, microalgae and phytoplankton contain chlorophyll, an accessory pigment used to facilitate photosynthesis and other metabolic processes. (Lababpour and Lee 2006). Leafy tissues and vegetables such as bell pepper, carrots and broccoli accumulate significant amounts of beta-carotene and lutein (Minguez-Mosquera and Hornero-Mendez 1993, Oliveira et al. 2009). Carotenoid biosynthesis is regulated in plants and algae by a metabolic pathway localized to plastids. The product of this pathway is phytoene, a simple carotenoid that serves as a precursor to more complex carotenoids that are synthesized later in other biochemical pathways (Cazzonelli and Pogson 2010). Phytoplankton, algae and other producers serve as valuable sources of carotenoids for larger consumers within an ecosystem.

Carotenoids exist as pure hydrocarbons (carotenes) or may be oxidized into xanthophylls and ketocarotenoids. Structurally, they allow for varying levels of light absorbance and appear blue, red, orange, or yellow, absorbing at wavelengths of 420- 480nm (Gilchrist and Lee 1972, Kour and Subramoniam 1992, Zadorozhny et al. 2007). Astaxanthin, for example is generally found to be in high abundance in the carapace of many aquatic crustaceans and usually it appears as a brown-red or reddish purple color to human eyes (Schiedt et al. 1993, Sagi et al. 1995, Wade et al. 2005). It is easily oxidized into astacene upon prolonged exposure to heat and light. Beta-carotene and other carotene compounds such as lycopene appear bright orange or dark yellow. Most of these carotenoids can exist either in esterified or un-esterified form, and can be complexed with other proteins or lipids. Canthaxanthin, echinenone and a number of others are considered to be metabolic intermediates of less oxidized carotenoids (Gilchrist and Lee 1972, Kour and Subramoniam 1992, Linan-Cabello and Paniagua-Michel 2002, Velu et al. 2003). The bonds and specific biochemical structure of each type of carotenoid dictate at what wavelength it absorbs light and what color it will reflect (Britton 1995).

Biological Function of Carotenoids

The physiological purpose of carotenoids varies depending on the organism. In some species, carotenoid color deposition in skin, integument, or feathers conveys information to potential mates via ornamental displays that indirectly represent quality of health and ability to resist disease. In birds, trade offs may

exist between health and plumage or beak pigmentation; carotenoids allocated for physiological functions may reduce their availability for ornamentation (Mayer et al. 2003, Cuervo et al. 2009, Surmacki and Kosicki 2009). In guppies, both sexes are attracted to small, orange objects and orange fruit that are rich in carotenoids (Rodd et al. 2002) and these preferences have been linked to female preference for carotenoid-based orange coloration of body spots on males (Houde 1987, Karino and Shinjo 2004).

Carotenoids are responsible for pigmentation patterns in crustaceans as well. Additionally, they may also serve as vitamin A precursors and have antioxidant properties (Miki et al. 1982, Latscha 1989, Miki et al. 1994). Carotenoids may play additional roles in stimulating the immune system, increasing stress tolerance, and enhancing embryonic and gonadal development in crustaceans (Olson 1993, Menasveta et al. 1994, Mantiri et al. 1995). Though compelling, most of these functions must still be supported by additional data (Linan-Cabello et al. 2002). In many species, carotenoids are deposited into egg yolk and reproductive tissues, and may enhance the quality of offspring (Safran et al. 2008). Pre-embryonic “maternal effects” are one way by which parents can deposit certain hormones or nutrients to their offspring to affect development and influence survival success. When taken as a whole, these data suggest that for many species, carotenoids are critical to many physiological processes, and it is possible that parasites often take advantage of this important host resource during infection.

Parasite Acquisition of Host Carotenoids

Acquisition of host carotenoids by parasites has been well documented in a variety of species. The presence of these compounds within a particular parasite often depends on their site of residence within the host's body. For instance, intestinal nematodes such as *Ascaris lumbricoides* and *A. suum* absorb lutein and beta-carotene directly from the host small intestine, (Czeczuga 1980) whereas *Fasciola hepatica* adults were found to contain mostly xanthophylls derived from the host's liver (Czeczuga 1980). Rediae of the trematode *Echinostoma trivolvis* in snails were found to contain beta-carotene and lutein derived from host tissues (Fried et al. 1993). Carotenoids were also isolated from the acanthocephalan *Pallisentis nagpurensis* infecting freshwater fish. In this example, the coloration of each adult worm was dependent on location within the digestive system contents; colorless worms were found in the unpigmented areas, and worms tinted yellow, orange or red were located in the colored gut contents (Ravindranathan and Nadakal 1971). Ectoparasites may also acquire host carotenoids. *Tinca tinca* fish infested with the gill copepod *Ergasilus sieboldi* had significantly lower carotenoid concentrations per gram of body weight than uninfected fish, and it appeared that the copepod acquired host carotenoids during its life on the fish (Czeczuga 1980b). In this case, the parasite was not just accumulating host carotenoids as it developed, but it was also depleting the reserves of the host.

Acquisition of host carotenoids may produce consequences, and in some instances is beneficial to the parasite by making the host more conspicuous. This occurrence has been well studied in various acanthocephalan-crustacean systems. For example, in *Gammarus* species, orange-pigmented *Polymorphus minutus* cystacanths are visible through the cuticle of the amphipod host, making infected individuals more conspicuous. (Gaillard et al. 2004). Parasites may also alter crustacean host cryptic coloration without directly adopting pigments. This was shown to occur in the isopod *Asellus aquaticus*, where 90% of individuals infected with a juvenile acanthocephalan were more darkly colored than uninfected isopods. Hypo-pigmentation of crustacean hosts was found to occur in the amphipod *Gammarus lacustris*, leading to cuticle depigmentation of infected individuals, revealing the host's underlying blue hemolymph. Depigmented, blue amphipods were 2.5 times more likely to be preyed upon by ducks than their brown, uninfected counterparts (Crompton and Nickol 1985).

Carotenoid Profiles of Acanthocephalans

Although cystacanths of many acanthocephalan species are not visibly pigmented, some species occurring in aquatic crustacean intermediate hosts are brightly colored (Denny 1969, Van Maren 1979 and Kennedy et al. 1978). For instance, *Polymorphus laevis*, *P. marilis*, *P. paradoxus*, and *P. minutus* cystacanths in *Gammarus* species mentioned above are bright orange. The compounds responsible for this pigmentation appear to be carotenoids derived from host tissue. Two studies have shown that the predominant carotenoid of

cystacanths infecting *Gammarus pulex* is esterified astaxanthin (Cheesman et al. 1967 and Gaillard et al. 2004). In many acanthocephalans, a single major carotenoid is detected rather than a mixture of pigments. For instance, astaxanthin is the only carotenoid found in *P. minutus* cystacanths and adults (Barrett and Butterworth 1968), and adult *Filicollis anatis* only contain beta-carotene (Barrett and Butterworth 1973). This suggests that although parasites are exposed to and can potentially obtain an array of host-derived carotenoids, only a subset of the carotenoids may be acquired by the parasite (Barrett and Butterworth 1973).

Carotenoids of *Emerita analoga*

Emerita analoga acquires carotenoids directly from its diet, which consists of phytoplankton and particles of organic matter collected from surrounding water (Gilchrist and Lee 1972). Phytoplankton contains chlorophyll *a* and xanthophylls, accessory pigments used to facilitate photosynthesis and other metabolic processes of these autotrophic organisms (Lababpour and Lee 2006). After being ingested, it is thought that these carotenoids are metabolized and converted into different forms and deposited into various tissues. The presence of carotenoids in *E. analoga* was first described by a study that analyzed the carotenoid content of eggs, carapace, hemolymph and ovarian tissue. Gilchrist and Lee (1972) used thin layer chromatography to identify eight carotenoids including two carotenoproteins. The most prominent xanthophylls found in *E.*

analoga were astaxanthin, zeaxanthin, diatoxanthin, and alloxanthin. Some characteristics of these carotenoids are summarized in Table 1.

In whole crabs, astaxanthin was the predominating carotenoid in both males and females and was found in both esterified and unesterified forms. The major carotenes were alpha and beta-carotene. Other carotenoids isolated from whole crabs in small amounts included echinenone and canthaxanthin. The average total carotenoid content of egg tissue was found to be 79 ug/g, consisting mostly of beta-carotene and a mixture of xanthophylls. The ovaries closely matched the carotenoid profile of egg tissue, but with slightly higher levels of astaxanthin. The carapace was found to contain predominantly astaxanthin and xanthophylls secondarily, with only small traces of beta carotene (Gilchrist and Lee 1972).

Esophagus tissue, which during our dissections was found to be bright red had not been analyzed for its carotenoid content by any previous studies.

Besides extracting free carotenoid molecules from various tissues of *E. analoga*, two carotenoproteins (one appearing blue) were also isolated from *E. analoga* egg and hemolymph (Gilchrist and Lee 1972). Chemically, these compounds consist of a structured, stoichiometric relationship with a carotenoid molecule and an apoprotein (Wade et al. 2009). Specifically in *E. analoga*, they were found to be complexed with a mixture of xanthophylls and astaxanthin, and in eggs the complex also contained a lipid component. Blood-borne pigments that are visible through the telson of living crabs were found to be comprised of the same carotenoproteins found in mature ovaries and early-stage eggs. It was discovered that females with strongly pigmented blood were always collected

during the non-reproductive months during the winter and early spring seasons. It was suggested that as ovaries decreased in size, these pigments were released to the hemolymph and mobilized to other parts of the body such as the exoskeleton (Gilchrist and Lee 1972). This may signify that females preferentially allocate carotenoids into their eggs when reproductive, and then secondarily use them for other biological functions.

Dietary carotenoids are converted to other forms in the body by the use of evolved, enzymatic pathways. A classic example is the conversion of beta-carotene into Vitamin A in the human body. This is achieved via ligand binding of trans-retinoic acid, a molecule subsequently produced by initial cleavage of trans-beta-carotene by beta-carotene monooxygenase (Grune et al. 2010). Interestingly, of the 50 carotenoids found in the average human diet, only a few are detectable in plasma, and only beta-carotene and beta-cryptoxanthin can be converted to Vitamin A (Castenmiller and West 1998). This suggests that enzymatic conversion of specific dietary carotenoids may be essential in order for many animals to efficiently utilize these compounds and incorporate them into their tissues.

