DISTRIBUTION AND FUNCTION OF THE HEMOLYMPH PROTEINS, HEMOECDYSIN AND HEMOCYANIN, IN RELATION TO THE MOLT CYCLE OF THE JUVENILE DUNGENESS CRAB, *CANCER MAGISTER*, AND SIZE-SPECIFIC MOLTING AND REPRODUCTIVE CAPABILITY OF THE ADULT FEMALE *CANCER MAGISTER*

by

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A THESIS

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An Abstract of the Thesis of

Clete A. Otoshi for the degree of Master of Science in the Department of Biology to be taken March 1994

Title: DISTRIBUTION AND FUNCTION OF THE HEMOLYMPH PROTEINS, HEMOECDYSIN AND HEMOCYANIN, IN RELATION TO THE MOLT CYCLE OF THE JUVENILE DUNGENESS CRAB, CANCER MAGISTER, AND SIZE-SPECIFIC MOLTING AND REPRODUCTIVE CAPABILITY OF THE ADULT FEMALE CANCER MAGISTER

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The Dungeness crab, Cancer magister, contains a non-respiratory hemolymph protein termed hemoecdysin. Hemolymph concentrations of this protein and the oxygen-carrying protein hemocyanin were quantitatively monitored through the molt cycles of juvenile crabs. Levels of hemoecdysin and hemocyanin were found to fluctuate greatly in correlation to the molt cycle. Native gel electrophoresis and SDS-PAGE were used to demonstrate that hypodermal tissue contained higher levels of hemoecdysin shortly after the molt than did other tissues. The hypodermis is known to synthesize new exoskeleton. Exoskeletal protein extraction and Western blotting techniques provided strong evidence that

hemoecdysin and hemocyanin function as integral exoskeletal proteins.

In addition, reproduction and molting studies were conducted on adult female C. magister. These experiments examined to what degree females with a carapace width ≥ 146 mm (recreational size limit) were still reproductive. Data showed that these females molted less frequently but retained the reproductive capability of the smaller crabs.

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TABLE OF CONTENTS

Chapter		Page
I.	HEMOECDYSIN AND HEMOCYANIN IN JUVENILE *CANCER MAGISTER*	1
	Introduction	1
	Materials and Methods	12
	Animal Maintenance	12
	Blood Sampling	13
	Tissue Extraction	14
	Exoskeletal Protein Extraction	15
	Native Gel Electrophoresis	16
	SDS Gel Electrophoresis	16
	A-5M Column Chromatography	17
	Electroelution	17
	Gel Analysis	18
	Western Blotting	20
	Results	21
	Discussion	58
	Summary	68
II.	REPRODUCTION AND MOLTING IN ADULT FEMALE *CANCER MAGISTER*	71
	Introduction	71
	Materials and Methods	75
	Crab Collection	75
	Crab Maintenance	76
	Data Collection	77
	Results	79
	Discussion	86
	Summary	90
III.	CONCLUDING SUMMARY	92
BIBLIOGRAPHY		93

LIST OF TABLES

Table		Page
1.	Data for Crab Size, Zoeal Hatch Date and Adult Death Date	80
2.	Data for Crab Size Before and After the Molt and Date of Death	82
3.	Data for Crab Size, Egg Extrusion Date and Final Egg Mass Size	84

LIST OF FIGURES

Figure		Page
1.	Typical Crustacean Exoskeleton and the Underlying Hypodermal Layer	3
2.	Changes in Integumentary Tissues from the Branchiostegite Region of the Land Crab Gecarcinus lateralis During a Molt Cycle	4
3.	Cumulative Data for Amounts of Cancer magister 25S Hemocyanin, Hemoecdysin and 16S Hemocyanin Measured in Java Scan Units	22
4.	Standard Curve Relating Java Scan Units to Micrograms of 25S Hemocyanin	23
5.	Cumulative Data for Amounts of Cancer magister 25S Hemocyanin, Hemoecdysin and 16S Hemocyanin Expressed as Milligrams Per Milliliter of Protein	24
6.	Individual Crabs Monitored for Hemolymph Levels of 25S Hemocyanin, Hemoecdysin and 16S Hemocyanin	26
7.	Time of Hemolymph Minimum Level, Start of Increase and Maximum Level of 25S Hemocyanin, Hemoecdysin and 16S Hemocyanin	35
8.	Hemolymph and Tissue Samples from a Crab Dissected 5-26 Hours After It had Molted	37
9.	Hemolymph and Tissue Samples from a Crab Dissected 2.5 Days After It had Molted	39
10.	Hemolymph and Tissue Samples from a Crab Dissected 4.5 Days After It had Molted	41

Page

11. Hemolymph and Tissue Samples from a Crab Dissected 6.5 Days After It had Molted 43

12. Hemolymph and Tissue Samples from a Crab Dissected 6.5 Days After It had Molted 45

13. Hemolymph and Tissue Samples from a Crab Dissected 7.5 Days After It had Molted 47

14. Western Blots of SDS-PAGE Separated Samples Using a Hemoecdysin Specific Primary Antibody 52

15. Western Blots of SDS-PAGE Separated Samples Incubated With Hemocyanin and Hemoecdysin Specific Primary Antibodies 54

16. Crab Size Distribution for Viability Study (I) 81

17. Crab Size Distribution for Molting Study (II) 83

18. Crab Size Distribution for Extrusion Study (III) 85

CHAPTER I

HEMOECDYSIN AND HEMOCYANIN IN JUVENILE CANCER MAGISTER

Introduction

It is estimated that there are 1.25 million described animal species. Approximately one million are arthropods (Kozloff, 1990). The complete numerical dominance of the arthropods leads one to wonder how such success was achieved. This extreme success is attributed, in large part, to a suite of adaptations which is referred to as arthropodization (Brusca and Brusca, 1990). The central adaptation of arthropodization was the formation of a hard, jointed exoskeleton. The exoskeleton gave these animals structural support as well as physical protection. It also provided physiological protection, functioning as a barrier against osmotic and ionic gradients. While the exoskeleton was extremely important in these regards it also created a problem in that it formed a barrier to the animal's growth. The arthropod's solution was to periodically molt its exoskeleton and create a new larger one. The relationship between the molt cycle of a crustacean, Cancer magister

Dana, the Dungeness crab, and its hemolymph proteins, will be the central focus of this chapter of the thesis.

A typical crustacean exoskeleton and underlying hypodermis layer is shown in cross-section, Figure 1. The hypodermis, which is sometimes referred to as the epidermis is, responsible for forming new exoskeleton. The four main layers of the exoskeleton are the epicuticle, exocuticle, endocuticle, and membranous layer. The epicuticle is composed of proteins, lipids and calcium salts, but no chitin (Travis, 1955; Welinder, 1975b; Stevenson, 1985). The exocuticle and endocuticle are calcified matrices of chitin and protein. The thin membranous layer contains chitin and protein but is not calcified.

The crustacean molt cycle was first subdivided into five distinct stages, A through E, by Drach in 1939 (Fig. 2). This staging was based on the hardness of specific regions of the exoskeleton. Then, in 1962, Skinner augmented these observations by correlating these changes with sequential structural changes in the hypodermis of the Bermuda land crab, Gecarcinus lateralis (Fig. 2). The following description of the physiological and morphological changes during each stage is modelled after a mature specimen of G. lateralis (Skinner, 1962).

Stage A and stage B are collectively termed metecdysis (Carlisle and Dohrn, 1953). These stages immediately follow ecdysis, or the molt, when the crab actually emerges from

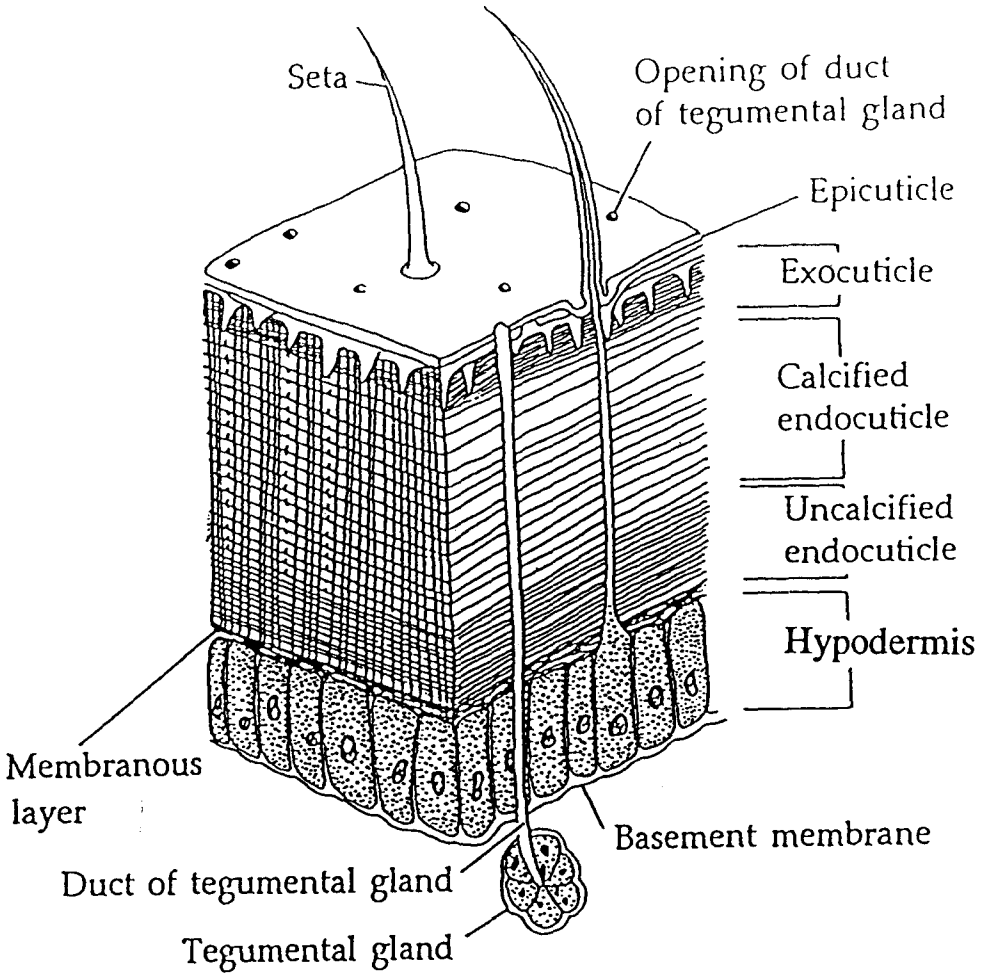


Figure 1. Typical crustacean exoskeleton and the underlying hypodermal layer (modified from Brusca and Brusca, 1990, p. 468.)

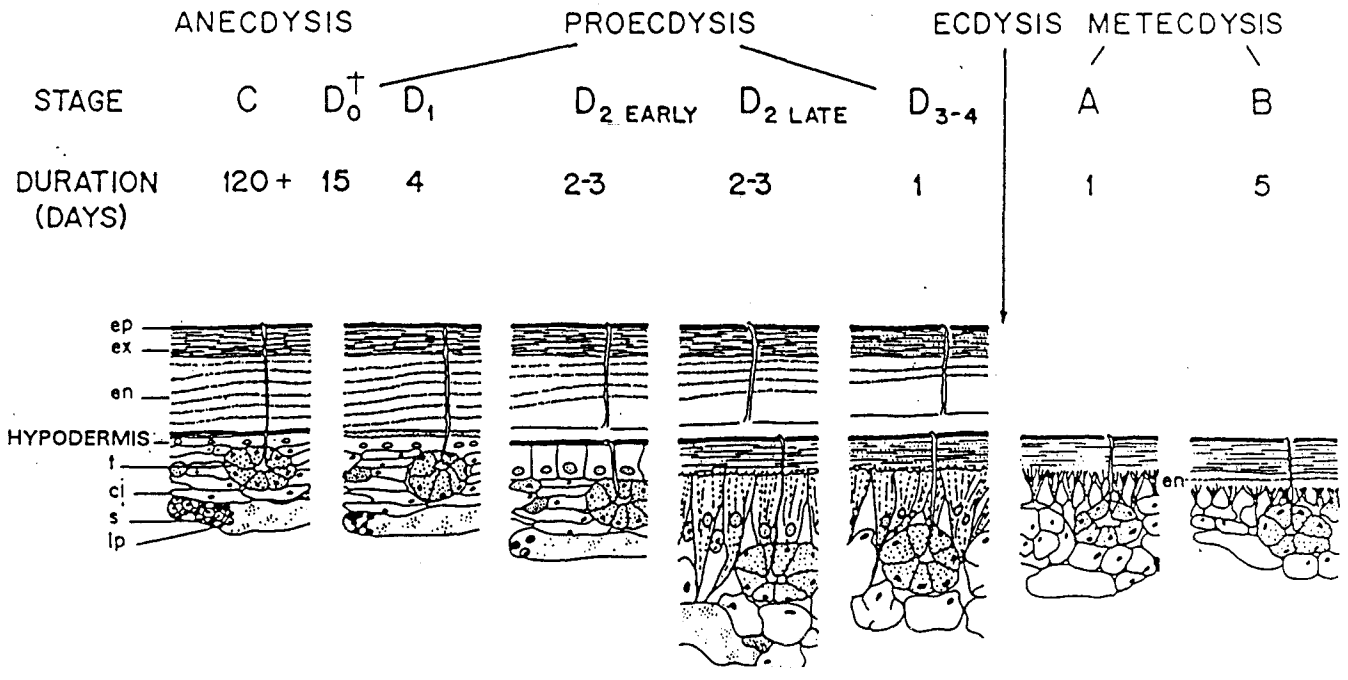


Figure 2. Changes in integumentary tissues from the branchiostegite region of the land crab Gecarcinus lateralis during a molt cycle. ep, epicuticle; ex, exocuticle; en, endocuticle; t, tegumental gland; cl, cell of Leydig; s, blood sinus; lp, lipoprotein cell (modified from Skinner, 1962, p. 644.)

the old exoskeleton. Stage A is characterized by a shrinking in the size of the hypodermal cells, dissolution of the gastrolith and ingestion of the exuviae, or old exoskeleton, by the crab. Stage B is distinguished by the initiation of endocuticle calcification and synthesis. Also, at some point during metecdysis the epicuticle and exocuticle are thought to harden by tanning (Vacca and Fingerman, 1975 a,b) and calcification (Travis, 1960). In stage C, muscle and endocuticle formation continue and membranous layer formation begins. Stage C_4 is a subdivision of stage C and is referred to as anecdysis. This stage lasts for approximately 150 days and is ucharacterized by a lack of physiological activity in the exoskeleton. Stage D, or proecdysis, signifies the beginning of the molting process again. Apolysis (Jenkin, 1966), or the separation of the hypodermis from the old exoskeleton, is the first sign of proecdysis. During this stage there is also gastrolith formation, regeneration of autotomized limbs, epicuticle formation, exocuticle formation and degradation of the old exoskeleton. All these events culminate in stage E, or ecdysis. By this time, the cast off old exoskeleton, or exuvia, has been hypodermally degraded by nearly 75% (reviewed in Skinner, 1985; Stevenson, 1985; Skinner et al., 1992). Upon emerging from its old exoskeleton, the crab is enclosed in a new, thin, pliable exoskeleton. Water uptake stretches the new

exoskeleton which consequently results in an expanded hemocoel (Bliss, 1963; Rao, 1968; Mykles, 1980). Because of this expansion, the animal after ecdysis is larger (Churchill, 1918). While there are species specific differences in the duration of the molt cycle, the major events and their sequence are very similar (Skinner, 1985).

Molting is a physiologically stressful process that requires a highly coordinated system at the molecular level. Proteins dissolved in the hemolymph are important in this regard. Hemocyanin is one such hemolymph protein which mainly functions as an oxygen transporter. This protein becomes extremely important before the molt, during stage D, when there is a 50 to 1900% increase in oxygen consumption by the whole animal (Poulson, 1935; Nyst, 1941; Scudamore, 1947; Edwards, 1950, 1953; Schneiderman, 1952; Bliss, 1953; Schneiderman and Williams, 1953).

The hemocyanin molecule contains two copper atoms at its active site and appears blue when the copper atoms become oxygenated. The basic building blocks of arthropod hemocyanin are heterogenous subunits of approximately 75,000 daltons with sedimentation coefficients of about 5S (Eriksson-Quensel and Svedberg, 1936; Van Holde and Van Bruggen, 1971). Six of these subunits self assemble to form hexameric molecules, and these hexamers can further associate to form two hexamer, four hexamer, six hexamer and eight hexamer molecules. Brachyuran crustaceans normally

have hexamer and two hexamer hemocyanins with sedimentation values of 16S and 25S, respectively (Van Holde and Miller, 1982). Hemocyanin is also found among the chelicerates. Only horseshoe crabs have eight hexamer (60S) aggregates. Most arachnids have four hexamer (33-37) aggregates, except for spiders like Cupiennus salei which has hexamer and two hexamer hemocyanin (Markl et al., 1979). Many molluscs also have a hemocyanin that shows amino acid content homology to the arthropod hemocyanin (Ghiretti-Magaldi et al., 1975) but varies greatly in structure from arthropod hemocyanin. The molluscan hemocyanin subunit is a large polypeptide chain of about 400,000 daltons. It is made up of 7 or 8 covalently linked 50,000 dalton units each containing two copper atoms that cooperate in the binding of one molecule of oxygen (Lontie et al., 1973; Brouwer et al., 1976). The subunits assemble into giant cylindrical molecules with molecular weights of up to 9,000,000 daltons.

A number of arthropods also produce substantial amounts of hemolymph proteins which are structurally similar to hemocyanin but do not act as oxygen carriers. Such proteins in crustaceans have remained largely uncharacterized. Conversely, in many insects these proteins have been well studied and have been shown to function in many ways. In a 1991 review, Telfer and Kunkel proposed the generic term "hexamerin" to refer to all of the approximately 500-kd hexamers of arthropods. This would include the hemocyanins

(VanHolde and Miller, 1982; Linzen et al., 1985), the insect storage hexamers (Scheller and Zimmerman, 1983; Levenbook 1985; Kanost et al., 1990; Telfer and Kunkel, 1991), and the less well recognized nonrespiratory hexamers in the hemolymph of Crustacea. Also included were the unusual hexamers with no antigenic similarity to the storage proteins that occur in great abundance in the hemolymph of several Lepidopteran insects.

Of the hexamerins, insect storage hexamers have been the most thoroughly studied and have been shown to play an important role in molting, metamorphic and reproductive processes. The insect storage hexamerins have been categorized depending on their methionine content and aromatic amino acid (tyrosine and phenylalanine) content (reviewed in Telfer and Kunkel, 1991). One such group that has been thoroughly studied are the arylphorins. Arylphorins (Telfer et al., 1983) have an aromatic amino acid content of more than 15 %. Calliphorin is one such protein that was originally isolated from extracts of Calliphora erythrocephala (Munn and Greville, 1969). Calliphorin also has a methionine content of more than 4% categorizing it as a methionine-rich protein as well. Such aromatic amino acid content and methionine content is more than twice the average for typical polypeptides as determined by King and Jukes (1969).

In 1984 Levenbook and Bauer showed that calliphorin, in

Calliphora vicina, was incorporated into adult tissue. Larvae were injected with ^{14}C-phenylalanine-labelled calliphorin, and the label was found to be widely distributed, with 46.5% in the thoracic muscles, 10.8% in the insoluble components of the exoskeleton, and lesser amounts in other tissue. Other studies have shown arylphorins to be incorporated into solvent soluble parts of the exoskeleton (Koeppe and Gilbert, 1973; Phillips and Loughton, 1976; Scheller et al., 1980; Kaliafas et al., 1984; Konig et al., 1986) and solvent insoluble sclerotized parts of the exoskeleton (Grun and Peter, 1983; Grun and Peter, 1984). In addition to these structural functions, insect hexamerins have been postulated to function as compounds that are metabolized for energy (Telfer and Kunkel, 1991), and as carrier molecules of riboflavin (Telfer and Massey, 1987), molting hormone (Enderle et al., 1983) or xenobiotics (Haunerland and Bowers, 1986).