Very little information exists regarding how *E. analoga* metabolizes dietary carotenoids to various usable forms, and an exact mechanism has not been extensively studied in any decapod crustaceans. However, Gilchrist and Lee (1972) found evidence that *E. analoga* metabolizes astaxanthin and other

intermediates from xanthophylls and carotene precursors at twice the expected level when fed radioactively labeled green algae. Although the bioconversion of carotenoids in *E. analoga* is not fully understood, proposed metabolic pathways for this species share similarities with previously studied crustacean models. For instance, in *Emerita asiatica*, it has been suggested that alpha carotene, beta carotene and zeaxanthin are converted into astaxanthin by a series of biochemical pathways, producing carotenoids such as echinenone, canthaxanthin and lutein as metabolic intermediates (Kour and Subramoniam 1992). A similar pathway has also been proposed for shrimp from the genus *Penaeus* and suggests that cryptoxanthin molecules serve as additional intermediates before echinenone and canthaxanthin are formed. (Figure 6).

Although the function of storing and mobilizing carotenoids in different tissues in *E. analoga* is unclear, the results of the Gilchrist and Lee (1972) study suggest that it may be linked to reproductive needs. In *E. asiatica*, yolk precursor proteins produced by adipose tissue or hemocytes are converted into lipovitellins, glycoproteins that are conjugated to carotenoid pigments. Generally, it is considered normal for crustacean oocytes to have stores of protein, lipids, carbohydrates, and other compounds that might support developing embryos, and bound carotenoids may offer additional support or protection (Tirumalai and Subramoniam 2001). The energetic cost of being parasitized in terms of reproductive output is an important component of *E. analoga* biology that has not yet been studied. If carotenoids do contribute significantly to embryonic development in this species, it is vital to investigate how increasing egg size

affects carotenoid deposition and ultimately reproductive potential. Trends in ovarian tissue carotenoids should also be studied because the nutrients deposited in this tissue may serve as the precursor for egg quality and development. This has been measured in the form of “maternal effects” in a number of species, including guppies (Grether et al. 2008), blackbirds and barn swallows (Safran R. et al. 2008, Newbrey and Reed 2009).

Besides reproductive tissues, carotenoid allocation and deposition into carapace tissue may affect the fitness of *E. analoga*. Carapace carotenoid concentrations have been quantified extensively in various crustacean species, including crayfish, lobster, shrimp, and hermit crabs (Czeczuga and Czerpak 1968, Nelis et al. 1984, Castillo and Negre-Sadargues 1995, Velu et al. 2003, Wade et al. 2005). Shell color in crustaceans is usually determined by the amount of astaxanthin that can be incorporated or complexed with crustacyanan proteins. This is ultimately responsible for cryptic coloration as the emission spectrum of astaxanthin will shift from red to purple or blue once built into this complex (Wade et al. 2009). Therefore, the amount of carotenoids that can be deposited into carapace tissue may determine ability to evade predators, and this may differ amongst crabs in different size categories or as reproductive status changes.

Methods of Carotenoid Analysis

There are five basic procedures in analytical chemistry used to characterize and quantify carotenoids in mixed biological samples. One of the simplest techniques is UV-Visible spectrometry (UV-Vis); however it is often difficult to quantify concentrations of individual carotenoids in a heterogeneous sample (Lababpour and Lee 2006). Typically, only the total carotenoid concentration of an aqueous sample can be determined using this method. A technique that was frequently used in older studies but is rarely relied on today is thin layer chromatography (TLC), which has been replaced by high performance liquid chromatography (HPLC). Thin layer chromatography was used by Gilchrist and Lee to analyze *E. analoga* carotenoids for the first time. Several older studies on crustacean carotenoids also used this technique (Czeczuga and Czerpak 1968, Castillo and Negre-Sadargues 1995). These studies are important because they have provided the framework for newer carotenoid analysis using HPLC. Unfortunately, TLC may not be as precise as HPLC due to carotenoid oxidation and subsequent loss from manual manipulation of samples. Therefore, comparisons between quantitative TLC and HPLC analytical results should be approached with caution.

Using HPLC has many advantages, namely the ability to equip these machines with built-in spectrometers, automated sampling protocols and refrigerated units for sensitive samples. Additionally, HPLC machines are often connected to mass spectrometry equipment, together referred to as liquid chromatography-mass spec (LC-MS). The most recent carotenoid studies use this technique, which

offers powerful carotenoid quantification and identification of unknown compounds in a mixed sample. Much of the information on the carotenoids of bird plumage, fish meat and vegetables has been obtained using very similar HPLC protocols and chemical extraction techniques (Minguez-Mosquera and Hornero-Mendez 1993, Moren and Hamre 2002, Isaksson et al. 2007, Juola et al. 2008). Most use reverse-phase C18 columns and acetonitrile or methanol for mobile phase separation. Therefore, these studies are easily reproducible and can be built upon readily. HPLC methods have also been utilized to successfully quantify the carotenoids of less commonly studied organisms, such as red coral, human serum, red yeasts, and quail retina (Zhu et al. 2006, Cvejic et al. 2007, Toomey and McGraw 2007, Weber et al. 2007).

One of the newer, more efficient techniques used to analyze carotenoids with an HPLC is known as matrix solid-phase dispersion, or MSPD. This methodology satisfies analytical conditions for even the most heat-sensitive samples, especially those that are only available in very small volumes. It operates on the same principle as classic chromatography, but involves mechanically blending the sample directly with a solid bonded-phase that would normally be contained within a column. The preparation can then be directly injected into an HPLC and readily analyzed. This procedure simultaneously accomplishes several steps necessary to prepare solid or semi-solid samples for analysis using chromatography. Chemical extraction and saponification of carotenoids from solid samples is virtually eliminated. Liquid nitrogen can also be added to

particularly sensitive samples, so MSPD prevents gross sample loss and degradation. However, the materials and equipment for MSPD are expensive, and in many cases conventional extraction techniques with HPLC are suitable (Barker 2007).

PROJECT OBJECTIVES

The main purpose of this study was to expand on previous studies of *Emerita analoga* carotenoids by utilizing more sophisticated and sensitive methods of carotenoid analysis and to evaluate the effects of parasitism by the acanthocephalan, *Profillicolis altmani*. First, I sought to determine if infection significantly altered the allocation and/or production of carotenoids in carapace, egg, ovary and esophagus tissue. I measured total carotenoid concentration as well as the levels of individual carotenoids normally predominating these tissues, namely astaxanthin, diatoxanthin and canthaxanthin for carapace and beta-carotene and diatoxanthin for egg. Secondly, I investigated if infection by *P. altmani* cystacanths significantly affected the reproductive quality of females in terms of carotenoid content and reproductive quantity in terms of the weight of individually collected egg masses. Lastly, to determine if cystacanths are acquiring host pigments, I analyzed the carotenoid profile of the cystacanths themselves. Any carotenoids found were evaluated by comparing them to those of host tissue, and identification of unique compounds may suggest active metabolic conversion by the parasite.

The other purpose of our study was to further investigate the physiological and ecological characteristics of *E. analoga*. This was evaluated in terms of carotenoid content of different tissues across reproductive seasons, within different size classes and between ovigerous and non-ovigerous females. More

generally, I sought to confirm previous results concerning egg mass production and carapace length. By studying reproductive and seasonal effects in conjunction with the effects of parasitism, I attempted to separate inherent morphological and physiological differences between crabs from those that are caused directly by parasitism.

Finally, a goal was to make new contributions to the study of *E. analoga* physiology. I evaluated the carotenoid content of esophagus tissue, which had never previously been analyzed, as well as clearly document the internal and external anatomy of *E. analoga* tissues. To date, no color images of internal or external *E. analoga* anatomy had been published. This makes replication of past studies difficult because dissections become subjective and are usually based on limited, written descriptions. Photographic documentation of dissections may help future researchers to conceptualize the coloration of carotenoid-rich tissues and their placement within *E. analoga*.

PROJECT OVERVIEW

MATERIALS AND METHODS

1. Collections

Crabs were collected by hand twice a month from the swash zone (area of wave action on the beach) of Pismo Beach, California (N35°13.992' W120°64.465') from February to November 2008 using a shovel and coarse sieves. During each collection, approximately twenty to forty crabs from varying areas of the beach were haphazardly selected and sacrificed using dry ice. Juvenile crabs smaller than 11mm that were not sexually mature, injured and newly molted crabs were excluded from the study. Sacrificed crabs were stored in —20°C until dissections.

2. Processing

Following collections, crabs were assigned individual identification numbers and placed in sealable plastic bags. Carapace length, defined as the length between the eyes and the beginning of the telson (Eickstaedt 1969), was measured to the nearest 0.1 millimeters using a ruler or digital calipers. Gender was determined by lifting the telson and noting the presence of pleopods, tiny abdominal appendages found only on adult females that are used for securing a developing egg mass. Absence of pleopods indicated that the crab was male.

3. Dissections

In preparation for dissection, a crab was set in a glass Petri dish with a shallow level of distilled water. The eggs of each ovigerous female were carefully teased away from the pleopods and egg wet mass was measured to the nearest 0.01 gram. The whole body mass of each crab was also measured prior to dissection, to the nearest 0.1 gram. An incision was made extending from the anterior end to the posterior end of the carapace and a perpendicular incision was made near the eyes, creating two flaps that could be opened, exposing the dorsal anatomy of the crab. Cystacanths lying directly on top of the tissue were immediately removed and categorized as existing in the hemocoel. Ovarian tissue was carefully teased away from the other tissues and removed using tweezers. Any cystacanths found inside this tissue were also removed and recorded as existing in the ovaries. Cystacanths found in the digestive gland or towards the hindgut region of the crab were also categorized accordingly. Cystacanths from different locations within the crab were stored in separate tubes. Nearing the end of the dissection, when all cystacanths and ovarian tissue had been collected, total parasite load was calculated. The stomach of the crab was removed to reveal a small section of exposed esophageal tissue underneath. Tweezers were used to remove as much of this tissue as possible. Care was taken to effectively remove as much ovarian and esophagus tissue as possible during dissections; however in some instances the entire organ could not be removed. Lastly, the carapace was removed and cleaned of any epidermis or muscle tissue still clinging to the underside. All cystacanths and tissue samples were stored in 1.5 ml centrifuge tubes in -80°C until further use.

4. Extractions

All tissues from female crabs collected during summer and fall were individually extracted in HPLC grade acetone under low light conditions, such as working in a dimly lit laboratory or wrapping the samples in foil whenever possible due to carotenoid photosensitivity. The standard weight used from each tissue is summarized in Table 2. Once weighed, each sample was extracted in 1.5 mL acetone. Although it was easy to remove whole carapace and egg tissue, ovarian tissue could not be removed in its entirety from some females. This was especially true for non-reproductive females with atrophied ovarian tissue and for very large ovigerous females with well-developed ovaries. The same was true for esophagus tissue because it was difficult to remove from the small cavity of hardened tissue surrounding it. For this reason, standard amounts for each tissue were removed from each crab with the exception of a few very small crabs whose tissues were significantly below the average weight. In this case, adjustments were made later so that the dilution factor for these tissues was the same. Additionally, extraction procedures varied slightly for each tissue being tested and are described below.

4a. Carapace

Individual carapace samples were homogenized under liquid nitrogen using a mortar and pestle. The dry powder weight was subsequently taken and acetone

was added. The sample was vortexed several times to expose the entire sample to the acetone. After being centrifuged for two minutes, the pellet was re-suspended and centrifuged again for five minutes. The colored, acetone supernatant was removed and placed into a new 1.5 mL tube, and the pellet was discarded.

4b. Egg, ovary and esophagus

Liquid nitrogen was not used to prepare egg, ovary, or esophagus samples due to the small volume of these tissues and the sample loss associated with this process. However, egg samples were homogenized using a micropestle prior to weighing. Individual tissue samples were then weighed accordingly, the appropriate volume of acetone was added, and samples were treated in the same fashion as carapace tissue.

4c. Cystacanths

Four hundred cystacanths from ovigerous and non-ovigerous female crabs were randomly chosen to be pooled and homogenized under liquid nitrogen using a mortar and pestle and then extracted in 0.5mL of acetone. The sample was then vortexed and centrifuged using the procedure detailed for tissue samples. Re-suspension of the pellet using a metal spatula was a vital step in this process due to the sticky consistency of homogenized cystacanth tissue. As with the other samples, acetone supernatant was removed and the pellet discarded. Once

aliquoted to new 1.5 ml centrifuge tubes, all extracts were labeled and kept in —80 °C until subsequent UV-vis analysis.

5. UV-visible Spectrometer Analysis (UV-Vis)

UV-Visible data generated during this study were used to analyze total carotenoid concentration of tissues sampled from individual crabs. Prior to analysis, samples were removed from —80 °C and allowed to thaw momentarily in the dark. Semi-micro cuvettes with a 1mL fill volume were used along with micro cuvettes with a .5ml fill volume for the smallest extraction samples. Both cuvette models had a beam travel length of 1cm. During each session, the Logger Pro software was opened and the UV vis lamp allowed to warm up for 1 minute. A micro or semi-micro cuvette was filled with acetone and blanked before any samples analyzed using the same model of cuvette could be run. Immediately after data collection, samples were returned to their original tubes and covered with foil. After each session, samples were returned to -80C for storage. Raw data were exported to an Excel spreadsheet and the lambda max for each sample was recorded. Conversion from absorbance units to micrograms was done using the Beer-Lambert law equation $C = A / E \times L$, where C is the carotenoid concentration in micrograms, A is the absorbance for each sample, L is the beam pathlength in centimeters and E is an extinction coefficient value. When unknown carotenoids are analyzed, the average extinction coefficient of carotenoids, $2500 \text{ M}^{-1} \text{ cm}^{-1}$ may be used (Formula adopted from Britton 1985).

6. Sample pooling for HPLC analysis

6a. Carapace and Egg

HPLC data were used to identify the specific carotenoid profiles of *E. analoga* tissues and build upon the results generated by UV-Vis analysis. Individual samples previously used for this analysis were pooled according to reproductive status, carapace length, and parasite load. Crabs were divided into five size categories: 11-16.9mm, 17-21.9mm, 22-24.9mm, 25-28.9mm, and 29-35.0mm, respectively. Within these categories, crabs were grouped further into increasing parasite load ranges. These ranges varied for the five different size categories due to naturally occurring trends in parasitism.

For each tissue, one HPLC pool contained samples from 3-4 crabs and was given a unique HPLC identification number. The same volume of each crab's contribution to the pooled sample was used, so that each individual crab contributing to a given pooled sample was equally represented. Samples were pooled out of necessity due to limited HPLC run time and reagents.

6b. Esophagus and Ovary

Individual samples used for UV vis analysis were pooled according to parasite load and reproductive season. Four groups, each comprised of five individual samples of equal volumes, were pooled as follows: Unparasitized or highly parasitized crabs collected during the reproductive season (late spring through

summer) and unparasitized or highly parasitized crabs collected during the non-reproductive season (winter). Samples selected to represent the “highly” parasitized grouping for either season were from individuals who had the five highest parasite loads for the season in which they were collected.

6c. Cystacanths

The cystacanths extracted in 0.5ml acetone used for UV vis analysis were prepared for HPLC analysis in the same way as pooled tissue samples.

7. Saponification of Samples

After briefly vortexing the newly combined samples, saturated KOH-MeOH solution was added to saponify the extract in the amount of 20% of the total pool volume in order to chemically remove ester groups and lipid complexes. Smaller pools were treated in 1.5mL tubes, while larger pools were treated in 15mL tubes. After the addition of KOH-MeOH, samples were immediately covered in foil and left to incubate on an automatic shaker for 30 minutes in the dark. The strongly basic pH of the saponified extracts was neutralized with 1M HCl in the amount of 10% of the volume of the pool. (Previous experimental samples had shown that this volume of HCl effectively neutralizes the pH of saponified carotenoid extracts.) After neutralization, the pools were centrifuged for 10 minutes and the colored, saponified supernatant removed. The precipitate formed a pellet and was discarded. Pooled extracts were labeled and stored in -80°C until HPLC analysis.

8. HPLC Analysis

In preparation for analysis, 250 microliters of each pool were transferred to HPLC vials and were kept refrigerated at -20°C until they were ready to be loaded onto the HPLC auto sampler. Samples sat at room temperature in the auto sampler no longer than 10 hours (carapace) or 6 hours (egg, ovary, esophagus, and cystacanths) before being analyzed.

9. HPLC Apparatus

Samples were run on a Shimadzu model LC-20AT Prominence with a photodiode array detector. It was also equipped with an auto sampler and degasser. A 4.6 x 250 mm C18 5 angstrom reverse phase column from Hitachi was used to analyze all samples. All data processing and conditions controls were performed by EZ Start software.

10. Gradient System and Conditions

Flow rate was 0.4mL/min; injection volume 100 microliters and detection by UV absorbance was monitored at 280, 420, 450, and 480 nm. Separations of carotenoid extracts were carried out using a mixture of MTBE, methanol and deionized water as mobile phase. A mixture of 86: 10:4 v/v/v methanol/MTBE/water (solvent A) were used for 10 minutes, followed by a 40-minute linear mobile phase gradient from 86:10:4 to 10:90:0

methanol/MTBE/water v/v/v (solvent B). The column was equilibrated 3 minutes in acetonitrile and over 15 minutes was changed to solvent A, which was maintained in the column for 17 minutes. The sample was then ready to be injected.

11. HPLC Sample Identification

HPLC-grade standards of astaxanthin, beta-carotene, zeaxanthin, and canthaxanthin were purchased from Fisher Science or Sigma Aldrich. Standards and experimental samples were run in duplicate and peak areas having a difference of less than 30% were averaged together. Pools with peak areas more than 30% different or with retention times more than 1 minute off were run again until peak areas matched more closely. Peak retention times of tissue samples were compared with those of standards to identify major compounds. Separate calibration curves were established for all standards by injecting 6 increasing doses of pure standard. Peak area for these carotenoids could then be converted into quantities.

12. Statistical Analysis

All statistical tests were performed using Minitab version 15 and Microsoft Excel 2004 version 11.6.1 at an alpha level of 0.05. The effect of season on body size was analyzed using ANOVA. The relationship between carapace length and parasite intensity was analyzed by regression for males and females. For females, parasite intensity was analyzed by constructing an ANOVA with

reproductive status (ovigerous or non-ovigerous) and carapace length (divided into the same five categories used for HPLC pooling) as fixed effects for each season. Parasite prevalence was analyzed using Chi-squared tests with season, reproductive status and categorical carapace length as main effects. Data from males were not used in these analyses due to inadequate sample size.

Relationships between body weight, carapace length and egg mass weight of females were analyzed with regression. The relationship between total carotenoid concentration and the aforementioned factors was also analyzed with regression for carapace, egg, ovary and esophagus tissue. Comparisons of carotenoid concentration of tissues of females and ovigerous females during different seasons were analyzed using ANOVA. The effect of parasite intensity on carotenoid concentration was analyzed using ANCOVA with carapace length and reproductive status as categorical fixed effect, parasite intensity as the covariate and carotenoid concentration as the dependent variable. For analyzing egg mass data, the same ANCOVA was applied but an egg to body weight ratio was used as the dependent variable. This ratio was determined by dividing wet egg mass weight into wet body weight for each ovigerous female collected. Males were not used in any UV-Vis or HPLC analyses due to inadequate sample size.

RESULTS

***E. ANALOGA* POPULATION STRUCTURE AND TRENDS IN PARASITISM**

Crab Sex and Size Distribution

A total of 243 crabs collected from Pismo Beach from February through November of 2008 were dissected. Two hundred thirteen of the crabs collected during the entire sampling period were female; males were omitted from further analyses due to inadequate sample sizes of 5, 6, 4 and 15 collected during winter, spring, summer and fall. A total of 90 non-ovigerous females and 123 ovigerous females were collected across all seasons. During the winter season, only non-ovigerous females and males were collected; however, as summer season approached, the number of ovigerous females increased and the number of males collected declined. By fall, the number of ovigerous females declined and was nearly equal to that of non-ovigerous females, while the number of males collected increased compared to the other seasons (Figure 7). Spring, summer and fall were the most productive seasons to collect; during winter, only one collection effort was made due to inclement weather and storm patterns that disrupted the beach.

Ovigerous females were the largest crabs collected, followed closely by non-ovigerous females, while males were always significantly smaller than females in either reproductive category (Figure 8). The average carapace length of males

showed little variation, remaining within the range of 12-15 millimeters across seasons (Figure 8). Average carapace lengths of ovigerous females increased from spring through fall, while the size of non-ovigerous females fluctuated throughout the seasons. Spring was the only season in which ovigerous females and non-ovigerous females were similarly sized (average carapace length= 24 and 25 mm) and there was no significant difference in the average size of these groups ($P = 0.54$).