In 1987 Telfer and Massey compared the amino acid contents of certain insect storage hexamerins to hemocyanin. Similarities such as high aromatic amino acid content and low glycine, alanine and cysteine contents were found. In 1989, Willott et al. compared amino acid sequences of Manduca sexta (tobacco horn worm) arylphorin, Panulirus interruptus (spiny lobster) hemocyanin, Eurypelma californicum (tarantula) hemocyanin and Limulus polyphemus (horseshoe crab) hemocyanin. They determined that

arylphorin was as homologous to lobster hemocyanin as lobster hemocyanin was to the other hemocyanins. They further concluded that these two classes of proteins had evolved from a common ancestor.

There have also been studies which sought to determine relatedness among crustacean and insect exoskeletal proteins. Dennell, in 1947, showed similarities between the crustacean exoskeleton and the insect exoskeleton with respect to composition, deposition and hardening. Many insect and crustacean exoskeletal proteins have also been shown to share characteristics such as acidic isoelectric points and molecular weights of 31,000 daltons or smaller (O'Brien and Skinner, 1991). More recently, immunological crossreactivity between crustacean exoskeletal proteins and insect storage hexamerins has been demonstrated (Stringfellow and Skinner, 1988; Kumari and Skinner, 1993).

A protein has been identified in the hemolymph of the Dungeness crab, Cancer magister, that may be structurally and functionally homologous to the insect storage hexamerins. The molecule has an apparent molecular weight of 450,000 daltons, a $S_{20,w}$ of 16S and transmission electron microscopy shows that it has a hexameric shape (Terwilliger, 1992). Because this protein is found at high concentrations in the hemolymph and because it seems to be somehow functionally related to ecdysis it will be referred to as hemoecdysin. Hemoecdysin has been observed to reach

hemolymph concentrations comparable to hemocyanin. Hemoecdysin will be the focus of this study, which is divided into three parts examining different aspects of this protein in relation to the molt cycle of the crab.

One interesting initial observation was that the amount of hemoecdysin in the hemolymph was not constant. The first part of this study was to quantify these fluctuations in concentration during the crab's molt cycle. Fluctuations in 25S (two hexamer) hemocyanin and 16S (hexamer) hemocyanin were also monitored. Quantification of the amount of hemoecdysin throughout the molt cycle should give insight about the functional role of this protein.

The second part of this study sought to explore the function of the hemoecdysin. Approximately 30-50% of the exoskeletons of arthropods are composed of protein (Kumari and Skinner, 1993) and as previously mentioned, many insect storage hexamerins seem to be incorporated into the exoskeleton. A main objective was to determine whether hemoecdysin also has this structural function. Since the hypodermis (Fig. 1) is responsible for the secretion of the new exoskeleton, it would be an important tissue to examine. If the hemoecdysin were incorporated into the exoskeleton, one might expect higher concentrations of this protein in the hypodermis some time around the molt, when the new exoskeleton was being formed. To examine this hypothesis, the hypodermis was dissected from crabs at certain stages of

the molt cycle, and the amount of hemoecdysin contained was qualitatively determined.

The third part of this study sought to show direct evidence for the incorporation of the hemoecdysin into the exoskeleton. Proteins were extracted from exoskeleton and reacted with hemoecdysin specific antibodies to determine whether hemoecdysin does in fact have this structural function. In addition, the extracted proteins were reacted with hemocyanin specific antibodies to examine the possibility that hemocyanin may also be incorporated into the exoskeleton.

Materials and Methods

Animal Maintenance

Megalopa of Cancer magister (Dana) were collected with dip nets from the boat docks in Coos Bay, Oregon, during the first week of May, 1992. The megalopa metamorphosed to juvenile crabs and were raised in the laboratory. The juvenile crabs were housed separately in modified one quart plastic containers. Nylon mesh was hot glued over two inch square holes cut from two opposite sides of the containers. A glass weight was placed inside each container, and the containers were kept in a three inch deep water table supplied with running seawater pumped from the mouth of Coos

Bay on an incoming tide. Several airstones were placed throughout the water table. The crabs were fed chunks of mussel, Mytilus californicanus, three times a week. Unless otherwise specified, juvenile crabs with carapace widths (measured from within the tenth anterolateral spine) from 20 mm to 50 mm were used for all of the following experiments.

Blood Sampling

Needles were made by pulling apart 10 microliter glass pipettes over a flame to generate a fine point. A needle was attached to a length of 0.86 mm polyethylene tubing and then inserted into the arthrodial membrane between the fourth and fifth walking legs of a crab. The blood sample was collected by drawing the blood (sometimes it was necessary to orally suck) into the needle and tubing. Initially, ten crabs were randomly selected to monitor on a regular basis. Later, seven more crabs were monitored in the same manner for varying lengths of time. Six microliter samples were taken daily from each of the monitored crabs, and 1.5 μl of glycerin was added to each sample. Seven microliters of each of these samples were loaded into wells of a twenty well gel. Native gel electrophoresis (see below) was completed on the blood samples that same day.

Tissue Extraction

The juvenile crabs were chilled for 10 minutes on ice before dissection. All of the following steps were also completed on ice. Heart, hepatopancreas, hypodermis and leg muscle tissue were removed. Each tissue was immediately put into a separate 1 ml aliquot of column buffer (0.05 M Tris-HCl, pH 7.5, 0.1 M NaCl, 0.01 M $MgCl_2$, 0.01 M $CaCl_2$) containing 1.7 mM phenylmethanesulfonyl fluoride, a serine protease inhibitor (the PMSF was first dissolved in ethanol before being added to the solution). In order to rinse other tissues and especially contaminating hemolymph from the desired tissue, each sample was placed into another vial containing 20 ml of fresh column buffer. The samples were gently shaken for 30 minutes at room temperature. This washing step was repeated one more time. The tissues were weighed and transferred to 1.7 ml micro-centrifuge tubes that contained 50 μl of column buffer plus PMSF. The amount of tissue used for all dissections was as follows: hepatopancreas, 200 mg; hypodermis, 40 mg; heart, 20 mg; and muscle, 100 mg. Each sample was then homogenized to a fine slurry and spun, at 4° C, in a Brinkmann microcentrifuge for 2 minutes at 13,000 rpm. The supernatants were immediately prepared for native pH 7.4 and denaturating sodium dodecyl sulfate (SDS) gel electrophoresis (see below). Native gel electrophoresis was carried out that same day or the

following day. SDS samples were stored at -20° C until analyzed by SDS-PAGE.

Exoskeletal Protein Extraction

The dorsal carapace portion of the exoskeleton was always used so that contaminants such as hair bristles could be excluded. The exoskeleton was brushed clean and rinsed of any visible cellular material with deionized water. It was then dried at approximately 60° C until constant weight was achieved. The dried exoskeleton was then ground with mortar and pestle at room temperature until it was a fine powder. The proteins from the ground exoskeleton were then extracted with a 1% KCl solution, pH 7.5, containing 1 mM PMSF using 12 ml solution per gram of ground exoskeleton. The extraction mixture was incubated overnight, on a shaker, in a 4° C cold room. The mixture was then centrifuged, at 4° C, in a Brinkmann microcentrifuge for 5 minutes at 13,000 rpm. Millipore Spectrapor 4 dialysis tubing with a molecular weight cut off of 12,000 to 14,000 was prepared for use by placing the membrane in boiling deionized water with a pinch of EDTA for a few minutes. The membrane was then stored at 4° C until it was needed. The extraction supernatant was dialyzed against a 0.1 M Tris-HCl, pH 7.6 buffer at 4° C (4 changes, 1 L). A buffer change was completed only after a minimum of 6 hours had passed. The

dialyzed samples were then prepared for SDS gel electrophoresis.

Native Gel Electrophoresis

Native polyacrylamide gel electrophoresis (PAGE) in the absence of denaturants or reducing agents was performed as described by Davis (1964) and modified by Terwilliger and Terwilliger (1982). Slab gels, 5% acrylamide, (13 x 11 x 0.0015 cm) were made using 37:1 acrylamide to bisacrylamide. Electrophoresis was carried out at pH 7.4 using a 0.05 M Tris-Hcl/Tris-maleate buffer system at room temperature. Glycerin was added (20 %, vol./vol.) to all samples. A current of 35 mA was applied for 2.5-4 hours depending on resolution desired. The gels were stained in Coomassie brilliant blue R (Fairbanks et al., 1971) and destained in 10% acetic acid.

SDS Gel Electrophoresis

Samples to be analyzed by SDS polyacrylamide gel electrophoresis (SDS-PAGE) were diluted 1:1 in "incubation buffer" containing 0.1 M Dithiothreitol (DTT), 2.8 mM PMSF, 3.3% SDS, 1.6 mM Ethylene diaminetetraacetic acid (EDTA), 16% glycerin, 0.1 M Tris-Hcl (pH 6.8) and 0.016% bromphenol blue and heated immediately for 1.5 minutes at 100° C. SDS

polyacrylamide gel electrophoresis was carried out following the discontinuous system described by Laemmli (1970). All SDS gels were 7.5% polyacrylamide slab gels (13 x 11 x 0.0015 cm) with approximately 3 cm of stacking gel. A constant 100 V was applied, at room temperature, for 3.5-4 hours depending on the resolution desired. The gels were stained in Coomassie brilliant blue R (Fairbanks et al., 1971) and destained in 10% acetic acid (v/v).

A-5M Column Chromatography

A 115 cm x 2 cm glass column was set up in a 4° C coldbox. The stationary phase of the column consisted of Bio-Gel agarose beads with a pore size that separated molecules with molecular weights between 10,000 and 5,000,000 daltons (BioGel A-5M). Column buffer (see tissue extraction section) was used to elute a sample through the column. An ISCO fraction collector was used to collect the fractions dropwise into test tubes (2.8 ml/ test tube). The rate of flow through the column was always approximately 1 drop every 40 seconds. Fractions were analyzed spectrophotometrically for A_{280} and A_{340}.

Electroelution

Electroelution was used to isolate hemoecdysin. Cancer

magister hemolymph, combined with 20% glycerin, was loaded onto a one well, 5% polyacrylamide gel and native pH 7.4 PAGE was conducted. After electrophoresis, approximately 1 cm strips were cut from the two sides of the gel, Coomassie stained and destained. The strips were then realigned with the gel. The hemoecdysin band was excised using the stained strips as a guide. The hemoecdysin strip was cut into small pieces and the protein was electroeluted from the gel pieces using Schleicher and Schuell's Elutrap system. The buffer used contained: 0.025 M maleic acid, 0.025 M NaOH and 0.025 M Tris-OH, pH 7.4. The Elutrap was run overnight in a 4° C cold room at a constant 120 V.

Gel Analysis

The native gels were analyzed using Jandell's Java software package. This system allowed quantification of the density of the desired protein band by directly scanning the gel so that comparisons of protein bands between different gels could be made. Fluctuations in densities of hemoecdysin, 25S hemocyanin and 16S hemocyanin bands were all quantified using this scanning technique. A dried, transparent gel was placed on a light table and a Pulnix TM-7CN camera with an AF Micro Nikkor 60 mm lens was mounted 29 mm above the gel and light table. The lens F-stop was set at 16. This camera and lens setup was linked via a single

multi-conductor cable to a Sanyo Hi-resolution HR4512 black and white monitor. The Sanyo monitor was connected to an HD 486 computer. Using this setup, the dried gel image was transmitted on to the Sanyo monitor. The Java program allowed the scanning of a specific protein band that appeared as part of the gel image. This scanning technique yielded a number which was correlated to the density of staining of the protein band. This arbitrary number was based on the way the program was calibrated. The numerical values will be referred to as Java scan units. The values of the Java scan units ranged from 0 to 1,615.

A standard curve was prepared using BioGel A-5M column purified Cancer magister 25S hemocyanin so that Java scan units could be correlated to micrograms of protein. Concentration was determined using the A_{340} (wavelength at which oxyhemocyanin absorbs) of the purified 25S hemocyanin and the A_{340} extinction coefficient (Nickerson and Van Holde, 1971). A range of known amounts of 25S hemocyanin was loaded on to a 5% polyacrylamide gel and native gel electrophoresis was carried out. A standard curve was then generated by analyzing the resulting bands with the Java gel scanning program. The 25S hemocyanin standard curve was also used for 16S hemocyanin and hemoecdysin. Hemoecdysin and 16S hemocyanin standard curves were not created because of the difficulty in isolating large enough amounts of these proteins.

Western Blotting

Monoclonal and polyclonal antibodies were developed at the University of Oregon's monoclonal antibody facility. Hemolymph, tissue samples and extracted exoskeletal proteins were prepared for SDS-PAGE as described above. The samples were electrophoresed into 12 well 7.5% slab gels for 4 hours. Completed gels and two Millipore Immobilon polyvinylidene difluoride (PVDF) blotting membranes were cut into 10.2 cm x 7.3 cm rectangles. The membranes were prewetted in 100% methanol for approximately 3 minutes. Both the SDS gels and the membranes were incubated for 15 minutes in "transfer buffer" which contained 2.5 mM Tris-HCl, pH 8.3, 20% methanol, 19 mM glycine, 0.01% SDS and 10 mM EDTA. The proteins from the gels were then blotted onto the PVDF membranes using the Biorad minigel blotting apparatus. Transfer buffer was used for the blot which was run for one hour at 200 mA at room temperature. The protein blotted membranes were removed and blocked in phosphate buffered saline (PBS) (0.14 M NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4*7H_2O$ and 1.5 mM KH_2PO_4) containing 5% nonfat milk, pH 7.3, on a shaker for 1 hour at room temperature. The blots were then reacted, in specially designed trays, with primary antibodies raised against hemoecdysin and hemocyanin. These

antibodies were diluted in PBS plus 5% nonfat milk and were incubated on a shaker, for 2 hours, at room temperature. The blots were washed and then reacted with the secondary goat-anti mouse-biotin antibody complex diluted 1:5,000 in PBS plus 5% milk. This incubated was completed on a shaker, for 2 hours, at room temperature and then washed. Streptavidin-alkaline phosphatase diluted 1:1,000 in Tris Tween buffered saline (TTBS) (20 mM Tris-HCl, 0.5 M NaCl and 0.2% Polyoxyethylene sorbitanmonolaurate (Tween 20)) was reacted with the blots on a shaker for 1 hour at room temperature. After this reaction the blots were washed in TTBS and then 0.1 M Tris-HCl (pH 9.5). The final reaction was completed using Zymed's BCIP/NBT stains. Each blot was incubated with gentle shaking for up to 5 minutes, depending on the desired degree of stain.

Results

Apparent changes in the amounts of 25S hemocyanin, hemoecdysin and 16S hemocyanin in the hemolymph of juvenile Cancer magister that occur during molting are shown in Figure 3. Blood from three crabs were monitored daily from 5.5 days premolt until 5.5 days postmolt; blood samples from an additional nine crabs were collected from the day of molting (day zero) to 5.5 days postmolt. Since it is not possible to predict the exact day when a C. magister will

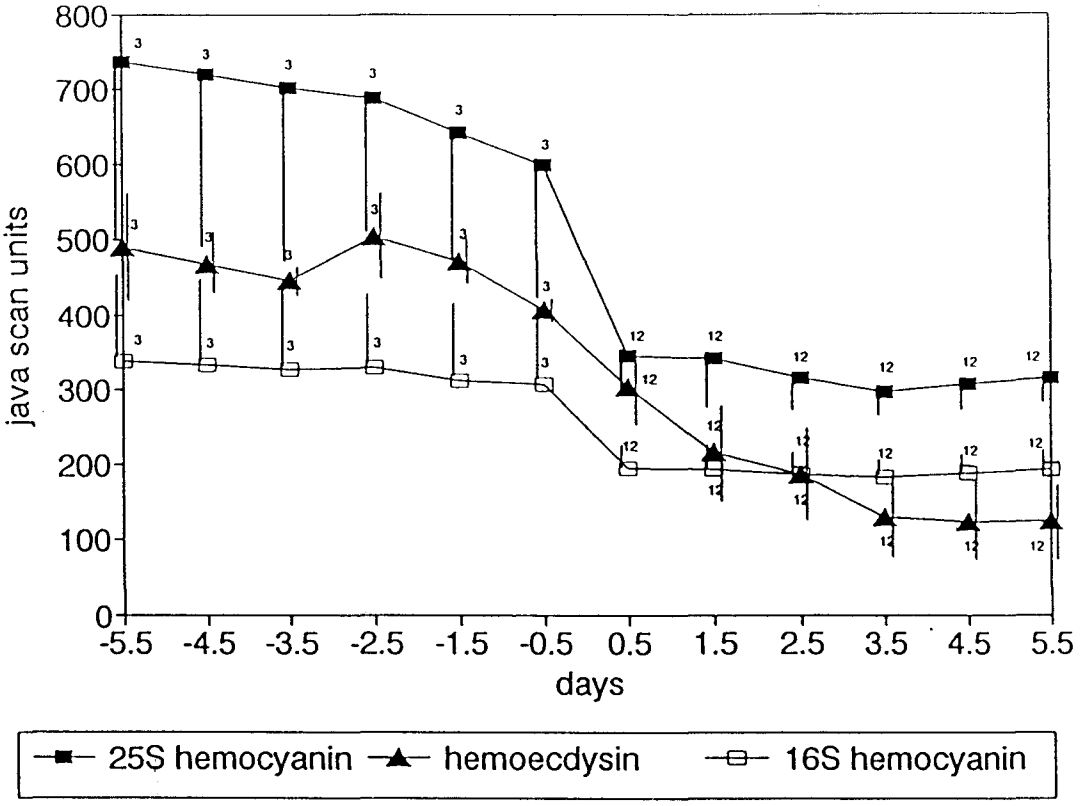


Figure 3. Cumulative data for amounts of Cancer magister 25S hemocyanin, hemoecdysin and 16S hemocyanin measured in java scan units. Crab hemolymph sampled from 5.5 days before a molt (n=3) until 5.5 days after a molt (n=12) occurring at day zero.

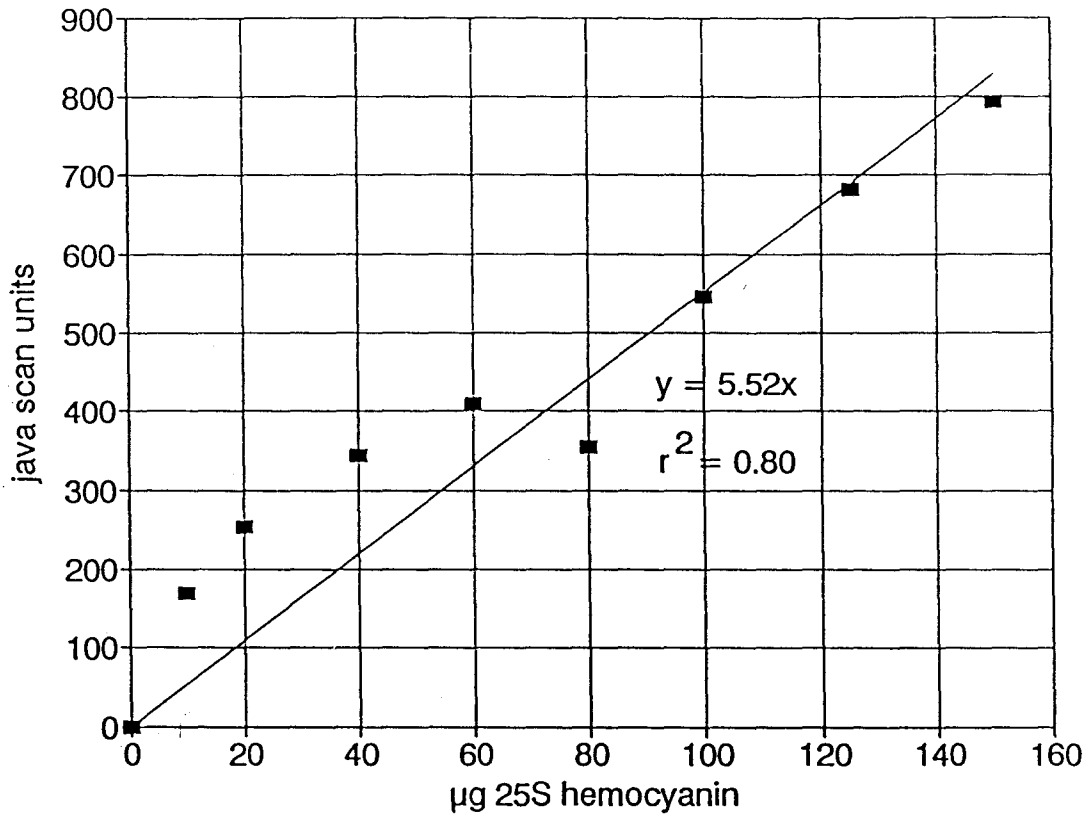


Figure 4. Standard curve relating java scan units to micrograms of 25S hemocyanin.