Effects of Crab Sex, Size, and Reproductive Condition on Parasite Intensity and Prevalence

From February-November, the infection rate of males decreased, but increased in females and was highest in fall (Figure 9). Ovigerous females had the greatest parasite prevalence across all seasons, ranging from 80 to 100%. During fall and summer, the parasite prevalence of females was significantly correlated with size ($\chi^2 = P < 0.001$). However during spring and winter, female size did not have a significant effect on parasite prevalence ($\chi^2 = P = 0.12$ and $P = 0.51$). During spring, ovigerous females had a greater parasite prevalence (80%) versus 55% of non-ovigerous females ($\chi^2 = P = 0.008$). The parasite prevalence of males was not statistically analyzed due to inadequate sample size. Reproductive condition could not be analyzed as an effect in winter because only non-ovigerous females were collected.

The parasite intensity in males did not change significantly across seasons ($P = 0.93$) but increased significantly in ovigerous females ($P < 0.001$) and varied

in non-ovigerous females ($P=0.18$; Figure 10). Increasing carapace length was significantly correlated with increasing parasite intensity in ovigerous and non-ovigerous females ($P < 0.001$) but not in males ($P=0.25$; Figure 11). Parasite intensity was also significantly correlated with increasing body weight ($P < 0.001$; Figure 12). Therefore, ovigerous females generally had the highest parasite loads because of their large size. This was found to only be a function of their size, as reproductive status did not have a significant effect on parasite intensity when analyzed by ANOVA ($P=0.41$).

Regression analysis of egg mass versus carapace length and body mass indicated that there was a significant positive relationship ($P < 0.001$ for both; Figure 13 and 14). Therefore, larger females may produce larger egg masses compared to smaller females. However, no significant changes in egg mass production were observed in response to increasing parasite intensities for females of any size ($P=0.89$; Figure 15).

Nematodes were found in 9 crabs collected during the summer months; the nematodes were located in the same tissues as acanthocephalan cystacanths. Nematodes from the genus *Proleptus* have previously been found in *E. analoga* along the California coast (Smith 2007). The nematodes were not identified and these crabs were omitted from further study.

***E. ANALOGA* ANATOMY, TISSUE CAROTENOID PROFILES, AND FACTORS AFFECTING CAROTENOID CONCENTRATION**

Observations of *Emerita analoga* Anatomy and Tissue Pigmentation

Crabs were observed prior to and during dissection for the presence of pigmented tissues, some of which have been previously shown to contain carotenoids (Gilchrist and Lee 1972). From these observations, the type of carotenoid that predominates each tissue was evaluated. Although one study had analyzed the carotenoid content of *E. analoga* tissues (Gilchrist and Lee 1972), information regarding the appearance and location of these tissues within the body is still scarce. Therefore, color photographs (Figures 16-20) help illustrate which tissues are pigmented, their overall appearance under a compound microscope, and where they are located.

Males and females of different sizes had similar coloration patterns. Externally, the dorsal carapace of *E. analoga* is a mottled grey-brown color that often appears slightly speckled, with thin striations running across (Figure 16). This coloration pattern is indicative of astaxanthin-like compounds, which when complexed with protein or other carotenoids can appear dark brown or purple (Wade et al. 2005). The ventral side of the carapace was off-white or beige colored with pinkish to orange colored sections around the legs and telson. These colors indicate the presence of carotenoids such as beta-carotene, which is orange, or canthaxanthin, which can appear as shades of red or pink (Britton

1985). Ovigerous females held a mass of eggs, ranging in color from bright orange to brown under their telson using thin, clear pleopods. The bright orange coloration indicates a high concentration of beta-carotene or xanthophylls, which can be yellow to orange in color (Britton 1985).

Upon opening up the dorsal surface of the crab, a thin membrane was found between the carapace and the underlying organs. The pigmentation pattern of this membrane matched the outer carapace, suggesting that it is a fresh layer of carapace tissue that hardens shortly after the older carapace is shed during a molt. This has been observed in other crustaceans such as hermit crabs and has been referred to as epidermis tissue (Castillo and Ngre-Sadargues 1995). After teasing this layer back, the heart, stomach, digestive glands and gills were made visible. In males and females, the digestive gland was a dark orange to brown colored tissue with a slightly feathery texture. (Figures 17 and 18). The pigmentation of this tissue indicated the presence of carotenoids such as carotenes and xanthophylls. The foregut, midgut and hindgut were located underneath the digestive gland tissue. These tissues were clear or off-white and did not appear to contain much pigment. Removal of the stomach revealed a bright red spot behind the oral cavity, presumed to be esophagus tissue (Figure 19). The bright red color suggested this tissue was composed of astaxanthin or canthaxanthin, carotenoids that are red (Wade et al. 2005). In females, the ovarian tissue extended through the digestive gland and around the gill tissue. Ovarian tissue varied greatly in color and size from crab to crab and differences were

most evident when females from reproductive and non-reproductive seasons were compared. During reproductive months, ovaries were large and bright orange, almost identical to the color of eggs. During non-reproductive months, they appeared atrophied and were often a dull-orange or yellow color, suggesting that fewer pigments are deposited during this time. An un-pigmented spermatophore was evident in some of the larger males, but overall, male gonads were difficult to visualize during both reproductive and non-reproductive seasons.

Some cystacanth stage acanthocephalans inside crabs were also obviously pigmented. Orange and pink cystacanths of varying intensities were found free in the hemocoel and embedded in ovarian and digestive gland tissues (Figure 20). Un-pigmented cystacanths were also found, often in the same crab with their pigmented counterparts. Pigmented cystacanths were not associated with any particular host tissues.

Carotenoid Profiles of *E. analoga* Tissues

Carapace The wavelength for the maximum absorbance of the carapace acetone extract was 480nm, similar to that of pure astaxanthin when analyzed using UV-Vis (Figure 21). Subsequent HPLC analysis showed the same maximum absorbance wavelength, suggesting that astaxanthin is the predominant carotenoid of carapace, comprising approximately 58% of this

tissue (Figure 29). Three of the predominant peaks were identified as esterified and unesterified astaxanthin and canthaxanthin based on retention times and comparison to known standards. The fourth predominant peak had a similar retention time and absorbance as zeaxanthin, but did not precisely match the retention time of the standard. A relatively small amount of beta-carotene was identified. Two smaller peaks with absorbance maxima at 420nm could not be identified, but may be smaller carotenoid molecules or degradation products (Figure 22).

Egg The wavelength of the maximum absorbance value of the egg acetone extract was 456nm, which is similar to that of beta-carotene or a xanthophyll such as zeaxanthin or diatoxanthin (Figure 23). Subsequent HPLC analysis showed the same wavelength at maximum absorbance. Two predominant peaks were identified as beta-carotene and the same unknown xanthophyll. These two compounds comprised 18% and 51% of egg tissue, respectively (Figure 30). Two smaller peaks were identified as unesterified astaxanthin and canthaxanthin. Three lesser peaks were not comparable to any known standards and may be keto-carotenoids or degradation products (Figure 24).

Ovary The UV-Vis and HPLC profiles of ovarian tissue were very similar to that of egg, with a wavelength of maximum absorbance at 455nm (Figure 25). HPLC results revealed the presence of two predominant peaks identified as beta-carotene and the unspecified xanthophyll mentioned before. Beta-carotene had

the largest relative percentage of the two, approximately 67% (Figure 31).

Several smaller peaks could not be identified; however two of those peaks had retention times similar to astaxanthin and canthaxanthin and may actually be trace amounts of these compounds (Figure 26).

Esophagus The UV-Vis profile of esophagus, specifically the maximum absorbance wavelength of 476nm, suggested a mixture of carotenoids that included astaxanthin and carotenes (Figure 27). HPLC analysis revealed that this tissue was comprised predominantly of unesterified astaxanthin and three lesser, unknown peaks that may also be keto-carotenoids (Figure 28). Although one of these peaks had a retention time similar to beta-carotene, the peak area was not large enough to be considered significant. Relative to the other carotenoids in esophagus, astaxanthin comprised approximately 89% of this tissue (Figure 32).

Effects of *E. analoga* Size, Reproductive Status and Season

on Tissue Carotenoids

There was a wide range in carotenoid concentrations for each tissue analyzed, which varied with respect to crab size, reproductive status and season. Table 10 summarizes the minimum and maximum carotenoid concentrations found in each tissue using UV-Vis analysis, as calculated using the general extinction coefficient of $2500 \text{ M}^{-1} \text{ cm}^{-1}$.

Carapace There was a significant negative correlation ($P < 0.001$) between carapace carotenoid concentration and carapace length of female crabs (Figure 33). A similar trend ($P = 0.008$) was observed for female crabs collected during fall (Figure 34). During summer however, this correlation was not significant ($P = 0.16$; Figure 35). Similarly, there was a significant negative correlation ($P < 0.001$) between carapace carotenoid concentration and increasing wet body mass of female crabs (Figure 39). HPLC analysis of carapace astaxanthin indicated a significant negative relationship between astaxanthin concentration and carapace length ($P = 0.03$) for female crabs from all seasons (Figure 40). Analysis of carapace canthaxanthin and diatoxanthin indicated no relationship between these factors ($P = 0.79$ and $P = 0.14$; Figure 41 and 42). When compared to fall females, small summer females were found to have a significantly higher carapace carotenoid concentration than the smallest females from fall ($P = 0.01$). However, females from the other size classes were not different (Medium: $P = 0.77$ and Large: $P = 0.21$; Figure 36). Non-ovigerous fall females appeared to have a higher carapace carotenoid concentration than ovigerous fall females, but this difference was not statistically significant for any size class (Small: $P = 0.08$, Medium: $P = 0.69$ and Large: $P = 0.08$; Figure 37). Additionally, no significant differences were observed between fall and summer ovigerous females in any size class (Small: $P = 0.97$, Medium: $P = 0.17$ and Large: $P = 0.40$) (Figure 38).

Egg There was no correlation ($P = 0.44$) between egg carotenoid concentration and carapace length in female crabs collected during all seasons (Figure 43).

When analyzed seasonally, there was no significant correlation between these factors for fall ($P=0.56$) or summer ($P=0.74$) female crabs (Figure 44 and 45). When the data were broken down into size classes, only the eggs of small female crabs from fall were found to have a significantly lower egg carotenoid concentration than the eggs of female crabs from summer (Small: $P=0.01$, Medium: $P=0.11$ and Large: $P=0.71$; Figure 46). However, uneven sample sizes existed for the two “small” female groups ($n = 16$ and $n = 3$), so this analysis may not be accurate. There was no relationship between egg carotenoid concentration and wet body mass ($P=0.25$) in female crabs (Figure 47). However, a significant negative relationship ($P < 0.001$) between egg carotenoid concentration and egg mass weight was found, and the carotenoid concentration of smaller egg masses (under 0.5 g) varied anywhere from 50-150 $\mu\text{g/g}$ (Figure 48). When analyzed using HPLC, no significant relationship ($P=0.42$) between egg beta-carotene concentration and carapace length was found (Figure 49). Similarly, there was no significant relationship between increasing carapace length and concentrations of astaxanthin ($P=0.08$), canthaxanthin ($P=0.08$) or diatoxanthin ($P=0.73$) (Figures 50-52).