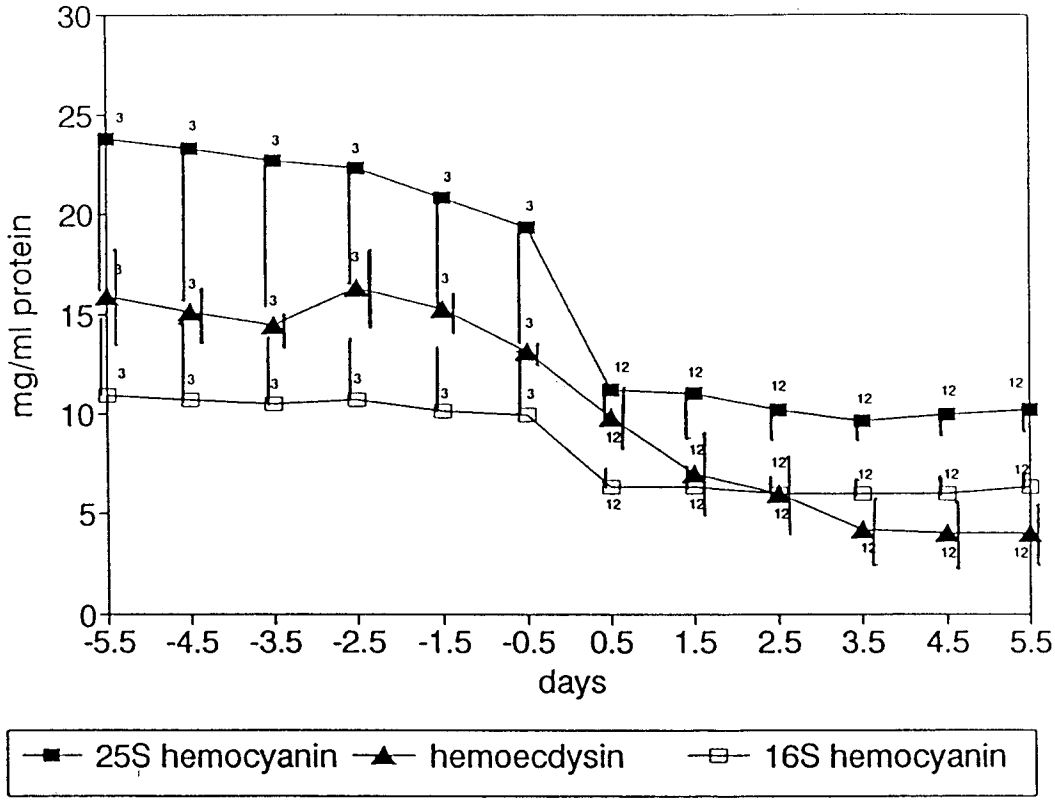


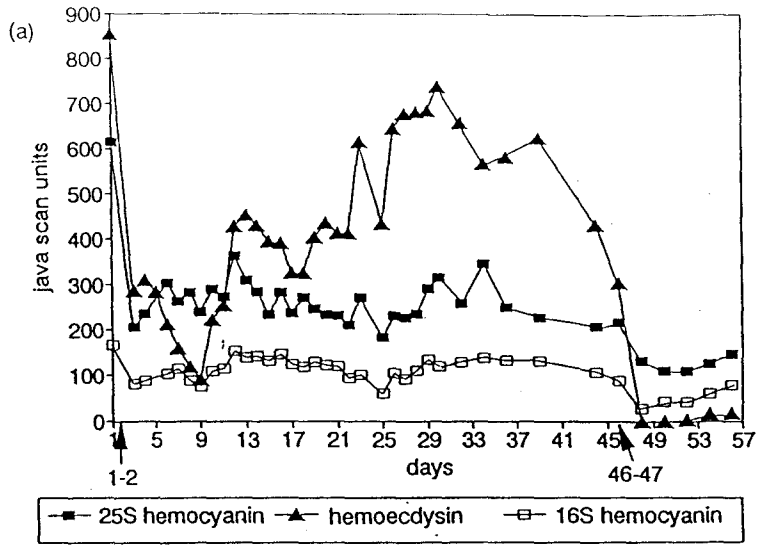
Figure 5. Cumulative data for amounts of Cancer magister 25S hemocyanin, hemoecdysin and 16S hemocyanin expressed as milligrams per milliliter of protein (from the regression in Fig. 4). Crab hemolymph sampled from 5.5 days before a molt (n=3) until 5.5 days after a molt (n=12) occurring at day zero.

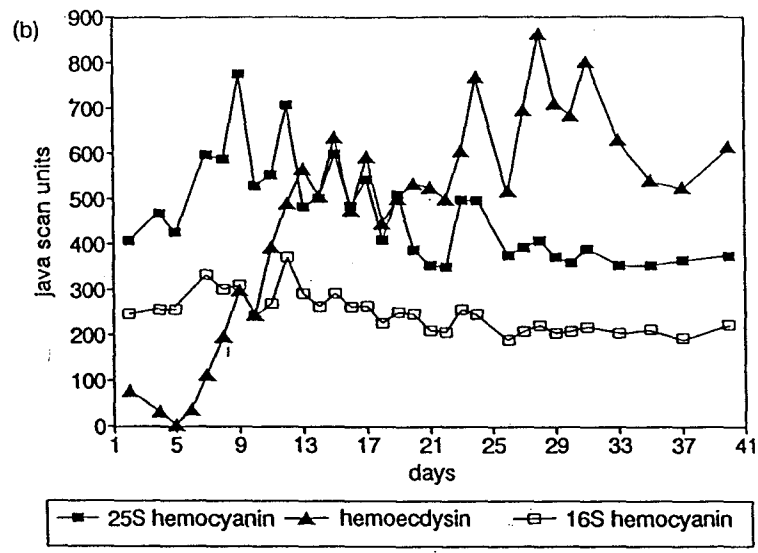
molt, there are fewer pre-molt data points.

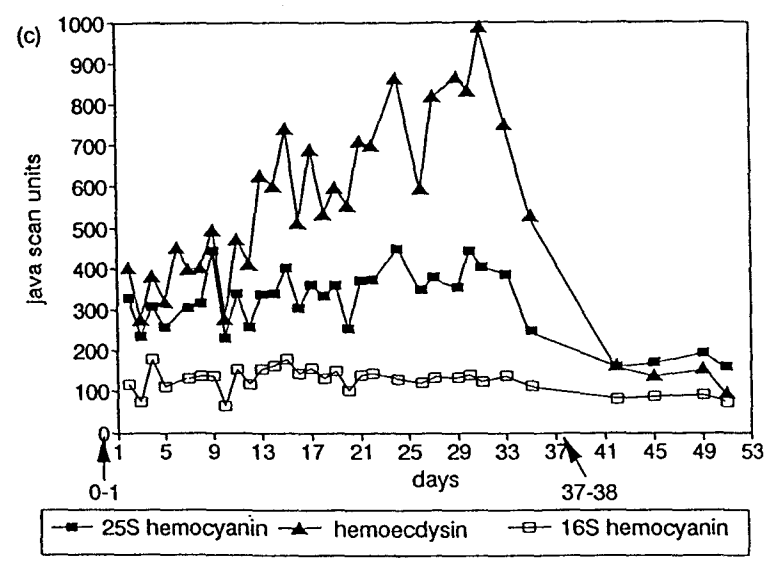
Figure 4 shows a standard curve relating java scanunits to micrograms of protein that was created using Biogel A-5M isolated 25S hemocyanin. A regression was made using only the first eight data points. The data above 150 μg protein appeared to have a non-linear relationship and were therefore not used in the regression. The 25S hemocyanin standard curve was used to convert java scan units (Figure 3) to micrograms of protein for 25S hemocyanin, 16S hemocyanin and hemoecdysin as seen in Figure 5. It is possible that hemoecdysin and hemocyanin have different affinities for Coomassie dye. If so, then using the 25S hemocyanin standard curve to convert hemoecdysin java scan units to micrograms of hemoecdysin would yield skewed results. The hemoecdysin amino acid content, however, has been found to be very similar to that of hemocyanin (N. Terwilliger, personal communication), particularly with respect to histidine, arginine and lysine (hemoecdysin = 16.0 residues/100 residues; hemocyanin = 16.2 residues/100 residues), the amino acids believed to be strongly involved in the Coomassie reaction. This would suggest that the affinity of hemoecdysin for Coomassie dye is comparable to that of hemocyanin.

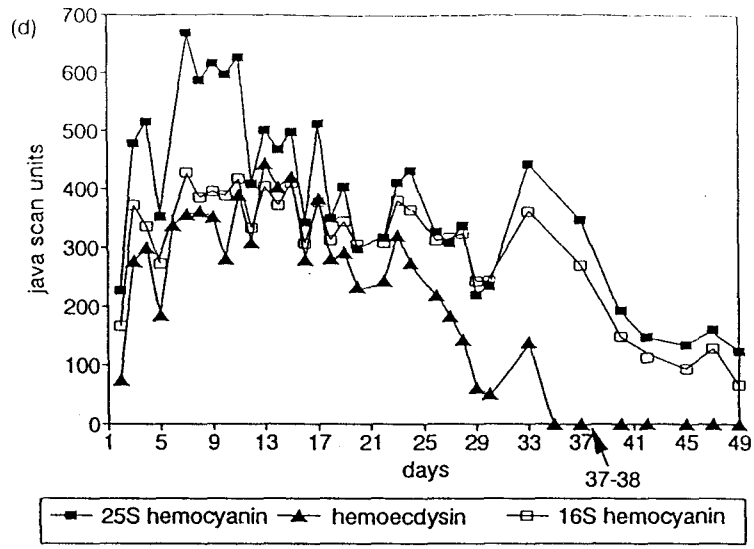
Figures 6a-q show hemolymph protein monitoring data from 17 crabs, with each graph representing an individual crab. The arrows on the x-axis are indicators of a molt;

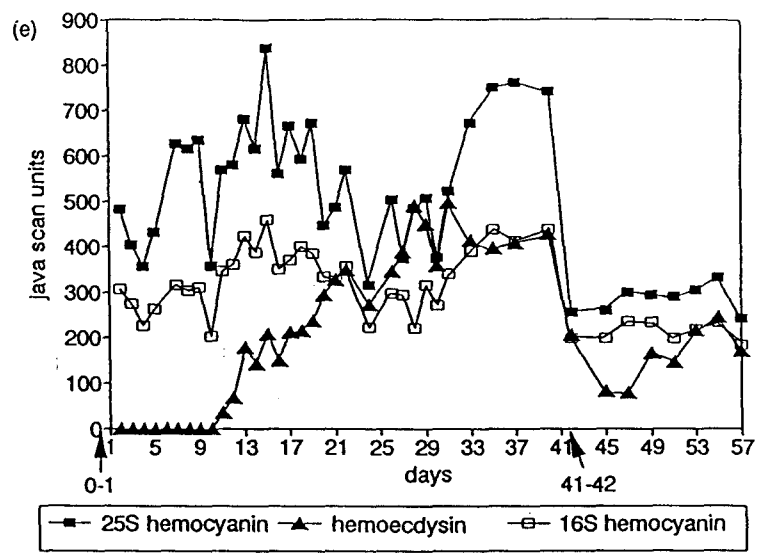
Figure 6. Individual crabs (a-q) monitored for hemolymph levels of 25S hemocyanin, hemoecdysin and 16S hemocyanin. Arrow on x-axis indicates a crab molt; numbers under the arrow represent days between which the molt occurred.

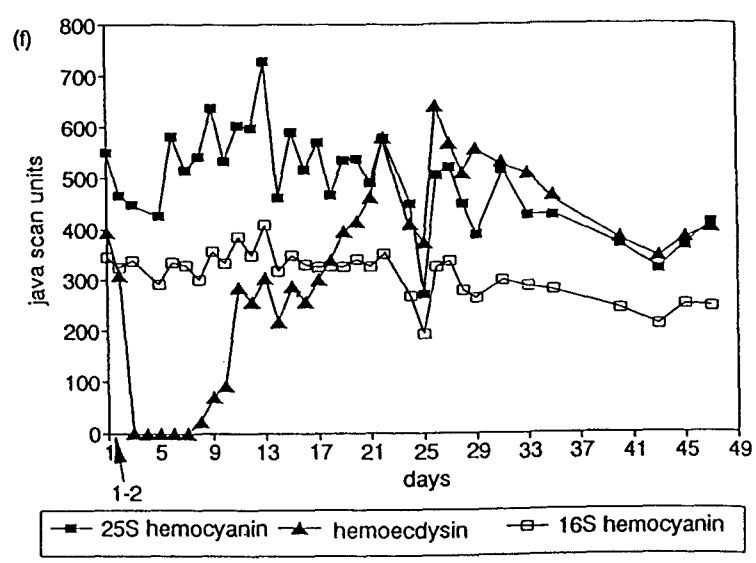

(a)
java scan units
900
800
700
600
500
400
300
200
100
0
1 5 9 13 17 21 25 29 33 37 41 45 49 53 57
days
1-2
46-47
25S hemocyanin
hemoecdysin
16S hemocyanin

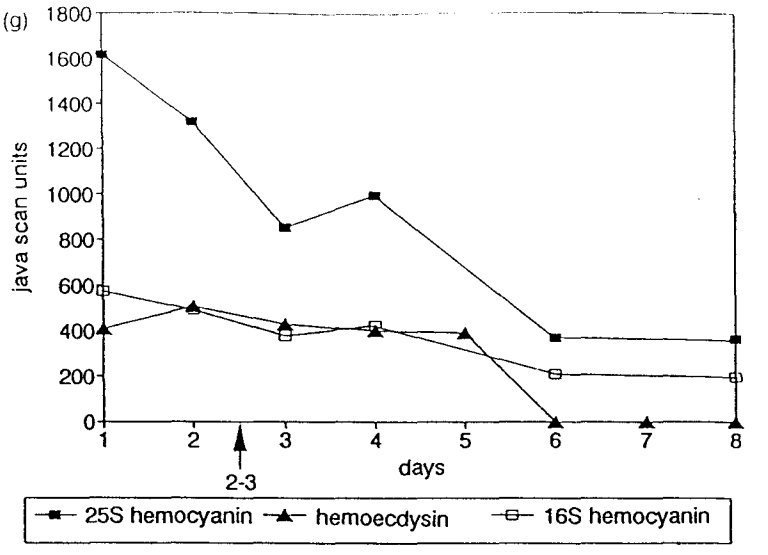

(b)
java scan units
900
800
700
600
500
400
300
200
100
0
1 5 9 13 17 21 25 29 33 37 41
days
25S hemocyanin
hemoecdysin
16S hemocyanin

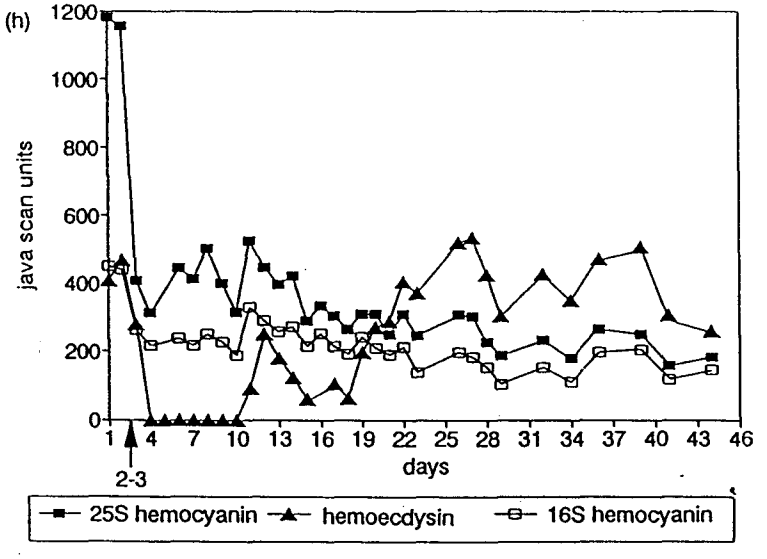

(c)
java scan units
1000
900
800
700
600
500
400
300
200
100
0
1 5 9 13 17 21 25 29 33 37 41 45 49 53
days
0-1
37-38
25S hemocyanin
hemoecdysin
16S hemocyanin