Ovary There was a distinct difference in ovarian carotenoid concentration between summer and fall female crabs (Small and Medium: $P < 0.001$, Large: $P < 0.01$). Carotenoid concentration in small summer crabs was almost three times higher than those collected during fall, and almost two times higher in medium and large crabs collected during fall (Figure 53). Ovary beta-carotene analysis

using HPLC indicated similar trends, with ovigerous females from summer having on average more ovarian carotenoids (Figure 57). Analysis of average ovarian carotenoids of fall ovigerous females compared to non-ovigerous females indicated no differences between these groups ($P=0.92$; Figure 54). During summer, there was no significant relationship between ovary carotenoid concentration and carapace length ($P=0.13$; Figure 56). However during fall, increasing carapace length was significantly positively correlated with ovary carotenoid concentration ($P=0.02$; Figure 55).

Esophagus Regression analysis indicated a significant positive relationship ($P < 0.001$) between esophagus carotenoid concentration and carapace length for all female crabs (Figure 58). When analyzed seasonally, the relationship was still significant for fall ($P=0.002$) and summer ($P=0.003$) female crabs (Figure 59 and 60). The esophagus carotenoid concentrations of summer females in various size classes did not differ significantly from fall females in the same size classes (Small: $P=0.16$, Medium: $P=0.16$ and Large: $P=0.96$; Figure 61), suggesting that season does not affect esophagus carotenoid concentration. Similarly, esophagus carotenoid concentration did not differ significantly between ovigerous and non-ovigerous females within each size class (Small: $P=0.28$, Medium: $P=0.39$ and Large: $P=0.13$; Figure 62). Non-ovigerous females from both seasons appeared to have higher esophagus astaxanthin concentrations, but this was not analyzed for significance (Figure 63).

Carotenoids of *P. altmani* Cystacanths

The UV-Vis profile of pooled cystacanths had a wavelength of maximum absorbance similar to that of astaxanthin, at 475nm (Figure 64). Subsequent HPLC analysis demonstrated that cystacanths do contain astaxanthin and canthaxanthin. The predominant carotenoid in cystacanths appears to be the same xanthophyll present in crab tissues. One unknown peak, almost as large as the astaxanthin peak, had an absorbance pattern similar to a xanthophyll. A very small amount of beta-carotene was also identified. Three other lesser peaks were identified as unknown ketocarotenoids (Figure 65). The concentrations of the xanthophyll, astaxanthin and unknown ketocarotenoids were relatively equal in cystacanths, comprising 41%, 22% and 30% of the pooled specimens (Figure 66), unlike *E. analoga* tissues where one carotenoid typically predominated over the others.

Effects of Parasitism on *E. analoga* Carotenoids

For each tissue, changes in total carotenoid concentration and individual predominating carotenoids in response to increasing parasite load were analyzed in response to increasing parasite load within different size classes. Seasonality was not taken into consideration for HPLC analysis due to the pooling schema, nor was it taken into consideration for UV-Vis analyses with the exception of ovarian tissue, which we found varied significantly in concentration between summer and fall. Although they were initially analyzed separately, data from ovigerous and non-ovigerous females were pooled because previous analyses of

carotenoids from these groups yielded no significant differences. No significant relationship between total carotenoid concentration and parasite load were observed for carapace (P 0.18; Figure 67), egg (P 0.42; Figure 71), fall and summer ovary (P 0.62 and P 0.25; Figure 76 and 77) or esophagus (P 0.18; Figure 78) of female crabs in any size class. HPLC analysis indicated no significant relationship between parasite load and the three predominant carotenoids of carapace (Astaxanthin: P=0.88, Canthaxanthin: P=0.50, and Diatoxanthin: P=0.64; Figures 68-70) or the four predominant carotenoids from egg (Astaxanthin: P=0.53, Beta-carotene: P=0.78, Canthaxanthin: P=0.20, and Diatoxanthin: P=0.07; Figures 72-75) for crabs of any size class.

DISCUSSION

Crab Sex and Size Distribution

Theoretically, our collections should serve as a representative "snapshot" of *E. analoga* population structure during a given season. *E. analoga* from Pismo Beach are sexually dimorphic, with ovigerous females being the largest crabs collected during all seasons. The average size of males does not change significantly across any seasons, likely because the molt increment of males is much smaller than that of females and their maximum size is smaller also. This may also indicate that size is not important for the reproductive success of *E. analoga* males. Overall, there were lower collection numbers for males despite attempting to sample at various parts of the swash zone, but this may be due to changing population structure, and a previous study has suggested that this is dependent on season (Barnes and Wenner 1968).

The increase in ovigerous *E. analoga* females during spring and summer indicates that the reproductive season begins during spring and reaches its peak during the summer months, similar to previous studies of *E. analoga* populations (Barnes and Wenner 1968, Eickstaedt 1969). Our collection data indicated that the number of ovigerous females nearly doubled between spring and summer, suggesting that a large subset of non-ovigerous spring females reproduced and became ovigerous during summer. The fact that the average size of ovigerous and non-ovigerous females is similar during this time is another clue that spring

marks a reproductive transition period for females. As fall season approached and reproductive activity slowed, previously ovigerous females may have released their last zoeae and became non-ovigerous or died. The population of large, ovigerous females that remains during fall could consist of older, larger females that are multiparous during the reproductive period, allowing them to remain ovigerous for longer in the year. This has been evidenced previously in a study that found females may often produce up to five egg batches per year (Efford 1969).

Effects of Crab Sex, Size, and Reproductive Condition on Parasite Intensity and Prevalence

Along with increasing egg mass weight, larger females also had the highest cystacanth intensities during all seasons. Because juvenile acanthocephalans cannot be released from the crab, but rely on ingestion of the crab host for transmission to the definitive host, older crabs would be expected to accumulate cystacanths over time as they develop (Crompton and Nickol 1985), and it is likely that shorter life spans of males contributes to their low parasite prevalence and intensities compared to females. The importance of size, not reproductive condition in determining female parasite intensity is best represented by spring collection demographics; during this season, ovigerous and non-ovigerous females are similarly sized and also share similar parasite intensities. However, parasite prevalence during this season was markedly higher in ovigerous

females and reproductive condition was found to be a significant effect rather than size.

It is possible that there is a change in homeostasis as females become ovigerous, and they are more susceptible to infection because the energetic costs of reproduction affect immune system function. High levels of sex hormones (testosterone or estrogen) have been shown to contribute significantly to sex-based differences in the intensity and prevalence of parasites (Eloi-Santos 1992, Walker et al. 1997, Aguilar-Delfin 2001, Klein 2004). It could also be that more sexually mature females ingest more food in preparation for egg production, thus increasing the chance that they will consume acanthocephalan eggs from the water. However, it takes hours to several days for *P. altmani* larvae to develop into cystacanths once ingested (Crompton and Nickol 1985), and therefore it is impossible to know when females acquire parasites and what reproductive stages they were in when infection occurred. The data from spring suggests that reproductive condition may be one component of parasite acquisition, but size is a more powerful indicator across seasons and within reproductive categories.

Although we found that larger, ovigerous females were more heavily parasitized, it appears that increasing parasite intensity does not alter the egg mass production of ovigerous females in any size class. This suggests that infection by *P. altmani* cystacanths does not cause ovigerous females to re-allocate reproductive products away from their eggs, at least in terms of volume.

However, it is possible that increasing parasite intensity affects aspects of egg production in a way in which we did not measure. For instance, factors such as individual egg weight and egg mass lipid volume were not analyzed. The eggs of *Emerita asiatica* had reduced lipid and glycogen content as a result of parasitism by the acanthocephalan species *Pomphorhynchus laevis*, (Plaistow et al. 2001) and this could also apply to *E. analoga*. Also, physical changes in the reproductive tissues of infected males were not measured. Some parasites such as *Sacculina* species may castrate their hosts, using gonads as a nutrient sources and perhaps reducing reproductive behavior that is risky to host survival (Goddard et al. 2005). In the future, additional *E. analoga* males could be collected and studied for these effects.

Carotenoid Profiles of *E. analoga* Tissues

Past carotenoid analyses of other crustaceans such as lobsters, shrimp and crayfish have shown that carapace tissue often contains significant amounts of carotenoids such as astaxanthin, canthaxanthin and lutein (Czeczuga and Czerpak 1968, Castillo and Négre-Sadargues 1995, Velu et al. 2003, Wade et al. 2005). Crustacean eggs are commonly composed of astaxanthin and beta-carotene, as is the case with *Emerita asiatica* (Kour and Subramoniam 1992). Our carotenoid analyses confirms that this is also true for *E. analoga* tissues. Qualitatively, our results closely matched those of Gilchrist and Lee when they used TLC to identify carotenoid compounds in *E. analoga* carapace, ovary and egg. Although they found zeaxanthin, diatoxanthin, and alloxanthin in *E.*

analoga, diatoxanthin predominated and shared a chemical structure and elution pattern similar to the other two, making them difficult to separate and quantify. For the purpose of our study, we tentatively referred to a xanthophyll peak found in all tissues as “diatoxanthin” because its elution pattern and absorbance was very similar to the one they described. However, analysis by mass spectrometry or comparison to a pure diatoxanthin standard would be needed for verification. Quantitatively, our results varied somewhat compared to those reported by Gilchrist and Lee. Specifically, we found a higher concentration of canthaxanthin in carapace, a lower concentration of beta-carotene in egg, and a higher concentration of diatoxanthin in egg (that persisted across size range and parasite intensity status) than in this previous study. However, HPLC analysis provided a different method of carotenoid detection and therefore our results will vary simply due to different analytical protocols. One of the smallest peaks found in the HPLC carotenoid profile of egg was quite persistent, but none of our standards matched its retention time. We suggest it may be echinenone, a carotenoid intermediate that was briefly discussed by Gilchrist and Lee and shared the same elution properties as our unknown peak (Gilchrist and Lee 1972). Peaks of equally small area, near the minimum threshold of detection by the HPLC, suggest the presence of canthaxanthin and astaxanthin in ovarian tissue; we did not analyze these further because of their very low concentrations. However, some studies do venture to make preliminary HPLC peak identification assessments using equally low concentrations (Gaillard M. et al. 2004).

Additional HPLC studies are needed to provide quantitative carotenoid data that are comparable to our results, and will permit validation of these findings.

Many HPLC egg samples had to be re-analyzed 4-6 times due to inconsistent repeatability between duplicate samples. We found that egg samples were less stable than carapace samples, which typically required only duplicate or triplicate runs. Specifically, the beta-carotene concentration of the egg samples varied widely between repeats. Repeatability errors were not encountered for ovarian beta-carotene analyses, suggesting inherent differences between the beta-carotene of these two tissues. This was unexpected, as we would predict that ovarian tissue carotenoids should closely mirror those of egg; indeed, the two shared very similar UV-Vis profiles, suggesting they contain roughly the same ratio of beta-carotene. However, egg carotenoids are often bound and stabilized stoichiometrically within larger lipid and protein complexes (Tirumalai and Subramoniam 2001, Safran et al. 2008). Egg-specific proteins or lipids bound to beta-carotene in this tissue could affect its stability more so than carotenoids found in ovarian tissue. However, protein composition of egg and ovarian tissue could not be compared during our analysis because samples were not treated in a way where proteins could be recovered.

The carotenoid content of esophageal tissue of this species had not previously been analyzed, however it had been noted by our research group as being bright red in coloration. Although we have verified with HPLC analysis that this tissue consists predominantly of astaxanthin, analysis by mass spectrometry might also

be valuable. Ideally, any future samples should be analyzed using liquid chromatography-mass spectrometry (LC-MS) to ensure a more thorough identification of all compounds. One reason this type of analysis would be valuable is the fact that the UV-Vis and HPLC profiles of esophagus differed slightly; Although the UV-Vis profile suggested a mixture of different carotenoids, HPLC data revealed only astaxanthin as the predominant carotenoid. Initially, it was thought that degradation of beta-carotene or other carotenes as discussed earlier could have resulted in this HPLC carotenoid profile. However, it is also possible that even trace amounts of carotenes or xanthophylls absorbing at 450nm shifted the absorbance wavelength such that the UV-Vis profile differed from that of pure astaxanthin.

The xanthophyll peak presumed to be diatoxathin was found in all *E. analoga* tissue samples at varying concentrations, suggesting that it may be a reflection of availability from the crabs' diet. The opposite may be true for beta-carotene in ovarian tissue and egg, and likewise for astaxanthin in carapace. In these tissues, select carotenoids predominate and seem to serve a specific function, for instance cryptic coloration by use of astaxanthin bound to crustacyanin and reproductive needs by protecting developing embryos with antioxidants in the form of beta-carotene (Wade et al. 2009, Cheesman et al. 1967, Miki et al. 1994). The purpose of high concentrations of astaxanthin and canthaxanthin in esophageal tissue is less clear. Canthaxanthin is thought to be a metabolic intermediate of astaxanthin in some crustaceans, so it is less surprising to find

the two existing together in the same tissue. However, it is not known if astaxanthin has a physiological function within the esophagus, or if excess amounts produced by the hepatopancreas are simply secreted or released into this organ at a later time. It could serve as a repository for dietary carotenoids that the crab has recently ingested, and astaxanthin predominates in the food samples that are taken in. Many other marine species have been known to store astaxanthin in their cuticle, tegument or exoskeleton (Velu et al. 2003, Moren and Hamre 2002) and *E. analoga* may be no exception.

Effects of *E. analoga* Size, Reproductive Status and Season on Tissue Carotenoids

Once the carotenoid profile of each tissue was established, season, reproductive condition and size were tested as factors that could effect carotenoid concentration. Our study has shown that season has no effect on the total carotenoid concentration of egg or carapace. Any seasonal differences in the carotenoid concentration of these tissues were due to size demographics for that collection period. Size measured by carapace length was the most significant factor predicting carotenoid concentration of carapace; larger crabs had significantly lower carapace carotenoid concentrations. The carotenoid found to be driving this trend was astaxanthin, which is not surprising because it is the predominant carotenoid in carapace. On average, non-ovigerous females had higher carapace carotenoid concentrations than ovigerous females, but the difference was not significant in any size class. This discrepancy could be due to

ovigerous females allocating a higher concentration of carotenoids to reproductive tissues, leaving less for the hemolymph and carapace. However, our results indicate that regardless of reproductive status or season, sufficient carotenoids are still allocated to the carapace of large crabs to maintain cryptic coloration.

Egg mass weight was the most significant factor predicting the total carotenoid concentration of egg tissue; heavier egg masses (generally produced by larger crabs) seem to have lower egg carotenoid concentrations. This correlation seems to be driven by the wide carotenoid concentration range of eggs produced by small crabs. Interestingly, egg carotenoid concentration never reached levels below 40 ug/g, even for the largest egg masses. This may be the minimum amount of carotenoids allocated to eggs during their development. Other carotenoids could be distributed to eggs in variable concentrations based on dietary availability or other unknown factors. Another possibility is that the eggs from small and medium-sized females were collected during different stages of development. One previous study had found that *E. asiatica* eggs had varying carotenoid concentrations during each stage of zoeal development (Kour and Subramoniam 1992). The developmental stage of each *E. analoga* egg mass collected during this study was not determined, in part because there is debate as to how many developmental stages exist and how each one should be characterized (Johnson and Lewis 1942, Efford 1970). An interesting follow-up

study would be to replicate what Kour and Subramoniam had done with *E. asiatica* eggs using modern methods of carotenoid analysis.

Egg mass samples analyzed using HPLC were pooled in size categories according to carapace length, which was found to have no significant correlation to egg carotenoid concentration. In the future, egg samples should be pooled according to egg mass weight, not carapace length for subsequent HPLC analysis because this would better indicate how specific egg carotenoids vary as female size increases.

The results of our study have also shown that unlike carapace and egg, the carotenoid concentration of ovarian tissue varied significantly between seasons and also with reproductive status. Total ovarian carotenoid concentration was significantly higher in ovigerous females than non-ovigerous females collected during summer. HPLC data confirmed these results and showed that ovarian beta-carotene increased on average as much as four-fold during the summer months. The obvious changes in ovarian volume and pigmentation during the reproductive season indicated that this tissue is vital for reproductive success. By the time reproductive activity began to slow in fall, the ovarian carotenoid concentration of ovigerous and non-ovigerous females was no longer significantly different, suggesting that carotenoids and other resources were no longer being allocated to female reproductive tissues. However, ovarian tissue was often difficult to extract, particularly from females collected during fall that

were smaller in size. There was also a great deal of variation between individuals collected within the same season. This could be due to the reproductive condition of the female at the time of collection, for instance whether she had just released an egg batch or was just about to produce eggs in the ovary. Reproductive condition may explain the variation seen within both seasons as well as the lack of variation between ovigerous and non-ovigerous females collected during fall.

Neither season or reproductive condition had an effect on the total carotenoid concentration of esophagus. Size measured by carapace length was the most significant factor predicting carotenoid concentration of esophagus, and unlike carapace and egg, larger crabs had significantly higher esophagus carotenoid concentrations. Larger crabs may collect more phytoplankton on their antennae as they feed, allowing a higher concentration of carotenoids to be stored in this tissue. However, this could also be a result of our dissections and how difficult it was to remove esophagus tissue from very small crabs. These samples could have been lower in quality and this may have affected our analyses. Although HPLC analysis revealed that crabs collected during summer on average had a higher esophagus astaxanthin concentration than those collected during fall, this could be due to the fact that crabs collected during the summer season were larger than those collected during fall. Secondly, phytoplankton might have been more abundant during the summer months, when algal blooms due to increased water temperature are common. Increased food availability might equate to

increased feeding and subsequently more carotenoids are accumulated directly in the mouth and esophagus.

Carotenoids of *P. altmani* Cystacanths and the Effects of Parasitism on *E. analoga* Carotenoids

The uptake of host carotenoids by adult and juvenile acanthocephalans has been investigated in previous studies (Ravindranathan and Nadakal 1971, Barrett and Butterworth 1972, Gaillard et al. 2004) and is important to understanding what physiological effects they may have on their hosts. Showing that acanthocephalans purposely sequester carotenoids from host tissues would indicate that these molecules are somehow useful for their own development, important to host fitness, or both. During our dissections of *E. analoga*, we often found faint orange and/or pink pigmented *P. altmani* cystacanths embedded in various tissues or floating freely in the hemocoel. Gilchrist and Lee demonstrated that carotenoids are prevalent in *E. analoga* egg, carapace and hemolymph, and we were interested in knowing if *P. altmani* was capable of manipulating host carotenoid allocations to these tissues. For example, juvenile *P. minutus* acanthocephalans contained varying amounts of esterified astaxanthin (Gaillard et al. 2004). Our UV-Vis and HPLC analyses of pooled *P. altmani* cystacanths from various locations within *E. analoga* confirmed the presence of carotenoids in this species of acanthocephalan as well. Because cystacanths were often found embedded in ovarian tissue, the presence of substantial amounts of beta-carotene in their integument would not have been surprising; however, this was

not the case. The cystacanth carotenoid profile did not clearly match any one tissue exactly, however it was most similar to carapace.

We propose one hypothesis to explain the origin of carotenoids found in *P. altmani* cystacanths. As acanthors develop inside *E. analoga*, carotenoids may be passively incorporated into the parasite integument as they grow and mature into cystacanths. In this form, carotenoids may simply be stored and are not being actively metabolized in any way. Therefore, carotenoids identified in *P. altmani* cystacanths may be derived during their journey through the digestive tract and hemolymph of *E. analoga* before they encysted in the ovaries, digestive gland or hemocoel. This would explain why the cystacanth carotenoid profile did not match any one tissue of *E. analoga*, even though most cystacanths were found atop ovarian tissue, which is rich in beta-carotene. It would also explain why cystacanths had a significant diatoxanthin concentration, because we found this xanthophyll to be ubiquitous in all the *E. analoga* tissues we analyzed. However, we were not able to determine if *P. altmani* passively or actively allocates host carotenoids during its development inside *E. analoga*. The majority of the carotenoids found in cystacanths were identical in retention time and absorbance wavelength to those isolated from host tissues, suggesting that they are not metabolized by the cystacanth in a unique way. Interestingly though, a few minor, unknown compounds with differing retention times were present and could be studied further. Perhaps these compounds are indeed the result of host carotenoids being converted into usable forms that are unique to the

cystacanths. This has been suggested before in a previous study examining the carotenoid composition of *P. minutus* cystacanths, which were shown to have different carotenoids than their hosts, *Gammarus pulex* and *G. roeseli* (Gaillard et al. 2004).