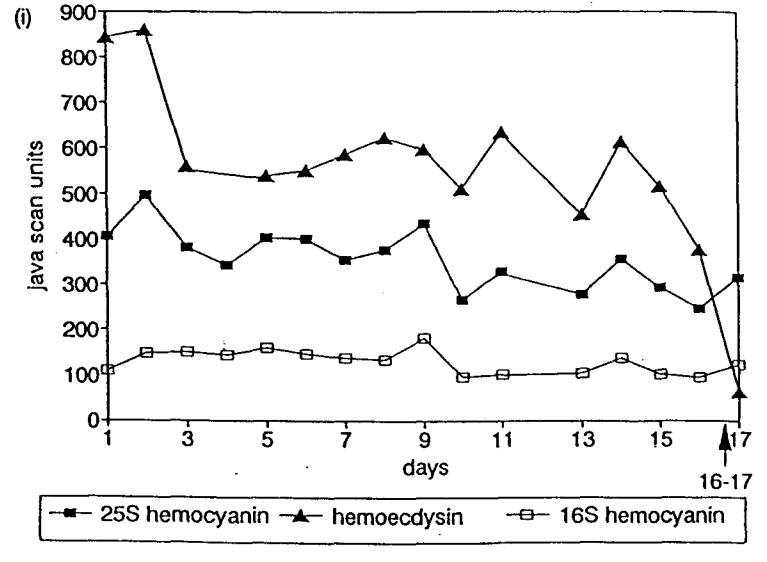

(d)
java scan units
days
37-38
25S hemocyanin
hemoecdysin
16S hemocyanin

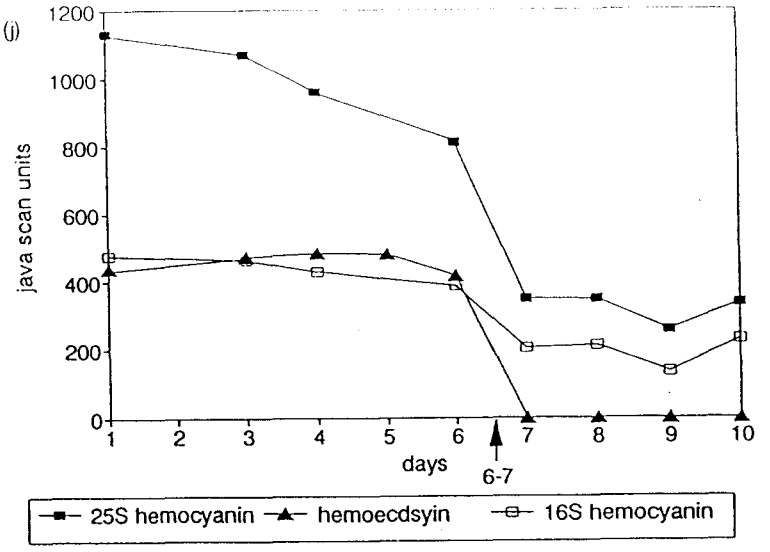

(e)
java scan units
days
0-1
41-42
25S hemocyanin
hemoecdysin
16S hemocyanin

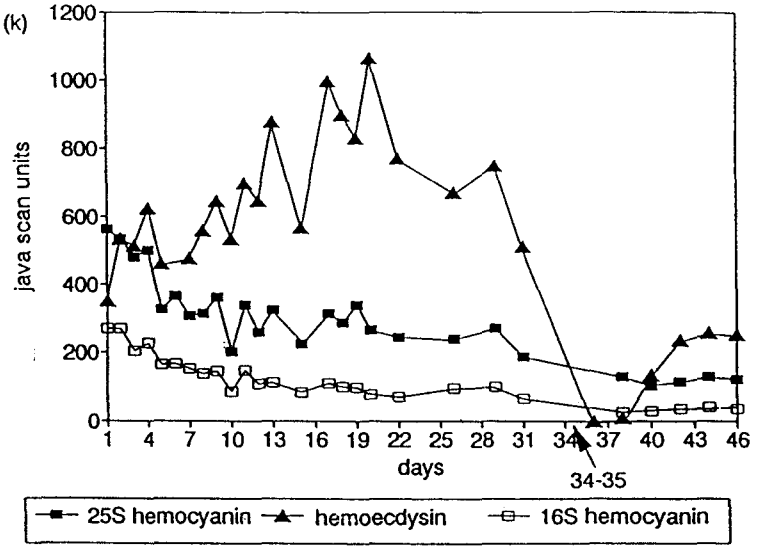

(f)
java scan units
days
1-2
25S hemocyanin
hemoecdysin
16S hemocyanin

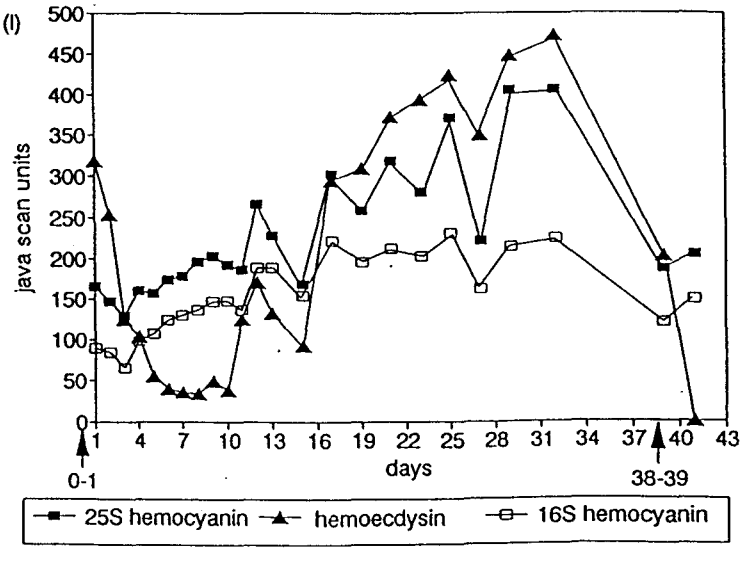

(g)
java scan units
1800
1600
1400
1200
1000
800
600
400
200
0
1
2
3
4
5
6
7
8
days
2-3
25S hemocyanin
hemoecdysin
16S hemocyanin

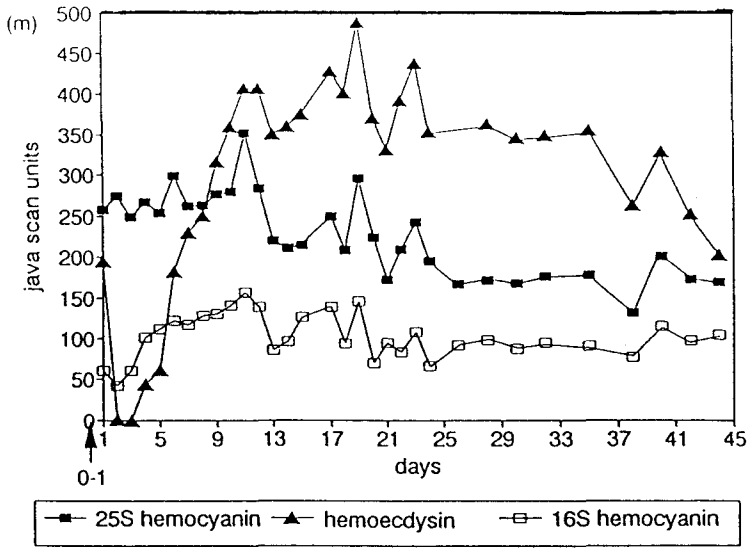

(h)
java scan units
1200
1000
800
600
400
200
0
1
4
7
10
13
16
19
22
25
28
31
34
37
40
43
46
days
2-3
25S hemocyanin
hemoecdysin
16S hemocyanin

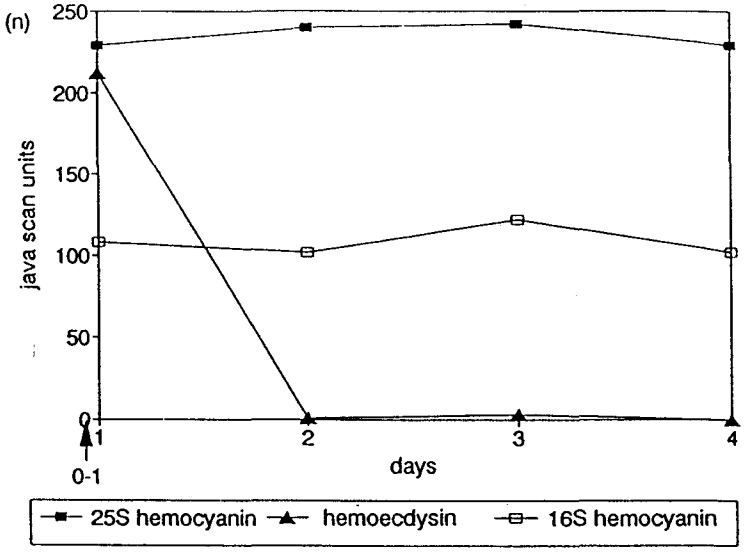

(i)
java scan units
900
800
700
600
500
400
300
200
100
0
1
3
5
7
9
11
13
15
17
days
16-17
25S hemocyanin
hemoecdysin
16S hemocyanin

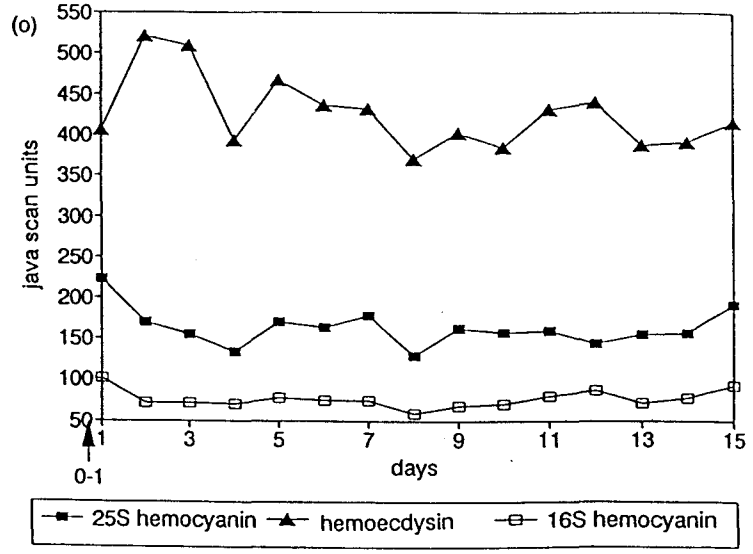

(j)
java scan units
1200
1000
800
600
400
200
0
1 2 3 4 5 6 7 8 9 10
days
6-7
25S hemocyanin
hemoecdsyin
16S hemocyanin

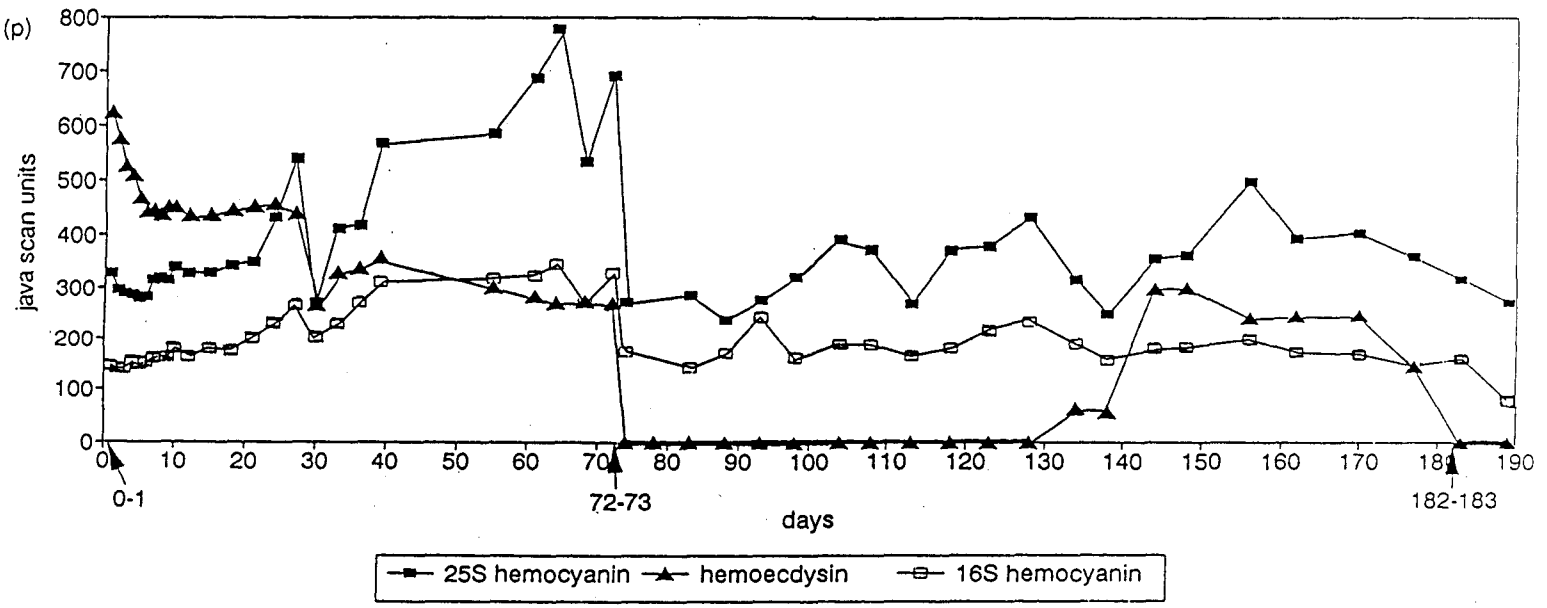

(k)
java scan units
1200
1000
800
600
400
200
0
1 4 7 10 13 16 19 22 25 28 31 34 37 40 43 46
days
34-35
25S hemocyanin
hemoecdysin
16S hemocyanin

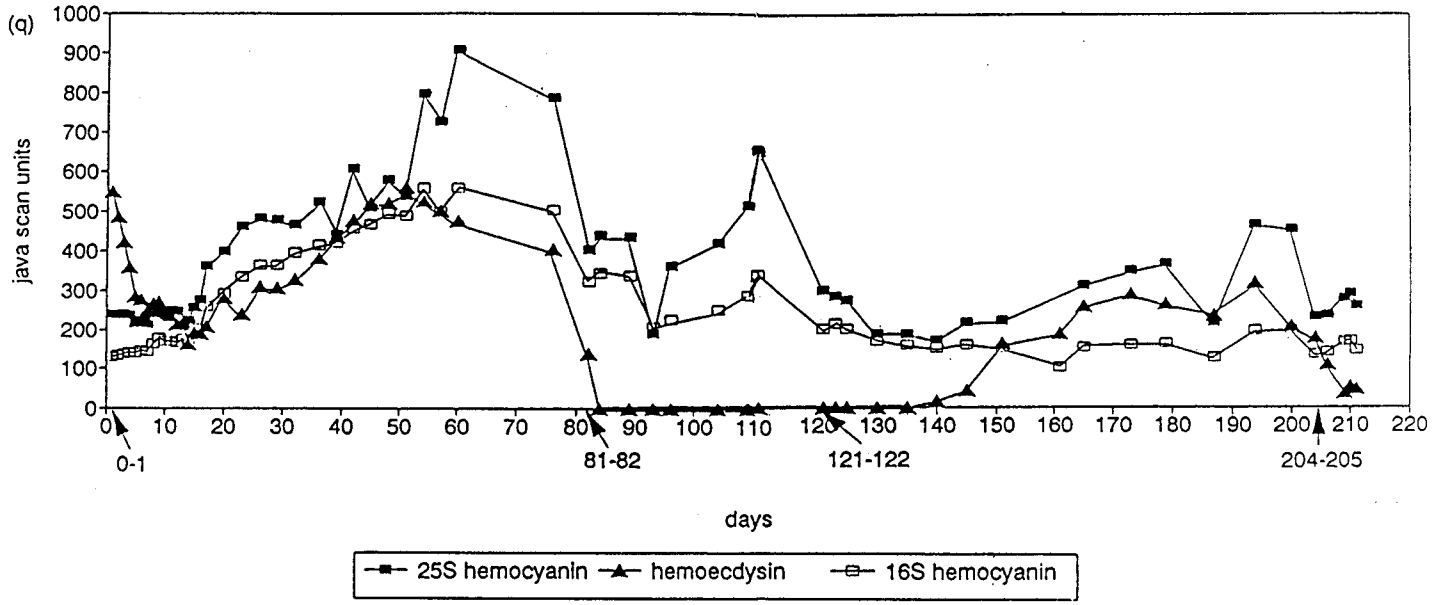

(l)
java scan units
500
450
400
350
300
250
200
150
100
50
0
1 4 7 10 13 16 19 22 25 28 31 34 37 40 43
days
0-1
38-39
25S hemocyanin
hemoecdysin
16S hemocyanin


(m)
java scan units
500
450
400
350
300
250
200
150
100
50
0
1
5
9
13
17
21
25
29
33
37
41
45
days
0-1
25S hemocyanin
hemoecdysin
16S hemocyanin


(n)
java scan units
250
200
150
100
50
0
1
2
3
4
days
0-1
25S hemocyanin
hemoecdysin
16S hemocyanin


(o)
java scan units
550
500
450
400
350
300
250
200
150
100
50
1
3
5
7
9
11
13
15
days
0-1
25S hemocyanin
hemoecdysin
16S hemocyanin


(p)
800
700
600
500
400
300
200
100
0
java scan units
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
190
0-1
72-73
182-183
days
25S hemocyanin
hemoecdysin
16S hemocyanin


(q)
java scan units
1000
900
800
700
600
500
400
300
200
100
0
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200 210 220
0-1
81-82
121-122
204-205
days
25S hemocyanin
hemoecdysin
16S hemocyanin

the numbers below the arrows show between which days the crab molted. These numbers show molting between two days because crab moltings occurred almost exclusively at night. Because the 25S hemocyanin standard curve loses a linear relationship above 150 micrograms of protein, it was not possible to convert the higher protein levels into micrograms of protein. Thus the graphs in Fig. 6 are expressed in java scan units. The value of presenting the individual crab monitoring data is to demonstrate the variability in hemolymph protein concentrations that exist between crabs. Furthermore, proportional differences in hemocyanin and hemoecdysin levels within and between crabs during molting are made clear.

Certain patterns in the fluctuations of the three proteins should be noted. The amount of hemoecdysin dropped to an undetectable level 62.5% of the time after a molt. There was only one instance where the hemoecdysin level did not drop significantly after a molt (Fig. 5o). The 25S and 16S hemocyanins always showed some decrease after a molt but never as great a decrease as the hemoecdysin levels. Figure 7 shows the minimum, initial increase and maximum concentrations of 25S hemocyanin, hemoecdysin and 16S hemocyanin. These concentrations were graphed against percent of molt cycle completed in order to standardize among crabs with differing molt cycle durations. Entire molt cycles were monitored nine times as seen in

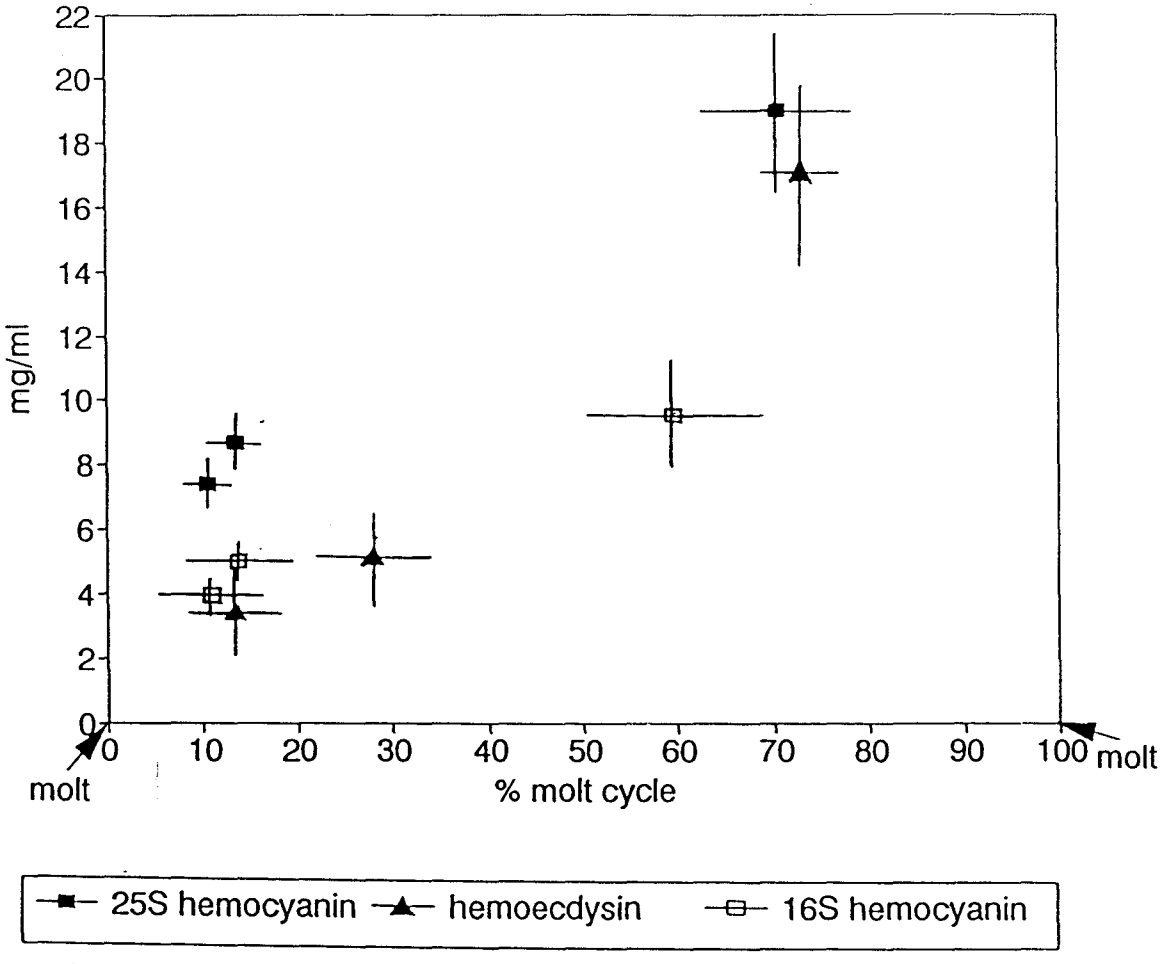
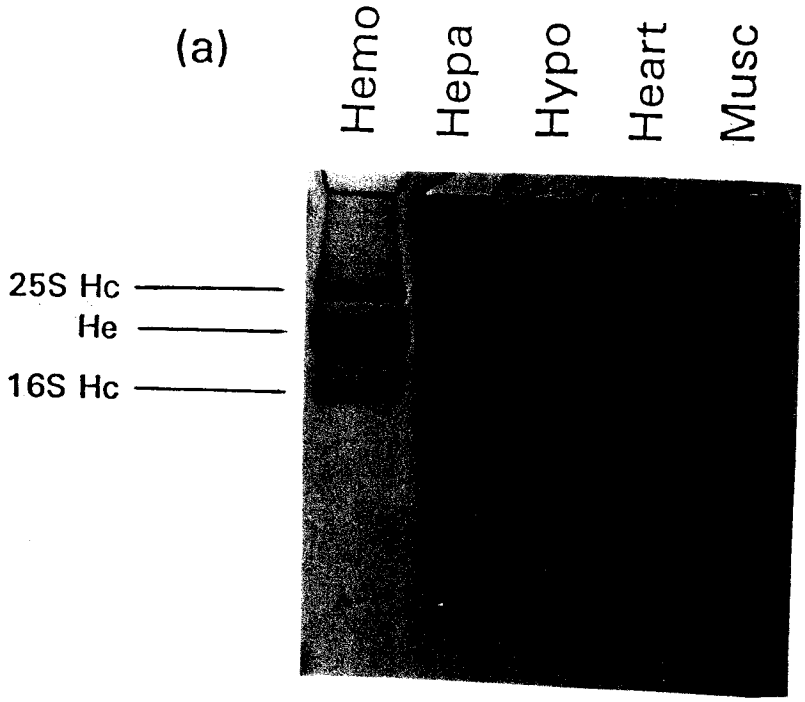


Figure 7. Time (as percentage of molt cycle) of hemolymph minimum level, start of increase and maximum level of 25S hemocyanin, hemoecdysin and 16S hemocyanin (n=8).