In the future, more pools of homogenized cystacanths could be analyzed and if possible, cystacanths should be grouped according to location of embedment within the crab. In this way, it could be determined if cystacanths embedded in different tissues were allocating carotenoids directly from that tissue, or if carotenoids were derived earlier during development and do not match any particular host tissue, as we have proposed.

Regardless of whether or not *P. altmani* actively or passively acquires carotenoids from *E. analoga*, the presense of these compounds in cystacanth integument indicated that loss of host carotenoids was possible. We were interested in determining how significantly increasing parasite load affected the total carotenoid concentration of carapace, egg, ovary and esophagus as well as the concentrations of specific carotenoids predominating these tissues. Within three different size classes, neither carapace, egg, ovary or esophagus were shown to have significantly reduced total carotenoid concentrations as a result of parasitism. Analyses of predominant tissue carotenoids by HPLC revealed that parasite intensity also did not significantly affect the concentration of specific carotenoids such as beta-carotene in egg and astaxanthin in carapace. This

disproved our theory that although total carotenoids were not affected, individual carotenoid levels might be affected due to altered host metabolism, feeding, or interruption of biochemical pathways in the hepatopancreas caused by cystacanth infection. Ideally, tissue samples should have been analyzed individually using HPLC as was done using UV-Vis, however time constraints made that type of analysis impossible and we had to group samples together. Still, our methodology should have provided a rough estimate of any major changes in predominating tissue carotenoids. It is evident from this study that there are no changes in carotenoid allocation or concentration associated with *P. altmani* infection of *E. analoga* tissues. If changes are occurring as a result of infection, they are either very subtle and could not be traced using our methods, or they are effectively countered by increased feeding or shifts in metabolism that this study could not identify.

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APPENDIX A: FIGURES AND LEGENDS

Figure 1. Geographic distribution of *Emerita analoga* populations in North and South America
(Map taken from Efford 1976.)

Figure 2. Polymorphus cystacanth with proboscis everted. Adapted from Pichelin et al. 1998.

Figure 3. Life cycle of *Profilicollis altmani*. (Provided by Julie Anderson, Dept of Biological Sciences Cal Poly San Luis Obispo)

Figure 4. Taxonomic key outlining key evolutionary characteristics of phylum Acanthocephala.

Adapted from Near (2002).

Figure 5. Phylogenetic relationship of species within each order of Acanthocephala based on 18S rRNA gene sequence analysis. Adapted from Near 2002.

Carotenoid	λ Max	Coloration
Astaxanthin	~ 480nm	Red-Purple
Beta-Carotene	~ 450nm	Orange
Canthaxanthin	~ 480nm	Orange-Red
Zeaxanthin	~ 450nm	Yellow

Table 1. Absorption spectra as λ Max (energy absorbed as a function of wavelength) and pigment qualities of the major carotenoids identified in *Emerita analoga* tissues using thin layer chromatography (Gilchrist and Lee 1972).

Figure 6. A depiction of metabolic pathways for carotenoids in the shrimp *Penaeus* from Linan-Cabello et. al. 2002. Proposed *E. analoga* carotenoid bioconversion pathways are very similar to this schema.

Figure 7. Number of *E. analoga* males, females, and ovigerous females collected at Pismo Beach, California during each season in 2008.

Figure 8. Seasonal changes in *E. analoga* size distribution (average carapace length) of males, females and ovigerous females (+/- SE bars).

Figure 9. Seasonal rates of *P. altmani* acanthocephalan infection for *E. analoga* males, females and ovigerous females.

Figure 10. Average parasite intensity for *E. analoga* males, females and ovigerous females during different seasons (+/- SE bars).

Figure 11. Correlation between carapace length and parasite intensity for ovigerous and non-ovigerous *E. analoga* females and males collected across all seasons.

S = 0.827486 R-Sq = 79.9% R-Sq(adj) = 79.6%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	249.806	249.806	364.82	0.000
Error	92	62.996	0.685		
Total	93	312.801			

Figure 12. Carapace Length and Body Mass. Relationship between wet body mass and carapace length of all *E. analoga* females collected during all seasons.

S = 0.396852 R-Sq = 21.1% R-Sq(adj) = 20.2%
 Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	3.8679	3.86789	24.56	0.000
Error	92	14.4892	0.15749		
Total	93	18.3571			

Figure 13. Egg Mass and Carapace Length. Relationship between egg mass and carapace length of ovigerous *E.analoga* females collected during all seasons.

S = 0.347378 R-Sq = 39.5% R-Sq(adj) = 38.9%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	7.2553	7.25531	60.12	0.000
Error	92	11.1018	0.12067		
Total	93	18.3571			

Figure 14. Egg Mass and Body Mass. Relationship between egg mass and wet body mass of ovigerous *E. analoga* females collected during all seasons.

Figure 15. Egg Mass Production. Effects of parasite load on egg mass production of *E. analoga* females. Size class determined by carapace length. Egg to body ratio expressed as wet weight. Differences between groups were analyzed using ANCOVA ($P=0.89$).

Figure 16. Dorsal external anatomy of *Emerita analoga*. Note grey-brown pigmentation patterns and striations on carapace.

Figure 17. Internal anatomy of *Emerita analoga* displaying digestive gland and gill tissue. Note the orange pigmentation of digestive gland tissue.

Figure 18. Internal anatomy of *Emerita analoga* displaying ovarian tissue and partial epidermis tissue. Note the orange pigmentation of both tissues.

Figure 19. Internal anatomy of *Emerita analoga* with stomach removed, displaying red esophageal tissue.

Figure 20. Infected *E. analoga* with two cystacanths in hemocoel cavity.

Table 2. Assigned standard weights and dilutions for each tissue for acetone extractions.

Tissue/Sample	Mass in mg	Vol acetone in mL	mg/mL
Carapace	50.0	1.5	33.0
Egg	50.0	1.5	33.0
Ovary	10.0	1.5	6.67
Esophagus	2.0	1.5	1.33
Cystacanths (400)	407.5	0.5	815.0

Figure 21. UV-Vis profile of *Emerita analoga* carapace tissue. The profile is similar to that of astaxanthin.

Figure 22. HPLC profile of predominant carotenoids in saponified, pooled carapace tissue.

Figure Key:

- 1 Unknown keto-carotenoid
- 2 Unknown keto-carotenoid
- 3 Xanthophyll (Diatoxanthin)
- 4 Astaxanthin – Unesterified
- 5 Astaxanthin – Esterified
- 6 Canthaxanthin
- 7 Beta-Carotene

Figure 23. UV-Vis profile of *E. analoga* egg tissue. The profile is similar to that of beta-carotene.

Figure 24. HPLC profile of predominant carotenoids in saponified, pooled egg tissue.

Figure Key:

- 1 Unknown keto-carotenoid
- 2 Unknown keto-carotenoid
- 3 Xanthophyll (Diatoxanthin)
- 4 Astaxanthin – Unesterified
- 5 Canthaxanthin
- 6 Unknown carotenoid
- 7 Beta-carotene

Figure 25. UV-Vis profile of *E. analoga* ovarian tissue. The profile is similar to that of beta-carotene.

Figure 26. HPLC profile of predominant carotenoids in saponified, pooled ovarian tissue.

Figure Key:

- 1 Unknown keto-carotenoid
- 2 Unknown keto-carotenoid
- 3 Xanthophyll (Diatoxanthin)
- 4 Astaxanthin - Esterified
- 5 Canthaxanthin
- 6 Beta-Carotene

Figure 27. UV-Vis profile of *E. analoga* esophagus tissue. The profile is similar to that of astaxanthin.

Figure 28. HPLC profile of predominant carotenoids in saponified, pooled esophagus tissue.

Figure Key:

- 1 Unknown keto-carotenoid
- 2 Xanthophyll (Diatoxanthin)
- 3 Astaxanthin – Unesterified

Figure 29
Carapace

Figures 29-32
Relative percentage of
predominating
carotenoids of various
tissues.

Figure 30
Egg

Figure 31
Ovary

Figure 32
Esophagus

S = 12.1783 R-Sq = 10.8% R-Sq(adj) = 10.0%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	2127.8	2127.81	14.35	0.000
Error	119	17649.1	148.31		
Total	120	19776.9			

Figure 33. Carapace. UV-Vis analysis of carapace carotenoid concentration in response to carapace length of *E. analoga* females collected during all seasons.

S = 13.5988 R-Sq = 9.7% R-Sq(adj) = 8.4%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	1374.6	1374.61	7.43	0.008
Error	69	12760.0	184.93		
Total	70	14134.6			

Figure 34. Carapace. UV-Vis analysis of carapace carotenoid concentration in response to carapace length of *E. analoga* females collected during fall.

S = 9.49474 R-Sq = 4.1% R-Sq(adj) = 2.1%
 Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	186.45	186.455	2.07	0.157
Error	48	4327.21	90.150		
Total	49	4513.66			

Figure 35. Carapace. UV-Vis analysis of carapace carotenoid concentration in response to carapace length of *E. analoga* females collected during summer.

Figure 36. Carapace. Average carapace carotenoid concentration of both ovigerous and non-ovigerous *E. analoga* females of different sizes collected during summer and fall.

Figure 37. Carapace. Comparison of average carapace carotenoid concentration of ovigerous and non-ovigerous *E. analoga* females in different size classes collected during fall.

Figure 38. Carapace. Comparison of average carapace carotenoid concentration of all ovigerous *E. analoga* females collected during summer and fall. Carapace length for size class categories are 22.0-24.9mm for small, 25.0-28.8mm for medium, and 29.9-32.0 for large.

S = 9.99752 R-Sq = 39.0% R-Sq(adj) = 37.8%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	3384.57	3384.57	33.86	0.000
Error	53	5297.37	99.95		
Total	54	8681.94			

Figure 39. Carapace. UV-Vis analysis of carapace carotenoid concentration in response to wet body weight of *E. analoga* females collected during all seasons.

Source	DF	SS	MS	F	P
Size	4	7805	1951	2.98	0.031
Error	38	24853	654		
Total	42	32658			

S = 25.57 R-Sq = 23.90% R-Sq(adj) = 15.89%

Figure 40. Carapace. Carapace astaxanthin concentration of differently sized *E. analoga* females collected during all seasons, ovigerous and non-ovigerous combined. Size classes expressed by carapace length ranges. Differences in carotenoid concentration between size classes were analyzed using ANOVA (P = 0.03).

Source	DF	SS	MS	F	P
Size	4	256	64	0.43	0.789
Error	33	4954	150		
Total	37	5210			

S = 12.25 R-Sq = 4.91% R-Sq(adj) = 0.00%

Figure 41. Carapace. Carapace canthaxanthin concentration of differently sized *E. analoga* females collected during all seasons, ovigerous and non-ovigerous combined. Size classes expressed by carapace length ranges. Differences in carotenoid concentration between size classes analyzed using ANOVA (P=0.79).

Figure 42. Carapace. Carapace diatoxanthin concentration of differently sized *E. analoga* females collected during all seasons, ovigerous and non-ovigerous combined. Size classes expressed by carapace length ranges. Differences in carotenoid concentration between size classes analyzed using ANOVA ($P=0.14$).

S = 23.5204 R-Sq = 0.8% R-Sq(adj) = 0.0%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	333.9	333.892	0.60	0.440
Error	79	43703.6	553.211		
Total	80	44037.5			

Figure 43. Egg. UV-Vis analysis of egg carotenoid concentration in response to carapace length of *E. analoga* females collected during all seasons.

S = 17.8381 R-Sq = 1.4% R-Sq(adj) = 0.0%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	112.03	112.034	0.35	0.558
Error	25	7954.95	318.198		
Total	26	8066.98			

Figure 44. Egg. UV-Vis analysis of egg carotenoid concentration in response to carapace length of *E. analoga* females collected during fall.

S = 24.6942 R-Sq = 0.2% R-Sq(adj) = 0.0%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	67.3	67.346	0.11	0.741
Error	52	31709.9	609.806		
Total	53	31777.2			

Figure 45. Egg. UV-Vis analysis of egg carotenoid concentration in response to carapace length of *E. analoga* females collected during summer.

Figure 46. Egg. Comparison of average egg carotenoid concentration of *E. analoga* females collected during fall and summer in different size classes. Carapace length for size class categories are 22.0-24.9mm for small, 25.0-28.8mm for medium, and 29.9-32.0 for large.

S = 23.4147 R-Sq = 1.6% R-Sq(adj) = 0.4%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	726.1	726.093	1.32	0.253
Error	79	43311.4	548.246		
Total	80	44037.5			

Figure 47. Egg. UV-Vis analysis of egg carotenoid concentration in response to wet body weight of *E. analoga* females collected during all seasons.

S = 21.7930 R-Sq = 14.8% R-Sq(adj) = 13.7%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	6517.7	6517.72	13.72	0.000
Error	79	37519.8	474.93		
Total	80	44037.5			

Figure 48. Egg. UV-Vis analysis of egg carotenoid concentration in response to egg mass wet weight of *E. analoga* females collected during all seasons.

Figure 49. Egg. Egg beta-carotene concentration of differently sized *E. analoga* females collected during all seasons. Size classes expressed by carapace length ranges. Differences in carotenoid concentration between size classes analyzed using ANOVA ($P=0.42$).

Figure 50. Egg. Egg astaxanthin concentration of differently sized *E. analoga* females collected during all seasons. Size classes expressed by carapace length ranges. Differences in carotenoid concentration between size classes analyzed using ANOVA ($P=0.08$).

Figure 51. Egg. Egg canthaxanthin concentration of differently sized *E. analoga* females collected during all seasons. Size classes expressed by carapace length ranges. Differences in carotenoid concentration between size classes analyzed using ANOVA ($P=0.08$).

Figure 52. Egg diatoxanthin concentration of differently sized *E. analoga* females collected during all seasons. Differences in carotenoid concentration between size classes analyzed using ANOVA ($P=0.73$).

Figure 53. Ovary. Comparison of average ovary carotenoid concentration of *E. analoga* females in different size classes collected during different seasons.

Figure 54. Ovary. Comparison of average ovary carotenoid concentration of ovigerous and non-ovigerous *E. analoga* females collected during fall. Difference in ovarian carotenoid concentration of ovigerous and non-ovigerous females analyzed using Student's T Test ($P=0.92$).

S = 17.1590 R-Sq = 16.9% R-Sq(adj) = 14.1%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	1791.1	1791.13	6.08	0.020
Error	30	8833.0	294.43		
Total	31	10624.1			

Figure 55. Ovary. UV-Vis spectrometer analysis of ovary carotenoid concentration in response to carapace length of *E. analoga* females collected during fall.

S = 70.5435 R-Sq = 4.9% R-Sq(adj) = 2.8%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	11593	11593.4	2.33	0.134
Error	45	223937	4976.4		
Total	46	235531			

Figure 56. Ovary. UV-Vis spectrometer analysis of ovary carotenoid concentration in response to carapace length of *E. analoga* females collected during summer.

Figure 57. Ovary. HPLC analysis of average ovarian beta-carotene concentration during summer and fall of ovigerous and non-ovigerous *E. analoga* females. Beta-carotene concentration expressed as an average of five crabs within each seasonal and reproductive category.

S = 703.555 R-Sq = 16.1% R-Sq(adj) = 15.3%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	9586547	9586547	19.37	0.000
Error	101	49993993	494990		
Total	102	59580539			

Figure 58. Esophagus. UV-Vis spectrometer analysis of esophagus carotenoid concentration in response to carapace length of *E. analoga* females collected during all seasons.

S = 779.565 R-Sq = 18.3% R-Sq(adj) = 16.6%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	6415953	6415953	10.56	0.002
Error	47	28562909	607721		
Total	48	34978861			

Figure 59. Esophagus. UV-Vis spectrometer analysis of esophagus carotenoid concentration in response to carapace length of *E. analoga* females collected during fall.

S = 624.428 R-Sq = 16.1% R-Sq(adj) = 14.5%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	3885132	3885132	9.96	0.003
Error	52	20275320	389910		
Total	53	24160452			

Figure 60. Esophagus. UV-Vis spectrometer analysis of esophagus carotenoid concentration in response to carapace length of *E. analoga* females collected during summer.

Figure 61. Esophagus. Comparison of average esophagus carotenoid concentration of *E. analoga* females of any reproductive status in different size classes collected during summer and fall.

Figure 62. Esophagus. Comparison of average esophagus carotenoids of ovigerous and non-ovigerous *E. analoga* females collected during all seasons.

Figure 63. HPLC analysis of seasonal changes in astaxanthin concentrations of esophagus tissue from ovigerous and non-ovigerous *E. analoga* females collected during summer and fall.

Table 10. Summary of total carotenoid concentration ranges for each tissue collected during all seasons.

	Total Carotenoid Concentration (ug/g) of tissue
Carapace	20-85
Egg	40-125
Ovary - Fall	10-70
Ovary - Summer	40-300
Esophagus	1000-3000

Figure 64. Uv-Vis profile of 400 pooled *P. altmani* cystacanths found in *E. analoga* tissues. The profile is very similar to that of astaxanthin.

Figure 65. HPLC profile of predominant carotenoids in saponified, pooled cystacanths.

Figure Key:

- 1 Unknown keto-carotenoid
- 2 Unknown xanthophyll
- 3 Unknown keto-carotenoids
- 4 Xanthophyll (Diatoxanthin)
- 5 Astaxanthin – Unesterified
- 6 Canthaxanthin
- 7 Beta-carotene

Figure 66. Relative percentage of predominating carotenoids in pooled *P. altmani* cystacanths.

Figure 67. Carapace Total Carotenoids Effects of increasing parasite intensity on total carotenoid concentration of *E. analoga* females. Size class determined by carapace length. Differences between parasite load categories for each size class were analyzed using ANCOVA ($P=0.18$).

Figure 68. Carapace Astaxanthin Effects of increasing parasite intensity on astaxanthin concentration of *E. analoga* females. Size class determined by carapace length. Differences between parasite load categories for each size class were analyzed using ANCOVA ($P=0.88$).

Figure 69. Carapace Canthaxanthin Effects of increasing parasite intensity on canthaxanthin concentration of *E. analoga* females. Size class determined by carapace length. Differences between parasite load categories for each size class were analyzed using ANCOVA ($P=0.50$).

Figure 70. Carapace Diatoxanthin Effects of increasing parasite intensity on diatoxanthin concentration of *E. analoga* females. Size class determined by carapace length. Differences between parasite load categories for each size class were analyzed using ANCOVA ($P=0.64$).

Figure 71. Egg Total Carotenoids Effects of increasing parasite intensity on total carotenoid concentration of *E. analoga* females. Size class determined by carapace length. Differences between parasite load categories for each size class were analyzed using ANCOVA ($P=0.42$).

Figure 72. Egg Beta-Carotene Effects of increasing parasite intensity on beta-carotene concentration of *E. analoga* females. Size class determined by carapace length. Differences between parasite load categories for each size class were analyzed using ANCOVA ($P=0.78$).

Figure 73. Egg Astaxanthin Effects of increasing parasite intensity on astaxanthin concentration of *E. analoga* females. Size class determined by carapace length. Differences between parasite load categories for each size class were analyzed using ANCOVA ($P=0.53$).

Figure 74. Egg Canthaxanthin Effects of increasing parasite intensity on canthaxanthin concentration of *E. analoga* females. Size class determined by carapace length. Differences between parasite load categories for each size class were analyzed using ANCOVA ($P=0.20$).

Figure 75. Egg Diatoxanthin Effects of increasing parasite intensity on diatoxanthin concentration of *E. analoga* females. Size class determined by carapace length. Differences between parasite load categories for each size class were analyzed using ANCOVA ($P=0.07$).

Figure 76. Fall Ovary Total Carotenoids Effects of increasing parasite intensity on total ovarian carotenoid concentration of *E. analoga* females collected during fall. Size class determined by carapace length. Differences between parasite load categories for each size class were analyzed using ANCOVA ($P=0.62$).

Figure 77. Summer Ovary Total Carotenoids Effects of increasing parasite intensity on total ovarian carotenoid concentration of *E. analoga* females collected during summer. Size class determined by carapace length. Differences between parasite load categories for each size class were analyzed using ANCOVA ($P=0.25$).

Figure 78. Esophagus Total Carotenoids Effects of increasing parasite intensity on total esophagus carotenoid concentration of *E. analoga* females. Size class determined by carapace length. Differences between parasite load categories for each size class were analyzed using ANCOVA ($P=0.18$).

APPENDIX B: HPLC STANDARD CURVES