Figures 6a, c, e, l, p (2) and q (3). Values from eight of these molt cycles were used in computing the points shown in Figure 7. The second molt cycle in Figure 6q, from an older, 7th instar juvenile, was not used because of the abnormally short molt duration and undetectable hemoecdysin levels. The 25S hemocyanin is shown to reach a minimum concentration of 7.4 mg/ml at 10.5% of the molt cycle, the concentration begins to increase at 13.4% of the molt cycle, and the 25S hemocyanin reaches a maximum concentration of 19.0 mg/ml at 70.3% of the molt cycle. The hemoecdysin reaches a minimum concentration of 3.4 mg/ml at 13.4% of the molt cycle and stays at a low level for a number of days, the increasing concentration is 5.2 mg/ml at 27.9% of the molt cycle, and the maximum concentration is 17.1 mg/ml at 72.7% of the molt cycle. The 16S hemocyanin is shown to reach a minimum concentration of 3.9 mg/ml at 10.8% of the molt cycle, the increasing concentration is 5.0 mg/ml at 13.7% of the molt cycle and the maximum concentration is 9.6 mg/ml at 59.5% of the molt cycle.

Tissues obtained at different stages of the molt cycle were examined for the presence of hemoecdysin. Dissections were carried out on juvenile crabs that were at the following points in their molt cycle: just premolt, 2-20 hours postmolt, 5-26 hours postmolt, 2.5 days postmolt, 3.5 days postmolt, 4.5 days postmolt, 6.5 days postmolt, 7 days postmolt, 8.5 days postmolt, 13 days postmolt and 16 days

Figure 8. Hemolymph and tissue samples from a crab dissected 5-26 hours after it had molted. (a) pH 7.4 PAGE, Hemo, hemolymph; Hepa, hepatopancreas; Hypo, hypodermis; Heart, heart; Musc, muscle; (b) SDS-PAGE. Sample lanes in (b) same as in (a).


(a)
Hemo
Hepa
Hypo
Heart
Musc
25S Hc
He
16S Hc

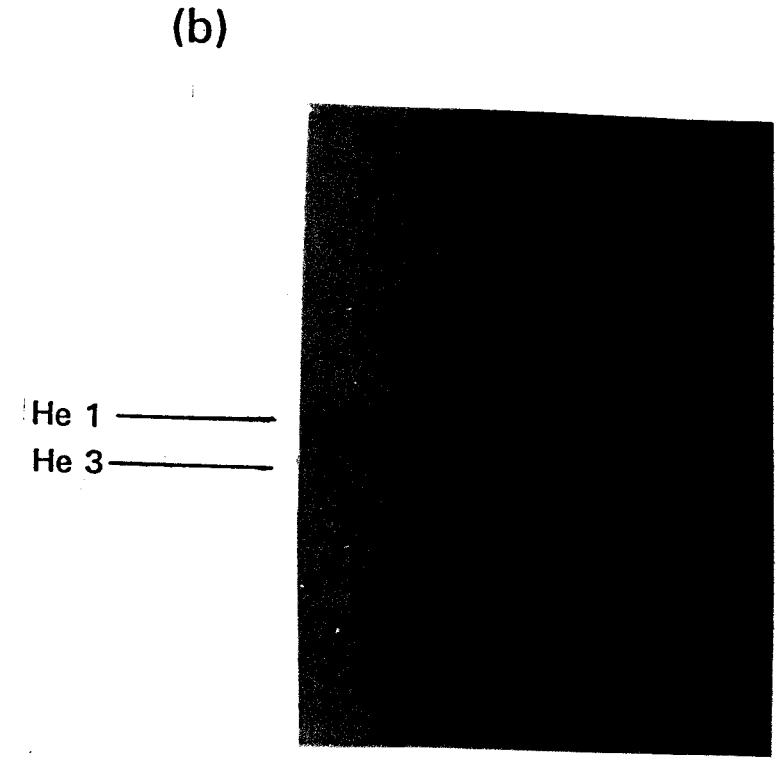
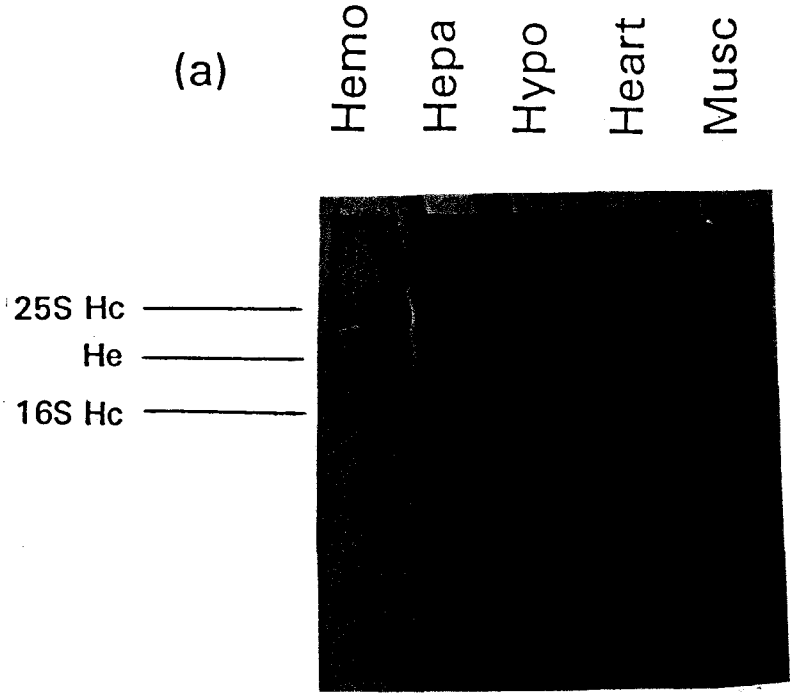

(b)
He 1
He 3

Figure 9. Hemolymph and tissue samples from a crab dissected 2.5 days after it had molted. (a) pH 7.4 PAGE, Hemo, hemolymph; Hepa, hepatopancreas; Hypo, hypodermis; Heart, heart; Musc, muscle; (b) SDS-PAGE. Sample lanes in (b) same as in (a).

(a)



(b)

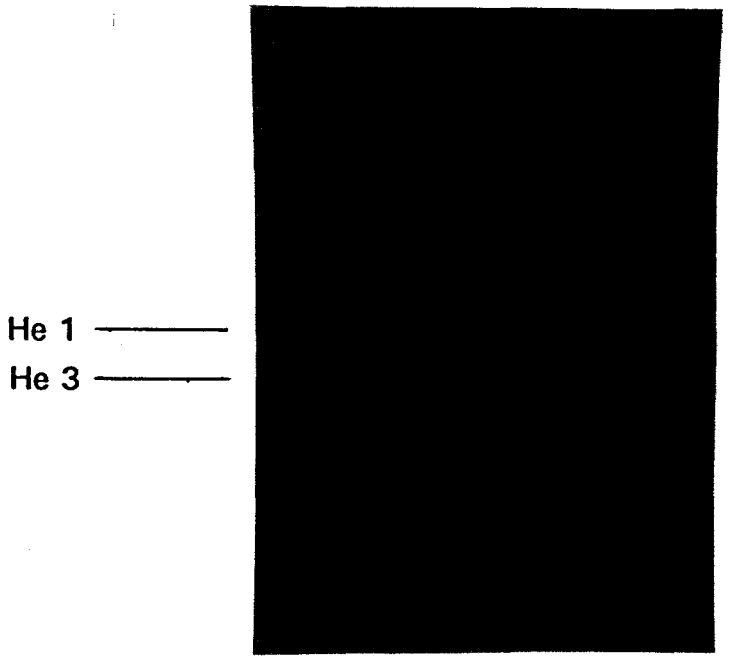
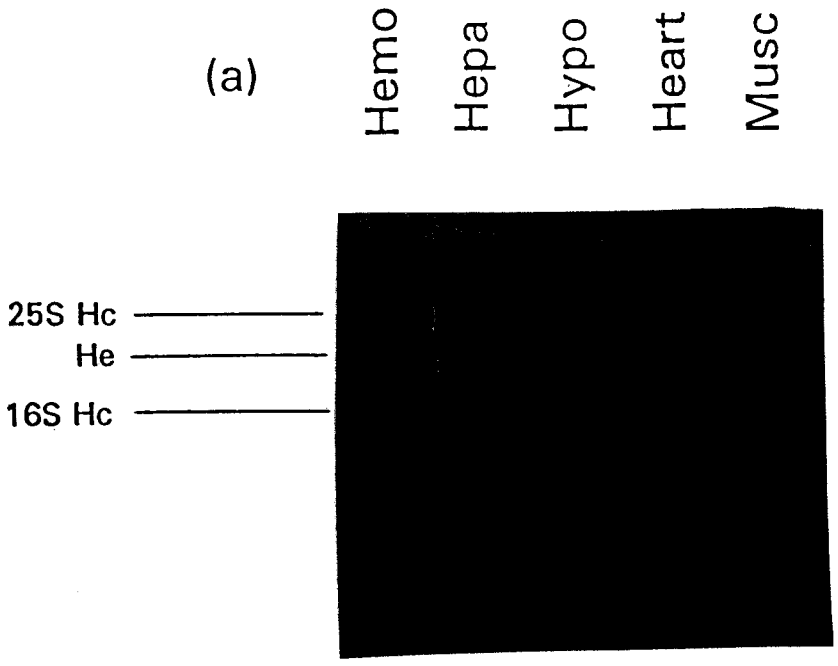


Figure 10. Hemolymph and tissue samples from a crab dissected 4.5 days after it had molted. (a) pH 7.4 PAGE, Hemo, hemolymph; Hepa, hepatopancreas; Hypo, hypodermis; Heart, heart; Musc, muscle; (b) SDS-PAGE. Sample lanes in (b) same as in (a).

(a)



(b)

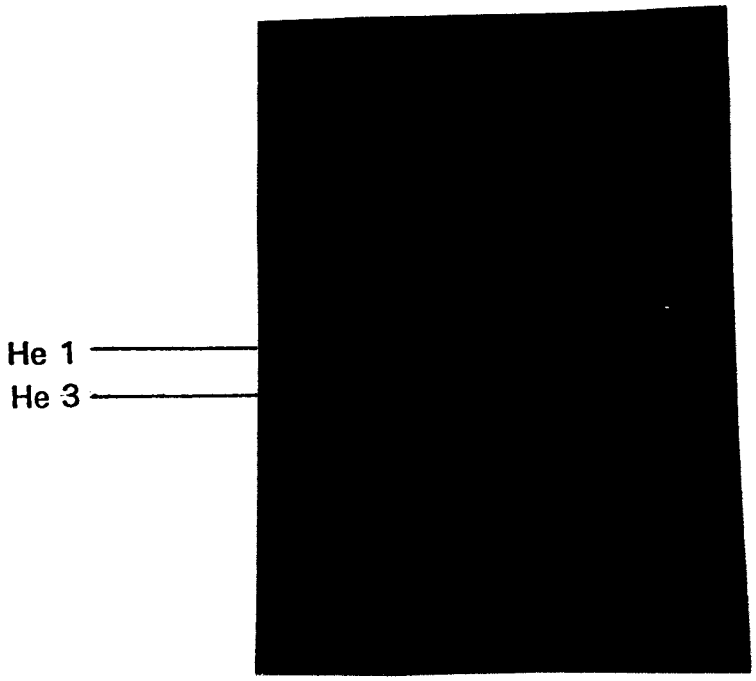
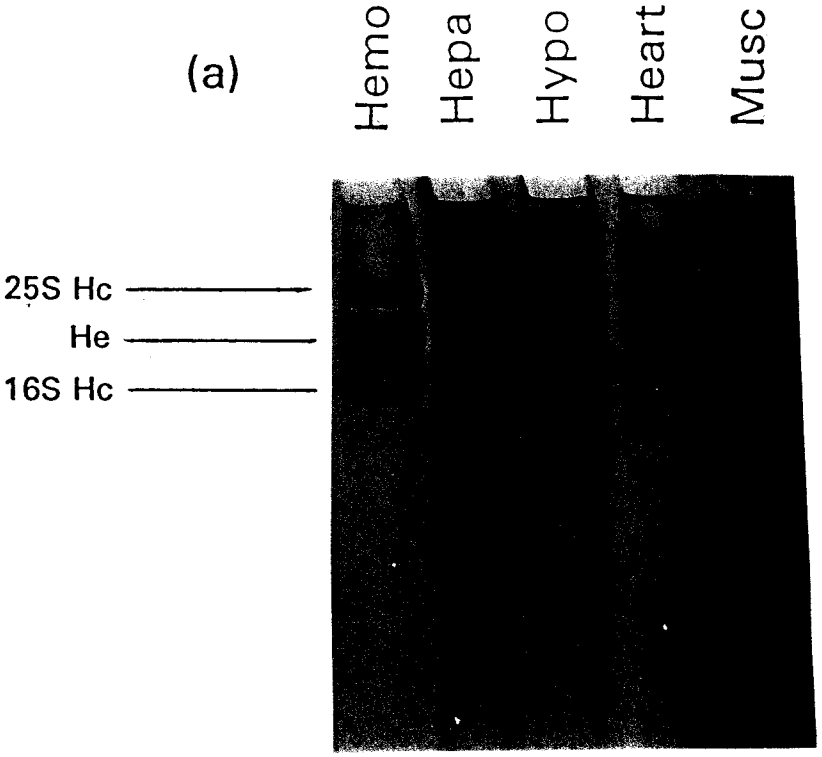


Figure 11. Hemolymph and tissue samples from a crab dissected 6.5 days after it had molted. (a) pH 7.4 PAGE, Hemo, hemolymph; Hepa, hepatopancreas; Hypo, hypodermis; Heart, heart; Musc, muscle; (b) SDS-PAGE. Sample lanes in (b) same as in (a).

(a)



(b)

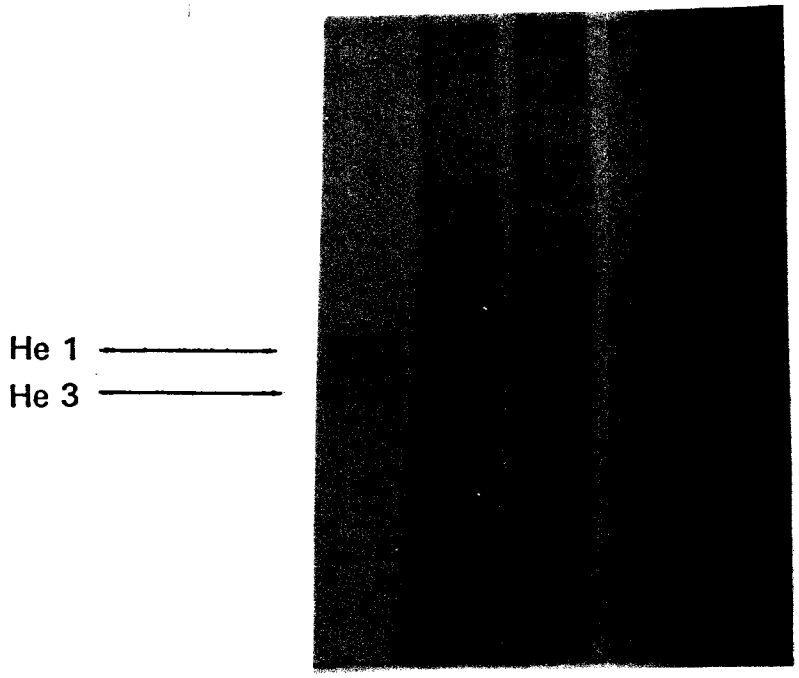


Figure 12. Hemolymph and tissue samples from a crab dissected 6.5 days after it had molted. (a) pH 7.4 PAGE, Hepa, hepatopancreas; Hypo, hypodermis; Hemo, hemolymph; Heart, heart; Musc, muscle; (b) SDS-PAGE. Sample lanes in (b) same as in (a).

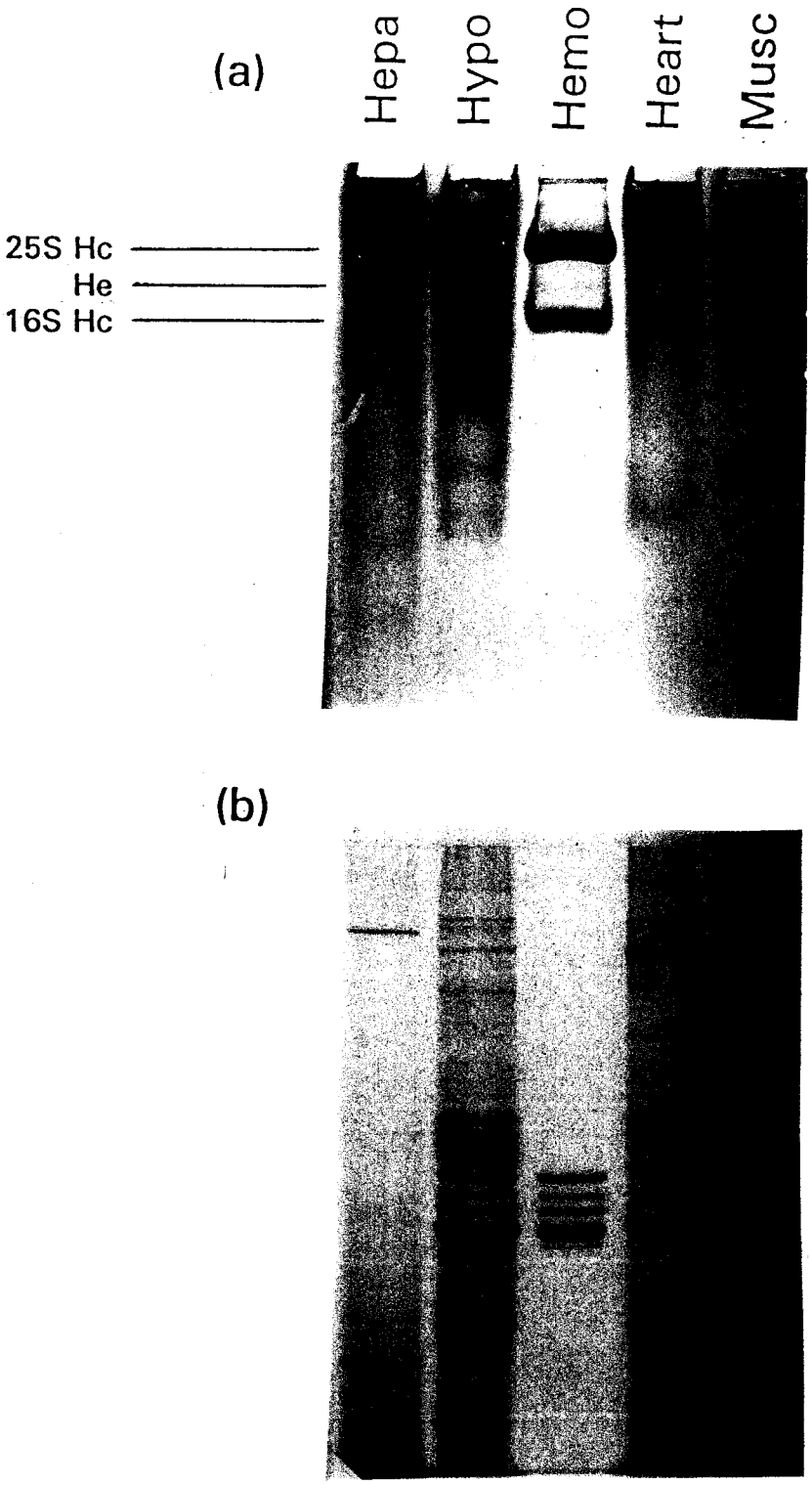
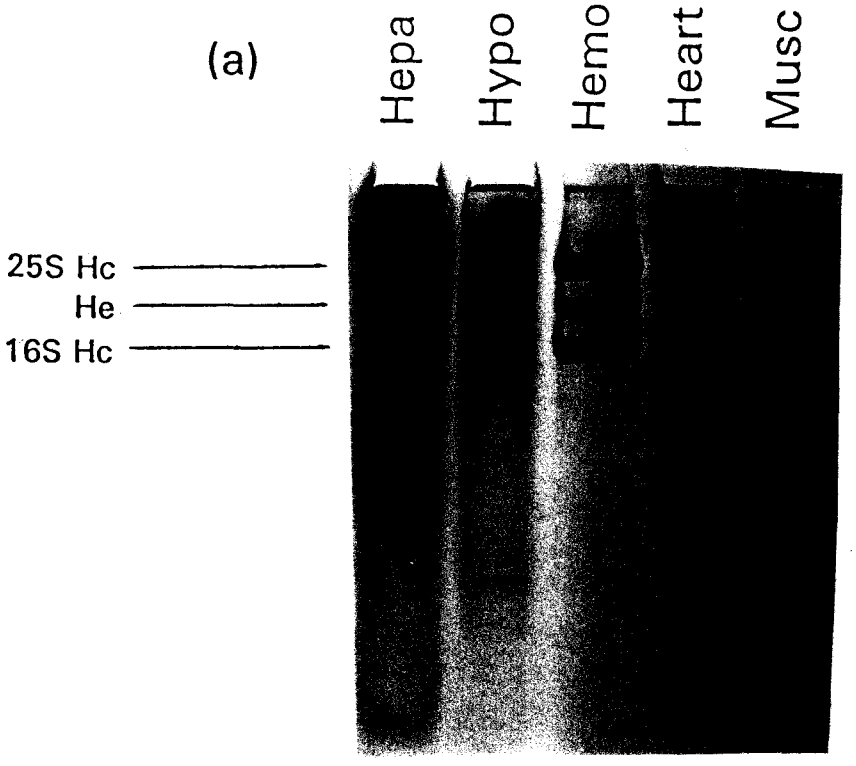
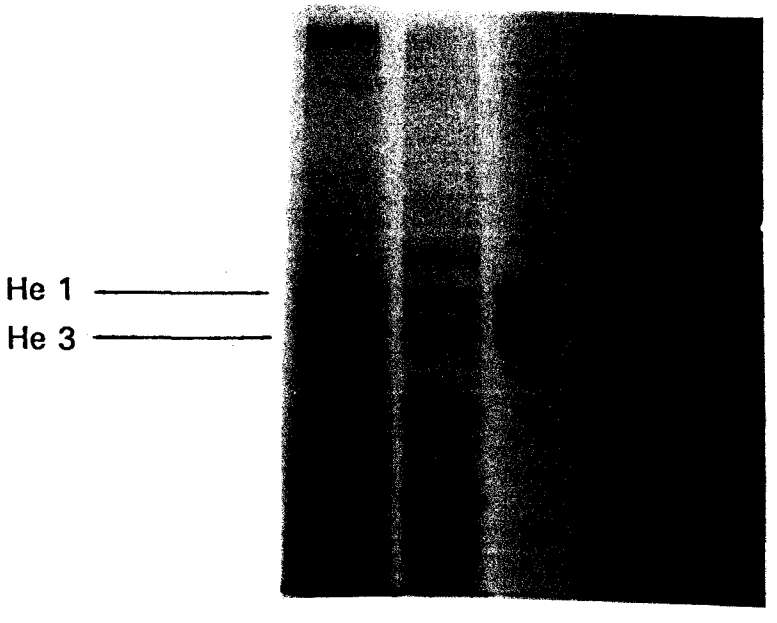

(a)
Hepa
Hypo
Hemo
Heart
Musc
25S Hc
He
16S Hc
(b)

Figure 13. Hemolymph and tissue samples from a crab dissected 7.5 days after it had molted. (a) pH 7.4 PAGE, Hepa, hepatopancreas; Hypo, hypodermis; Hemo, hemolymph; Heart, heart; Musc, muscle; (b) SDS-PAGE. Sample lanes in (b) same as in (a).


(a)
Hepa
Hypo
Hemo
Heart
Musc
25S Hc
He
16S Hc


(b)
He 1
He 3

postmolt. Figures 8-13 compare the protein concentration in the tissues from six of these time points (carapace width): 5-26 hours (37.3 mm), 2.5 days (28.7 mm), 4.5 days (32.2 mm), 6.5 days (33.3 mm), 6.5 days (75.7 mm) and 7 days (23.7). Hepatopancreas, hypodermis, heart, and muscle extracts as well as a hemolymph sample taken at the time of dissection are shown for each crab. Because analysis was completed on the same amount of each tissue from each crab dissection, the results are comparable. The tissue homogenates from each crab were analyzed with both pH 7.4 PAGE and SDS-PAGE. The hemolymph sample serves as a standard for each dissection; its major proteins are resolved into three bands with native pH 7.4 PAGE. This electrophoretic technique separates high molecular weight oligomers like hemocyanin and hemoecdysin on the basis of size and charge, but does not dissociate them into individual subunits. The slowest migrating band is 25S hemocyanin and the fastest migrating band is 16S hemocyanin. The band in the middle corresponds to hemoecdysin. This protein is present in the hemolymph of a premolt crab but usually disappears from the hemolymph after the molt, as demonstrated in Figure 6.

SDS-PAGE in the presence of dithiothreitol, a reducing agent, denatures and dissociates hemocyanin and hemoecdysin into subunits that have similar molecular weights. As shown in Figure 15a Cancer magister 25S hemocyanin has six

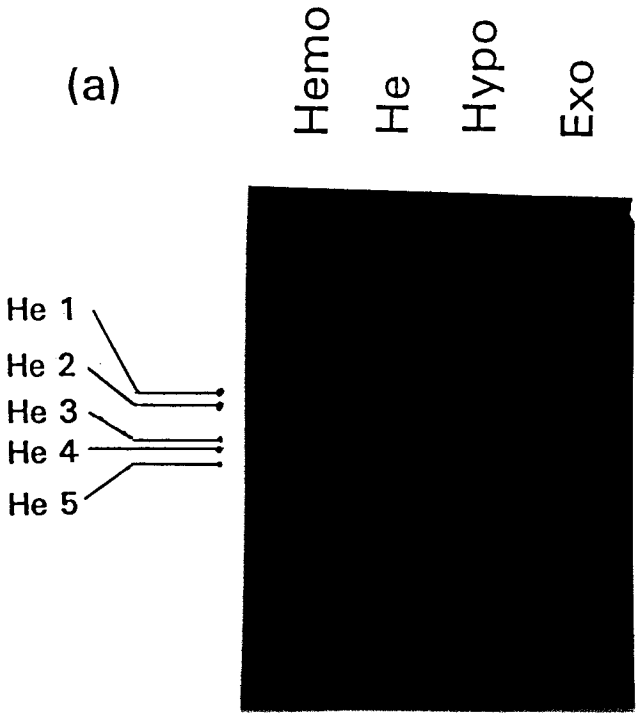
subunits that show up as distinct bands ranging in size from 67,300 daltons to 81,800 daltons (Larson et al., 1981). The SDS-PAGE pattern for hemoecdysin varies depending upon whether the sample was taken from hemolymph or from hypodermal tissue. There are five bands that appear for hemoecdysin. Hemolymph hemoecdysin characteristically shows bands 1, 2 and 4 on SDS-PAGE while hypodermal hemoecdysin electrophoreses as four bands, 1, 2, 3 and 5 (Fig. 14a). Bands 1 and 2 probably correlate to distinct subunits; it is not yet clear whether bands 3, 4 and 5 are subunits, post-translationally modified subunits or degradation products. In the SDS gels shown in Figures 8-13, the only bands labelled are bands 1 and 3 of hypodermal hemoecdysin because they are the easiest to distinguish and occur in the largest amounts. Identification of SDS-PAGE hypodermal hemoecdysin bands 1 and 3 in a tissue sample will serve to confirm the presence or absence of the corresponding pH 7.4 PAGE hypodermal hemoecdysin band.

Figures 8, 9 and 10 are 5-26 hours postmolt, 2.5 days postmolt and 4.5 days postmolt, respectively. Hypodermis tissue from all three show very strong hemoecdysin bands by pH 7.4 PAGE. These high concentrations of hemoecdysin in the hypodermis are confirmed by the presence of hemoecdysin bands 1 and 3 in the hypodermal tissue by SDS-PAGE. Figures 11 and 12 each represent a 6.5 day postmolt crab. While the Figure 11 crab has high levels of hemoecdysin in the

hemolymph, the crab depicted in Figure 12 has none. Both crabs show much less hypodermal hemoecdysin than do those shown in Figures 8, 9 and 10 crabs. The crab depicted in Figure 13 was dissected 7 days postmolt. The heart tissue shows a relatively strong hemoecdysin band. It is unlikely that this band is caused by hemolymph contamination since hemoecdysin levels in the hemolymph are very low. Furthermore, the hemolymph 25S and 16S hemocyanin bands are much stronger than the hemolymph hemoecdysin band. Therefore, if the heart tissue was contaminated by hemolymph, one would expect to see the same ratio of hemocyanin to hemoecdysin in the heart tissue sample as in the hemolymph. This does not seem to be the case.

Figures 14 and 15 are Western blots that were completed using antibodies that reacted specifically with either hemocyanin or hemoecdysin. Negative controls for the different antibodies were also completed. In one case, the primary antibodies were omitted from the protocol in order to test for any non-specific binding of the secondary antibody. No reactions were observed when these negative controls were performed. In another negative control, a set of six proteins unrelated to hemocyanin or hemoecdysin and that ranged in size from 42,700 daltons to 200,000 daltons were included on a blot along with tissue samples. The full Western blot protocol was carried out. This negative control tested for non-specific binding of the primary

Figure 14. Western blots of SDS-PAGE separated samples using a hemoecdysin specific primary antibody. (a) Hemo, hemolymph containing both hemocyanin and hemoecdysin; He, purified hemoecdysin; Hypo, hypodermis extract; Exo, exoskeletal extract, (b) Hemo and Exo, same samples as in (a); Exu, exuvial extract.


(a)
Hemo
He
Hypo
Exo
He 1
He 2
He 3
He 4
He 5

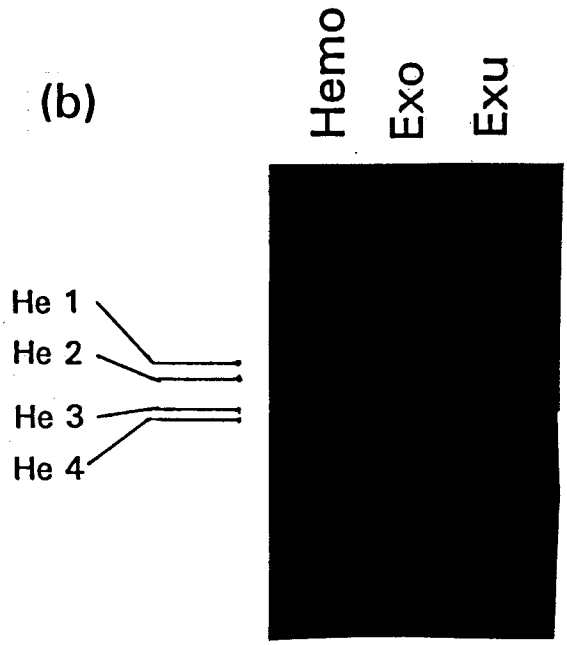
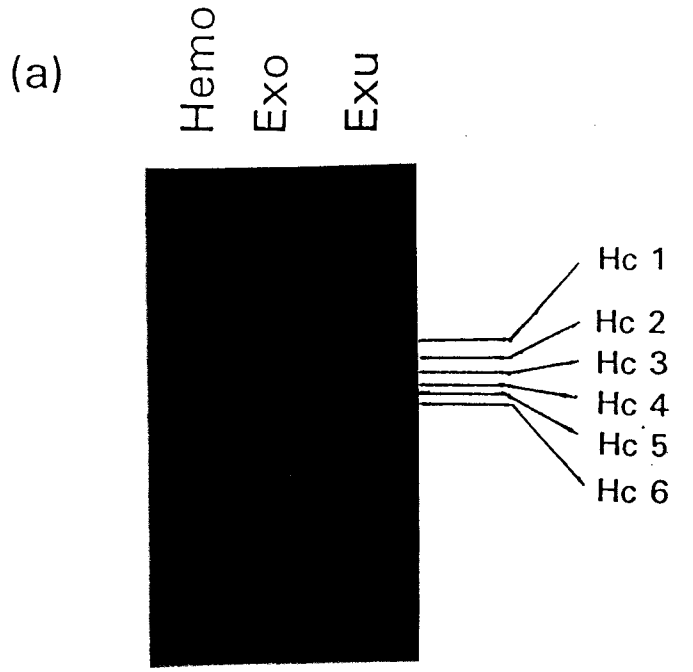
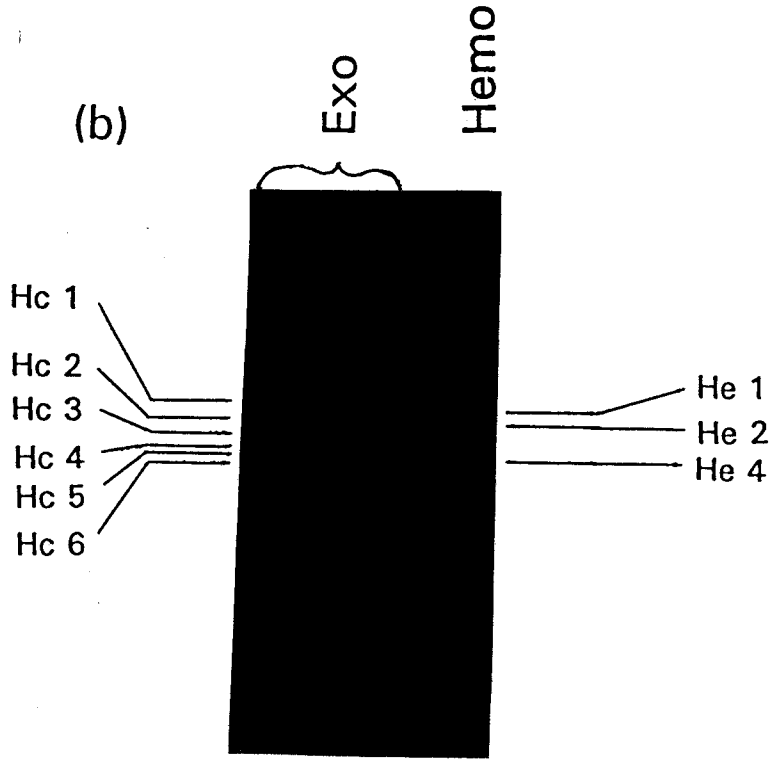

(b)
Hemo
Exo
Exu
He 1
He 2
He 3
He 4

Figure 15. Western blots of SDS-PAGE separated samples incubated with hemocyanin and hemoecdysin specific primary antibodies. (a) Hemo, hemolymph containing hemocyanin and hemoecdysin; Exo, exoskeletal extract; Exu, exuvial extract; same samples as in Figure 14b. Primary antibody specific for subunits 1-6 of Cancer magister hemocyanin was used. (b) slot blot, lanes 1-3, exoskeleton, lane 4, hemolymph containing both hemocyanin and hemoecdysin. Primary antibodies, lane 1, hemocyanin subunit 1-6 specific antibody; lane 2, hemocyanin subunit 4-6 specific antibody; lane 3, hemoecdysin specific antibody; lane 4 hemoecdysin specific antibody.


(a)
Hemo
Exo
Exu
Hc 1
Hc 2
Hc 3
Hc 4
Hc 5
Hc 6


(b)
Exo
Hemo
Hc 1
Hc 2
Hc 3
Hc 4
Hc 5
Hc 6
He 1
He 2
He 4

antibodies to other proteins. Only a very slight reaction with all of the antibodies (diluted 1:300) was detected with one protein, ovotransferrin (77,000 daltons).

Figure 14a shows hemolymph hemoecdysin, electroelution isolated hemolymph hemoecdysin, hypodermal hemoecdysin and exoskeletal hemoecdysin incubated with a hemoecdysin specific antibody. The hemolymph sample contains both hemocyanin and hemoecdysin. This sample serves as a positive control and demonstrates the specificity of the hemoecdysin primary antibody in that the antibody reacts with hemoecdysin and not hemocyanin. As previously mentioned, the SDS band pattern for hemoecdysin differs depending on whether the hemoecdysin is obtained from hemolymph or from hypodermis. The five main hemoecdysin bands are shown in this Figure. Hemolymph hemoecdysin (Hemo) shows bands 1, 2 and 4 while hypodermis hemoecdysin (Hypo) shows bands 1, 2, 3 and 5. The exoskeletal extract (Exo) shows bands 1 and 3. In Figure 14b the same hemolymph sample was used as in 14a except that the 14b sample is ten times concentrated. With this extra amount of protein, hemoecdysin band 4 becomes visible. The 14b exoskeletal sample (Exo) again shows bands 1 and 3 while the exuvia sample (Exu) shows no bands.

Figure 15a is a Western blot in which a hemocyanin specific primary antibody was used. The hemolymph sample (Hemo) shows a positive reaction for all six hemocyanin

subunit bands. The hemolymph sample used here is the same hemolymph sample used in Figures 14a and 14b. This demonstrates the specificity of the hemocyanin antibody in that it reacts with hemocyanin and does not react with hemoecdysin. The exoskeletal sample (Exo) shows a reaction for hemocyanin subunits 3, 4, 5 and 6; however a white blotch appears where subunits 1 and 2 should be. This blotch appears consistently in the same area for the exoskeletal extracts as also seen in Figures 14a and 14b. It may be due to the presence of a protein band that does not react with the antibody. Figure 15b is a Western blot displayed in a different slot blot format. Lanes 1-3 each contain the same exoskeletal protein extract but have been reacted with different antibodies, each with a unique specificity. Lane 1 has been incubated with a hemocyanin specific antibody and all six subunit bands appear. Lane 2 was incubated with an antibody that reacts only with subunits 4, 5 and 6 of hemocyanin; all these bands are visible. Lane 3 was incubated with a hemoecdysin specific antibody and appropriately shows a reaction for hemoecdysin band 1. Lane 4 is a hemolymph sample that contains hemocyanin and hemoecdysin and was reacted with a hemoecdysin specific antibody. This serves as the standard and shows reactions for hemoecdysin bands 1, 2 and 4. The main point in showing this blot is to demonstrate that hemocyanin subunits 1 and 2 are present in exoskeletal

extracts, along with hemoecdysin. It is not known why there was no white blotch in this blot's exoskeletal samples.

Discussion

This study shows very distinct patterns in the fluctuations relative to the molt cycle of 25S hemocyanin, 16S hemocyanin and hemoecdysin in the hemolymph of juvenile Cancer magister crabs. In 1985 Mangum et al. measured Callinectes sapidus molt stage specific hemocyanin concentrations spectrophotometrically. It was determined that the hemolymph hemocyanin concentration reached a maximum of 42.5 mg/ml during stage D. This pre-molt peak was followed by an 80% decrease upon molt to ~ 8.7 mg/ml during stage A of metecdysis. In 1987 Engel conducted a similar experiment with C. sapidus and reported that the hemolymph hemocyanin reached a concentration of approximately 55 mg/ml in stage D and then decreased by 60% upon crab molt. The reported values in mg/ml (Fig. 1, Engel, 1987) decreased from a high of 55 mg/ml during stage D to 16 mg/ml during stage A, a 70% decrease.

The study presented here also monitors hemocyanin but is unique in that the 25S hemocyanin and the 16S hemocyanin were monitored separately. Furthermore, protein levels in hemolymph of individual C. magister were monitored

throughout entire molt cycles. It was determined here that the total hemocyanin reached an average maximum concentration of 28.6 mg/ml and then decreased to a minimum concentration of 11.3 mg/ml, a 2.5 fold decrease (Figure 7). This decrease is much less than the 5 fold decrease observed by Mangum *et al.* but is comparable to the reported decrease in hemocyanin concentration for *C. sapidus* by Engel. There was little difference between the proportional decreases in *C. magister* 25S hemocyanin and 16S hemocyanin, a 2.6 fold decrease and a 2.4 fold decrease respectively. Such similar decreases would suggest that the factor responsible for the decrease whether it be catabolism, sequestration, excretion, synthesis or a combination, does not distinguish between the two forms of hemocyanin. Engel and Brouwer (1991) showed that degradation is an important factor immediately after the molt in the blue crab, *Callinectes sapidus*. They observed a transient pulse of non-hemocyanin copper in the digestive gland within 90 minutes after the molt. They correlated this copper pulse to catabolism of hemocyanin and the stripping of copper from hemocyanin. Such catabolism was said to be the cause of the approximate 60% decrease in hemocyanin.

A much more dramatic decrease was demonstrated in the hemolymph concentration of *C. magister* hemoecdysin. Hemoecdysin shows an approximate 5 fold decrease in concentration after the molt. This decrease is

proportionally much more severe than the decrease in either 25S or 16S hemocyanin. Furthermore, the hemoecdysin remains low for twice as many days of the molt cycle as do the hemocyanins (Fig. 7).

A significant factor to consider when discussing protein concentrations in the hemolymph is water intake immediately at the molt. Upon molt, seawater is taken up by the crustacean from the surrounding environment. For the lobster Homarus americanus, the hemolymph volume doubles (Mykles, 1980). A similar hemolymph dilution factor apparently occurs in C. sapidus that is not large enough to account for the huge 5 fold decrease in hemocyanin concentration at the molt (Mangum et al., 1985). If there were a similar two-fold dilution of the hemolymph at the molt for C. magister, it would account for most of the 2.5 fold decrease in total hemocyanin. Dilution, however, is not the only factor causing the decrease in C. magister hemolymph proteins. If it were, one would see a proportionally equal decrease in the concentration of hemoecdysin and hemocyanin. This is not the case as there was a 5 fold decrease in hemoecdysin and only a 2.5 fold decrease in hemocyanin.

It is important to note the large variation in protein concentrations between individual crabs. Figures 6a-q show data that were collected on 17 different crabs monitored through one or more molt cycles. Large differences in the

maximum protein concentrations are obvious even for those crabs monitored through successive molts (Fig. 6p, q). Such differences point out the extreme physiological variation that exists between and within crabs. Such variation in hemolymph protein concentrations in crustaceans and chelicerates has been noted many times in the literature (for review see Truchot, 1992).

The purpose of examining different crab tissues was to obtain a qualitative idea of the distribution and amount of hemoecdysin present after the molt, since earlier experiments had demonstrated that this protein decreases dramatically in the hemolymph. In 1986 Bielefeld et al. completed a study looking at the stage-specific polypeptide patterns in the hypodermis and hepatopancreas tissues of the crayfish Astacus leptodactylus. Their study showed the molt cycle correlated appearance of certain proteins in these tissues. If hemoecdysin were incorporated into the exoskeleton, one might expect fluctuations in hypodermal hemoecdysin that correlated to the formation of the new exoskeleton. Several studies have demonstrated that insect hemolymph hexamerins are sequested immediately after the molt into the midgut gland, an organ equivalent to the crustacean hepatopancreas (reviewed in Telfer and Kunkel, 1991). The tissue dissection data presented here intended to determine if there were increased levels of hemoecdysin in the hypodermis or hepatopancreas. If high levels did

exist, it would be of interest to know exactly when, after the molt, the increase occurred.

The gel electrophoresis data showed that there were high levels of hemoecdysin in the hypodermis tissue of certain C. magister crabs. Crabs that were dissected in early postmolt (5-26 hours, 2.5 days, 4.5 days postmolt) had large amounts of hemoecdysin in their hypodermis. Crabs that were dissected later in postmolt (6.5 days, 6.5 days and 7 days postmolt) had noticeably less hemoecdysin in their hypodermis. There was also a correlation between high hemoecdysin levels in the hemolymph and high hemoecdysin levels in the hypodermis. This is true for low concentrations as well; the 6.5 day postmolt crab (Fig. 10) had no hemoecdysin in the hemolymph or in the hypodermis. At first glance, this could suggest that the high levels of hypodermal hemoecdysin are caused by contamination from the hemolymph. However, the extremely large amounts of hypodermal hemoecdysin (Figs. 8a, 9a and 10a) in proportion to hypodermal hemocyanin excludes the possibility of hemolymph contamination.

The only other tissue that showed increased amounts of hemoecdysin is the heart tissue as demonstrated in Figure 13. The heart tissue sample showed a band for hemoecdysin stronger than the hemocyanin bands even though there was much more hemocyanin than hemoecdysin in the hemolymph. This may be of interest because the heart tissue has been

suggested as a site for synthesis of hemocyanin in the spider Eurypelma californicum (Kempter, 1986). Nonetheless, the level of hemoecdysin in this heart tissue sample is nowhere near that of levels found in certain hypodermal tissue samples.

The initial question asked whether the hypodermis was sequestering hemoecdysin for incorporation into newly formed exoskeleton. An important question was addressed by Koeppe and Gilbert in their 1973 study on the tobacco hornworm, Manduca sexta. They asked whether large hemolymph carrier proteins were able to traverse the two membranes of a hypodermal cell and enter the newly deposited pupal cuticle in an unaltered state. They demonstrated that the hypodermal cell was indeed able to take up hemolymph proteins larger than 40,000 daltons. Thus there is precedent for the potential uptake of hemoecdysin by hypodermal cells. That other crustacean tissues can endocytose large molecular weight proteins has been documented in the uptake of vitellogens and hemocyanin by oocytes (see Charniaux-Cotton 1985 and Terwilliger 1991 for reviews).

With the data gathered here it is not valid to assume that the high levels of hemoecdysin in the hypodermis of certain crabs are due only to sequestering of the protein. An equally valid argument is that the hemoecdysin is being synthesized by the hypodermis. Bielefeld et al. (1986) do

report such molt cycle correlated production of proteins by the hypodermis. In order to resolve what is actually happening to hemoecdysin in C. magister, a technique such as radioactive labeling would have to be employed so that hemoecdysin from the hemolymph could be visibly traced into the hypodermis.

As discussed in the Introduction, certain insect hexamerins have been shown to function as carrier molecules of molting hormone, riboflavin and xenobiotics. The hypodermis is known to be an ecdysteroid, or molting hormone, target tissue because of the existence of hormone receptor proteins within (Spindler et al., 1984). It is therefore possible that hemoecdysin is acting as a carrier molecule of molting hormone. Such a function is another possibility that would explain the high levels of hemoecdysin in the hypodermis at times near the molt. If hemoecdysin does bind molting hormone, it could also serve the purpose of clearing the hormone from the animal's system after the molt when ecdysteroid titers are known to drop rapidly (Hopkins, 1983). Figure 5 shows that the average juvenile crab loses most of the hemolymph hemoecdysin by the third day after the molt. If much of the lost hemoecdysin was excreted, while being bound to ecdysone, then the levels of ecdysone in the crab's system would be dramatically decreased.

In 1985 Vacca and Fingerman described two proteins in

Uca pugilator with molecular weights of ~ 150,000 daltons and > 400,000 daltons that carried tanning precursor molecules. Hemoecdysin has a molecular weight of ~ 450,000 daltons and a sedimentation coefficient of 16S (N. Terwilliger, personal communication). It would therefore be interesting to compare various structural aspects of hemoecdysin and this large carrier protein to see if they are similar.

Many studies have shown incorporation of hemolymph proteins into the exoskeleton of insects. Radiolabeled calliphorin has been traced into larval insect exoskeleton (Scheller et al., 1980). Similarly, intact calliphorin was demonstrated in the exoskeleton of larvae and pupae by immunoblotting of SDS extracts (Konig et al., 1986). Studies showing the direct relationship of hemolymph proteins and exoskeletal proteins have been common in the study of insects. This, however, has not been the case in the study of the crustacean exoskeleton. Although data has been collected characterizing the proteins of the crustacean exoskeleton there has been little focus in relating these proteins to hemolymph proteins.

This study presents strong evidence that hemoecdysin is modified in the hypodermis and then incorporated into the exoskeleton. As discussed earlier, hemoecdysin gives two banding patterns depending on whether it is obtained from the hemolymph or from the hypodermis. One possible

explanation for the two banding patterns is that the hypodermis takes up hemoecdysin and then an endogenous protease cleaves it, yielding the lower, smaller molecular weight bands 3 and 5. Band 1, predominant in hemolymph hemoecdysin, is much reduced and band 3 is much stronger in hypodermal hemoecdysin which suggests that the band 1 protein has been cleaved yielding the smaller protein band 3. The exoskeletal extracts always show bands 1 and 3 when reacted with a hemoecdysin specific antibody. Band 3 only appears in hypodermal and exoskeletal hemoecdysin and it therefore seems possible that the hypodermis is taking up the hemoecdysin, cleaving it and then incorporating it into the exoskeleton. Another possible explanation for the appearance of bands 3 and 5 is that the hemoecdysin is degraded during the extraction of the protein. A serine protease inhibitor was included when the tissue was homogenized, however there may have been another protease that was unaffected by this inhibitor and was responsible for degradation of the hemoecdysin.

Hemocyanin's most widely accepted function is in carrying oxygen. Hemocyanin has also been suggested to function as a protein reserve during times of nutritional need, such as the fasting period following molting in the spider crab Maja squinado (Zuckerkandl, 1960). The present study gives strong evidence that hemocyanin serves the additional function of being incorporated into exoskeleton.

Such a function would also serve as part of the explanation for the decrease of hemocyanin at periods after the molt, such as stage B, when the thick endocuticle layer is being formed.

It is also interesting that there were no hemoecdysin or hemocyanin antibody reactions with the exuvial extracts (Figs. 14b and 15a) as there were for the exoskeletal extracts. The exuvia is hypodermally degraded by 75% by the time it is cast off at the molt. This means that the membranous layer, endocuticle and part of the exocuticle have been enzymatically degraded. The fact that there was no antibody reaction that showed the presence of hemoecdysin or hemocyanin in the exuvia indicated that these proteins were incorporated into layers of the exoskeleton that had been degraded by the time molt occurred and the exuvia was cast off. In 1993 Kumari and Skinner completed a comprehensive comparison of exoskeletal proteins from different layers of the exoskeleton at different stages of the molt cycle. Exuvial extracts were also analyzed and, similarly, the loss of certain protein bands due to degradation was demonstrated.

The extraction procedure used in the exoskeletal and exuvial analyses provides information about how the hemoecdysin and hemocyanin may exist in the exoskeleton. Solubilization of the proteins in a 1% KCl solution suggests that hemoecdysin and hemocyanin were either unbound or bound

weakly by van der Waals' forces. The possibility remains that there are hemoecdysin and hemocyanin molecules in the exoskeleton bound by forces that a 1% KCl solution would not disrupt. In order to answer this question and get a more comprehensive idea of how the proteins of the exoskeleton are bound a technique such as Hackman's (1972) serial extraction should be employed. Hackman used a series of solvents on the insect exoskeleton of Agrianome spinicollis that included water, KCl, urea and NaOH. Protein in the water-soluble fraction was not bound, that in the KCl-soluble fraction was bound by weak bonds such as van der Waals' forces, that in the urea-soluble fraction by hydrogen bonds and that in the NaOH-soluble fraction was more firmly bound and possibly required hydrolysis for its removal. Percents of proteins bound by these different interactions could subsequently be determined.

Summary

Hemocyanin and hemoecdysin are the predominant proteins in the hemolymph of the Dungeness crab, Cancer magister. Hemocyanin exists in the hemolymph as both a two-hexamer (25S) molecule and a one-hexamer molecule (16S). The newly termed hemoecdysin protein, a one-hexamer molecule, has remained completely uncharacterized until recently. Hemoecdysin and hemocyanin levels in the hemolymph of

juvenile crabs have been shown to undergo extreme fluctuations in correlation with the molt cycle. Both proteins gradually increase in concentration before the molt and then decrease immediately after the molt. Hemocyanin was shown to undergo a 2.5 fold decrease while hemoecdysin was shown to undergo a much more dramatic 5 fold decrease. Hemoecdysin levels remain low for a much greater portion of the molt cycle than do hemocyanin levels.

Hepatopancreas, hypodermis, heart and muscle tissue homogenates were examined, and hemoecdysin was shown to occur at high levels after the molt, only in hypodermal tissue. Juvenile crabs dissected 5-26 hours postmolt, 2.5 days postmolt and 4.5 days postmolt showed high hemoecdysin level in hypodermal extracts. Crabs dissected later in the postmolt at 6.5 days postmolt, 6.5 days postmolt and 7 days postmolt showed much reduced levels of hypodermal hemoecdysin.

A major function of the hypodermis is to secrete new exoskeleton. A partial explanation for the high levels of hypodermal hemoecdysin might be that the hypodermis is incorporating the hemoecdysin into the exoskeleton. Hemoecdysin and hemocyanin specific antibodies were incubated with exoskeletal extracts, and positive reactions were observed for both hemoecdysin and hemocyanin. Exuvia (exoskeleton that is cast off at molt) is degraded by 75% prior to the molt. Exuvial extracts were also tested, and

no reactions were observed for hemoecdysin or hemocyanin. These results suggest that the proteins are incorporated into layers of the new exoskeleton; these layers are then degraded before the molt.

CHAPTER II

REPRODUCTION AND MOLTING IN ADULT FEMALE *CANCER MAGISTER*

Introduction

The harvesting of the Dungeness crab, *Cancer magister*, is a large fishery that extends along the Pacific coast from central California to Alaska. Because the Dungeness crab is both commercially and recreationally important, numerous studies have been conducted on many aspects of the biology and ecology of this species. Surprisingly few studies, however, have focused on the reproductive biology of the female Dungeness crab (Wild, 1980; Wild, 1983; Freese and O'Clair, 1985; Hankin *et al.*, 1985; Hankin *et al.*, 1989). Studies have focused on the male Dungeness crab largely because, with the exception of British Columbia, only males may be legally commercially harvested. The female is obviously important in the perpetuation of the species, and knowledge of its biology is extremely important in determining management policy.

This study focuses solely on the biology of the female Dungeness crab, *Cancer magister* Dana, and directly addresses

the implications of a new Oregon law. Starting in 1993, females as well as males with a carapace width of ≥ 146 mm (measured from inside the 10th anterolateral teeth) were declared legal for harvesting by recreational fishermen. This new law was based on speculation that there were not substantial numbers of female crabs larger than this size. It was also speculated that female crabs of this size were not reproductively successful enough to contribute significantly to the annual production of larval crabs.

Several reasons have been suggested about why large female crabs may not be able to reproduce successfully. In order to mate, the male must embrace and control the female, which is accomplished much more effectively if the male is significantly larger than the female (Butler, 1960; Snow and Neilsen, 1966). It has been theorized that today, many female crabs may not be able to mate because of the decreased opportunity to find sufficiently large male partners (Smith and Jamieson, 1991). Smith and Jamieson have suggested that the intensive and highly efficient fishing of the large males (carapace width of ≥ 154 mm in British Columbia and ≥ 159 mm in the U.S.) has significantly decreased the pool of potential mating males. They further proposed that because of such intensive fishing female crabs greater than about 140 mm would have difficulty finding sexual partners.

The mating process is also usually coordinated with the

molting, or ecdysis, of the female crab. The male injects the sperm, using specialized gonopods, into the spermathecae of the freshly molted female. In order to grow, arthropods must molt; that is, they shed their hard exoskeletons and synthesize new larger ones. "Terminal molt" refers to a final molt of a crustacean after which it is no longer capable of molting. It is unknown what may cause such a condition although degeneration of the Y-organ responsible for synthesizing the molting hormone ecdysone has been suggested. Other possible causes are overproduction of molt inhibiting hormone or decrease of energy sources (reviewed in Skinner, 1985). Terminal molt was first described in the lobster Homarus gammarus (Couch 1837). Since then, several decapod crustaceans have been suggested to enter terminal anecdysis. This idea, however, has always been a subject for debate and is far from being established. If the Dungeness crab does enter a terminal molt phase, is this to say that they are then unable to mate and therefore reproduce? This question and the idea that the large females may not be able to find a suitably sized mate both suggested that perhaps large females, $\geq$ 146 mm, are incapable of successful reproduction.

One interesting strategy that would allow the large female an alternative avenue of reproduction is the possibility of sperm storage. It has been reported that females are able to store sperm for up to two and a half

years (Hankin 1989). If so, females would be able to successfully fertilize oocytes for two years after mating. More information about frequency and success of this storage technique is necessary to fully understand the implications.

The present study used Dungeness crabs collected from Coos Bay, Oregon, in 1993. The study consisted of three parts that examined aspects of the reproduction and molting of female crabs that are ≥ 146 mm in carapace width. Part I took place from January to March, 1993, and sought to determine the viability of egg masses spawned by female crabs ≥ 146 mm, the minimum recreational size limit. Female crabs that were ≥ 146 mm and possessed egg masses or "berries" were collected. A range of smaller females with egg masses were also collected as controls. The crabs and egg masses were monitored through development until hatching into zoeae occurred.

Part II of this study took place from April until July, 1993, and examined the molting of female crabs. Crabs ranging in size from 110 mm to 166 mm were used, and the data of interest included molt increment, molt timing and incidence of molt in relation to the size of the crab. Such laboratory data could be compared to data that has been collected in the field (Hankin 1989). This part of the study also hoped to address the possibility of a terminal molt in C. magister. A number of very large crabs (> 150mm) were included to see whether or not they would molt.

Part III of this study took place from October until December, 1993, and sought to determine what proportion of female crabs ≥ 146 mm spawned egg masses. Crabs without egg masses were randomly collected in early October, which was just before the females in Coos Bay area normally go into berry. A size range of 25 female crabs ≥ 146 mm and a size range of 25 female crabs < 146 mm were obtained and each crab was held in a separate aquarium. These crabs were maintained and checked routinely to see if and when they would go into berry.

There are many unanswered questions about the reproduction of the female Dungeness crab. These questions are very pertinent to understanding the dynamics of the crab fishery and are key in determining how to manage the resource. This study hopes to shed light on some of the above questions and to provide preliminary data for future studies.

Materials and Methods

Crab Collection

(I) The female crabs with egg masses were obtained from a local Coos Bay crab fisherman. A permit was authorized to Bob Barnes, the captain of the Metta Marie. Specific

directions were given to take only females that had extruded egg masses. The crabs that were collected were caught between Coos Bay and Winchester Bay in approximately 30 feet of water. Female crabs were brought in on two different dates: January 1, 1993 and February 5, 1993.

(II) The crabs for the molting study were obtained from the Charleston boat basin docks on March 22, 1993. Another set of crabs were collected from the docks on April 12, 1993, to replace crabs that had died. There were a total of 65 crabs used in this study. Crabs were selected only if they had an old shell appearance to guard against using crabs that had already molted this season. Criteria included shell hardness, cleanliness, coloration and barnacle growth.

(III) The crabs for the egg extrusion study were obtained at three different sites within Coos Bay. Smaller crabs were collected at different locations within the bay on: October 8, 13, 14 and 15, 1993. On October 19th Bob Barnes, a commercial crab fisherman, obtained most of the experimental crabs ≥ 146 mm near the mouth of the bay in approximately 40 feet of water.

Crab Maintenance

(I), (II) and (III) The crabs were kept in 15 gallon glass and wooden aquaria. Each aquarium was subdivided into

two separate compartments by a wooden divider. Because running seawater was supplied to only one of the two compartments a 2 inch hole was drilled in the center of each divider to allow water to circulate. Each tank had approximately 2 inches of sand, and one airstone was supplied to each tank. The crabs were fed two times a week with either squid, Loligo opalens, or fish heads, Sebastolobus alascanus, depending on availability.

Data Collection

For all parts of the study, each crab was numerically marked on the upper right portion of its carapace for easy identification and as a means to organize the data that was collected.

(I) The seawater temperature and salinity were taken during the day, two times a week. The temperature ranged between 6° C and 10° C and the salinity ranged between 29 and 33 parts per thousand. This part of the study had a sample size of 31 crabs. Initial data such as carapace width, egg mass color, shell hardness and missing appendages were recorded when the crabs were first received. The crabs were checked daily to see if larval zoeae had hatched and if there were any adult mortalities. Egg development is easy to monitor; there is a distinct and gradual color change associated with development. When the eggs are first

extruded, they are bright orange due to the high yolk concentration. As the eggs further develop, they turn brown. Prior to hatching, eyespots can be observed.

Such developmentally correlated color changes were monitored twice a week. This monitoring was done so that even if the female died before the eggs reached hatching stages it would be known whether or not the eggs were undergoing development.

Egg samples that differed in coloration from the original orange color were preserved in Bouin's fixative (71.4% aqueous saturated picric acid, 23.8% formalin and 4.8% glacial acetic acid). The fixed eggs were eventually examined by light microscopy to confirm whether changes in egg color were in fact due to egg development.

(II) The seawater temperature and salinity was taken during the day two times a week. The temperature ranged from 11° to 16° C and the salinity ranged from 30 to 34 parts per thousand. This part of the study had a sample size of 65 crabs. Crab carapace width and shell hardness data were initially collected. The crabs were checked daily to see if they had molted. New carapace widths were recorded upon molting.

(III) The seawater temperature and salinity was taken during the day two times a week. The temperature ranged from 6° to 12° C and the salinity ranged from 32 to 35 parts per thousand. This part of the study had a sample size of

50 crabs. Crab carapace width and shell hardness data were initially collected. The crabs were checked every other day to see if eggs had been extruded. At the end of the experiment, on December 19, 1993, the size of the egg masses from the crabs that had extruded eggs were measured.

Results

(I) Of the 31 egg masses, 16 had hatched prior to receiving, 10 hatched during the experiment, and 5 of the crabs died before the eggs hatched. Throughout the experiment 16 crabs died. This was not extremely important as many of the crab's eggs hatched out before death. A summary of the results are presented in Table 1. The distribution of crab sizes is presented in Figure 16.

(II) The molting and mortality data is shown in Table 2. The distribution of crab sizes is presented in Figure 17.

(III) The crab size, egg extrusion date and final egg mass size data are given in Table 3. There were three egg mass size catagories: full, some and none. Full refers to a mass that appeared maximal in size. Some describes a mass approximately one-fourth the size of a full egg mass. None means that there were only a few eggs or no eggs at all. The distribution of crab sizes is presented in Figure 18. The proportion of crabs $\geq$ 146 mm that extruded eggs was

Table 1. Data for crab size, zoeal hatch date and adult death date.

size (mm)	zoeal hatch date	adult death date	size (mm)	zoeal hatch date	adult death date
135*		1/26	150*		1/27
140		1/26	150		1/22
140*		3/10	151		1/29
140*			151	1/20	
140*		1/23	153*		2/5
141*			153*		1/25
142	2/6	2/8	155	2/5	
144*			155*		
145*		1/20	154	2/11	
145	1/15		156*		
145*			157		1/24
145*			158	1/20	2/3
145		1/26	159	1/12	4/14
147*			161	2/10	
150*			161	2/11	
150	2/15	3/11			

* egg mass hatched partially or fully prior to receiving.

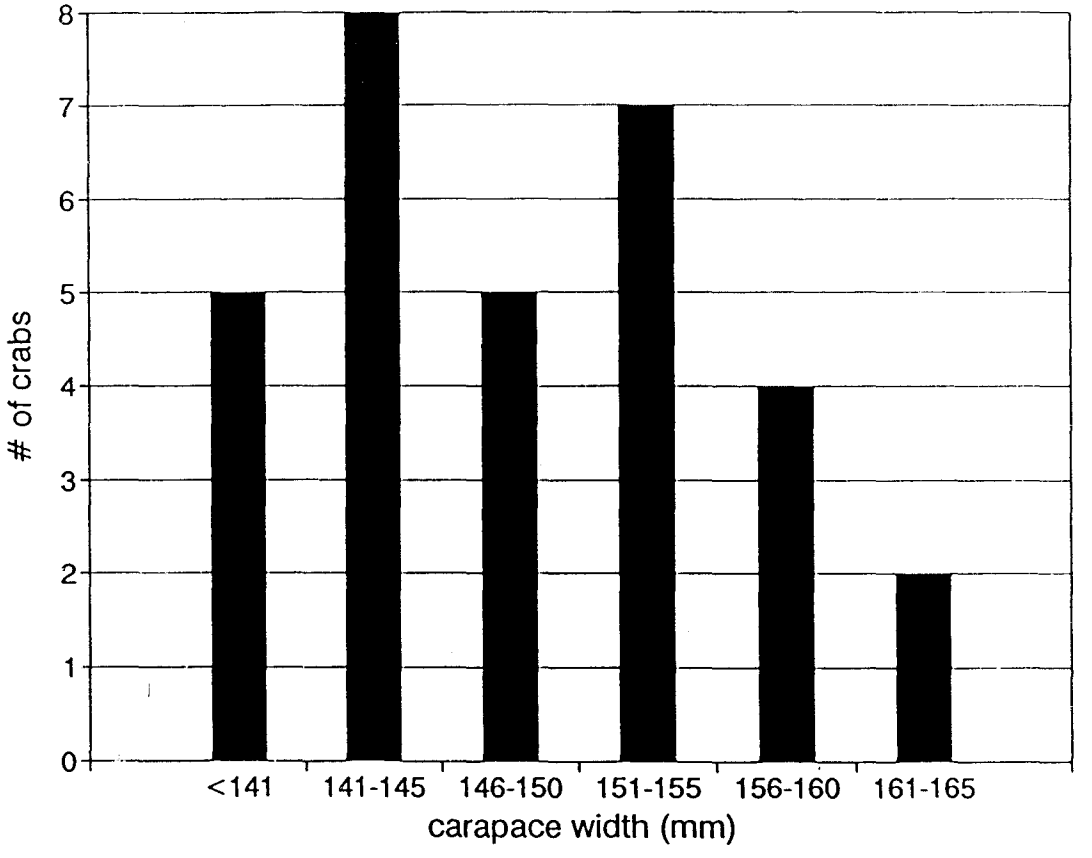


Figure 16. Crab size distribution for viability study (I).

Table 2. Data for crab size before and after the molt and date of death.

pre molt size (mm)	post molt size	death date	pre molt size (mm)	post molt size	death date	pre molt size (mm)	post molt size	death date
110		5/17	130	142		144		6/17
111		4/29	131		4/8	144		4/2
112		6/17	132		6/27	145		6/7
112		4/2	133		4/30	145		5/23
116	127		133	142		145		
116		5/20	133		5/11	146		7/1
117	127		134		6/10	147		4/28
119		7/1	134	143		148		4/8
121		5/24	135			151		7/9
122		4/27	135		6/7	152		7/11
122	140		135		5/17	153		
122		6/15	137	153		154		
124		4/26	138			154		
124		4/2	139	150	5/28	155		4/12
125	138	7/7	139		3/22	155		
125		4/28	139		5/31	156		
126			140			159		4/14
126			140		5/20	160		5/27
127	139	7/8	141	155	6/10	161		5/23
129		6/13	141	154		165		7/16
129		6/25	142			166		
130			144					

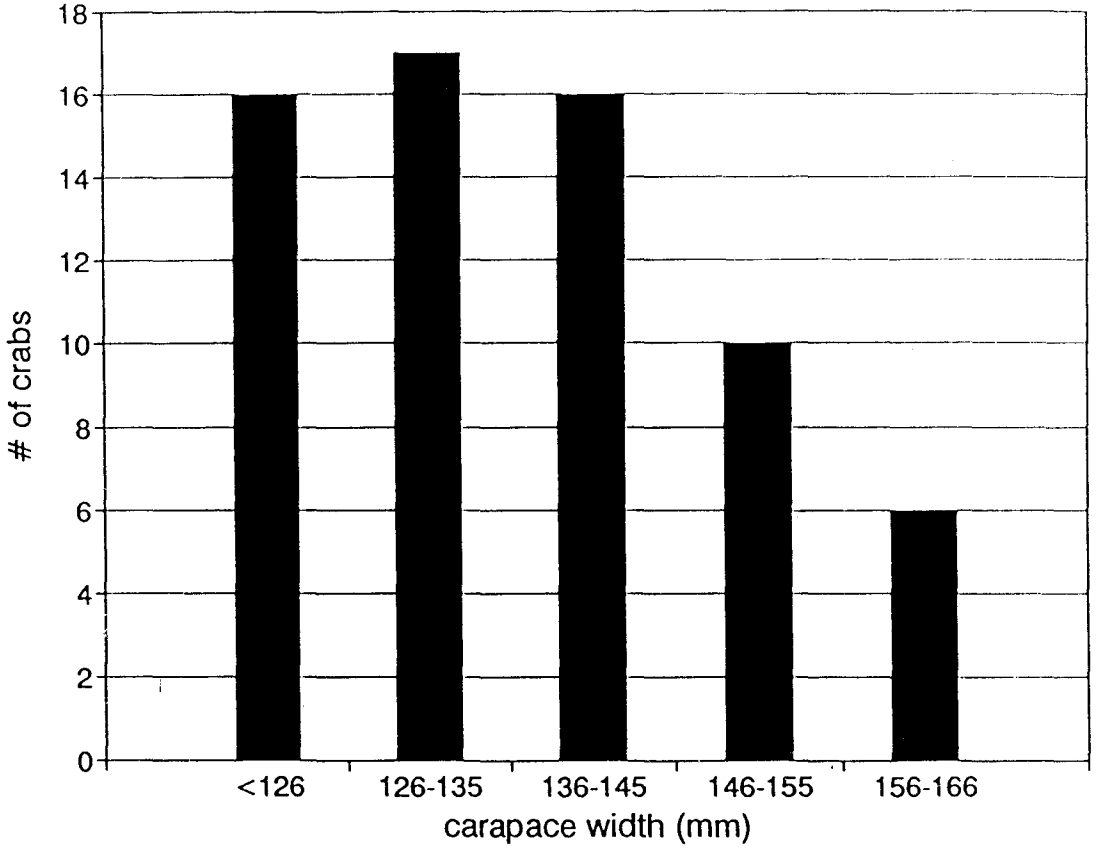


Figure 17. Crab size distribution for molting study (II).

Table 3. Data for crab size, egg extrusion date and final egg mass size.

size (mm)	extr. date	mass size	size (mm)	extr. date	mass size	size (mm)	extr. date	mass size
117	10/22	some	137	10/16	none	152	10/15	none
118			138	10/23	none	153	11/3	some
118			138	10/22	some	153	11/12	some
120*			139	10/20	none	154	10/18	none
120	10/28	none	140	11/9	some	154	10/29	none
122	10/19	some	140	10/19	full	156		
124			142	11/29	some	156	10/26	none
124	10/22	none	144*			157	10/31	none
125			146	10/16	none	158	10/27	none
127	12/3	none	147	10/20	some	158	10/24	none
127	10/28	none	147	10/29	none	159	11/23	full
128*	10/22	none	147	12/7	full	159	11/14	some
129	11/3	none	149	10/25	full	161	11/9	full
129	10/26	some	150	11/11	some	161	10/31	full
130			150	10/28	none	164		
130			151	11/15	some	166	11/9	some
131	10/15	none	151	10/27	some			

* died on 11/29, 11/6 and 12/14 respectively.

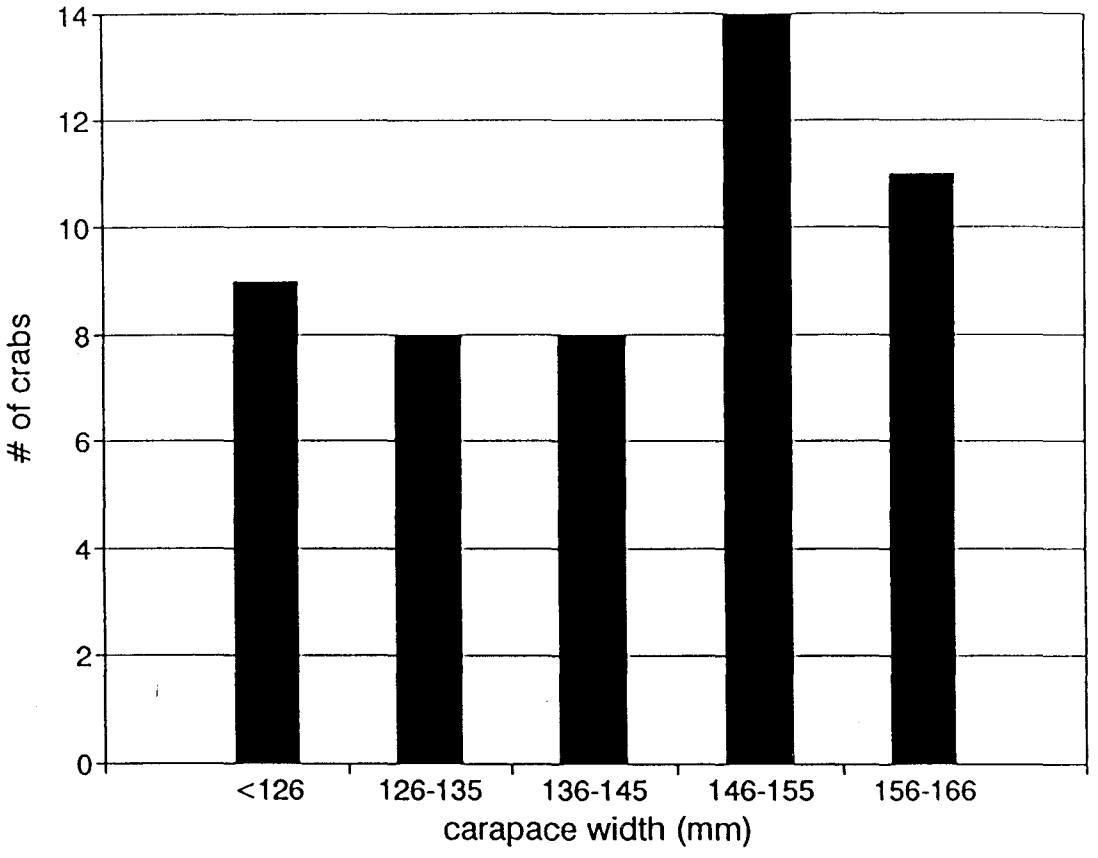


Figure 18. Crab size distribution for extrusion study (III).

significantly higher than the proportion of crabs < 146 mm that extruded eggs (Chi squared = 4.5, $p < 0.05$).

Discussion

(I) This part of the study took place from January 1, 1993 to March 20, 1993. Zoeal hatching normally begins sometime in December in the Coos Bay vicinity of the southern Oregon coast. Therefore many of the experimental crabs held egg masses that had already partially or completely hatched. These crabs with hatched egg masses were not immediately thrown out of the experiment because the egg masses on these crabs appeared full and were brown in color. Not until later, when the eggs were examined under microscope, was it determined that the putative egg masses were actually empty egg cases. Eggs from ten of the crabs, however, were observed to have hatched into zoeae in the lab. Of these ten crabs, eight were above the recreational harvest limit of 146 mm. Egg masses of the other 21 crabs either hatched out completely or partially prior to receiving or the crabs died before the eggs hatched. All of the crabs, therefore, ≥ 146 mm in size, had developing egg masses.

Part I shows that large female crabs do have the capability to successfully reproduce. With this information in hand, it would next be important to determine what

percentage of females ≥ 146 mm actually do extrude viable egg masses in the field and what percentage of the total female population is ≥ 146 mm. Such information would allow determination of the proportional contribution of these large females to annual larval crab production.

Analysis of Part I suggested the importance of increasing the sample size and beginning the observations earlier in the spawning season. Part III therefore included collecting random crabs without egg masses in October, holding the crabs to see what percentage of the crabs actually extruded egg masses and monitoring the complete cycle of egg development. Data correlating zoeal hatching numbers to female crab size would also be interesting. Results from Part I also indicated that for such a long term study it would be important to set up some form of sand filtration to prevent the sand layer from becoming anoxic.

(II) This study sought to collect laboratory data on different aspects of the molting of the female crab. The high mortality rate makes it difficult to make definitive statements. Even though the tanks were cleaned twice a week after feeding, it was difficult to keep the sand from becoming at least partially anoxic. This might have provided a stress on the animals that affected their normal molting pattern.

Among the 12 crabs that molted, none were above 141 mm. This adds to the data that large crabs are either more

likely to skip a molt or may go into terminal anecdysis.

Hankin's 1985 field and laboratory study is the only one that has been completed on female Dungeness crabs with a substantial sample size. The molt increments in this study were smaller overall than what Hankin observed/measured. This is likely because, in general, crabs from more northern latitudes and therefore colder temperatures, have been shown to have smaller molt increments (Mayer 1972; Hankin 1985). The timing of the annual molt in the Coos Bay area supposedly occurs in late spring. This is confirmed by this study as there was 1 molt in April, 9 molts in May and 2 molts in June.

It is interesting to note the high incidence of microsporidium parasite in crabs from the experiments. This parasite invades the muscle tissue and can take up to one year to finally kill the crab (Olson and Childers, 1993). In later stages of the disease, the white, stringy appearance of infected muscle distinguishes it from non-infected muscle. This muscle condition is visible through the arthrodial membranes at the joints of the crab's walking legs. Based on such visual inspection, approximately one third of the crab deaths in Part II could be attributed to this disease. A more thorough analysis would be required for a quantitative assessment of infection. If, however, such a high incidence of infection does occur in the wild, this parasite would have a tremendous impact on the overall

population of the crab.

(III) This part of the experiment explored what proportion of female crabs ≥ 146 mm extrude egg masses and is this proportion different from crabs < 146 mm. As shown in Table 5, 40 of the 50 crabs were observed to have extruded eggs. There were 25 crabs ≥ 146 mm and 25 crabs < 146 mm. Of the larger crabs, 92% extruded eggs while 68% of the smaller crabs extruded eggs. This serves as indirect evidence that a significant proportion of crabs ≥ 146 mm are successfully mating and reproducing and thus reemphasizes that large female crabs are important in the production of larval crabs. The final egg mass size data taken at the end of the experiment shows that only 6 of the 40 crabs that began to extrude eggs ended up carrying what appeared to be full egg masses. It was observed that in many cases eggs were dropped onto the sand without attachment of the eggs to the pleopods. Shields et al. report an average egg mass size of 3-4 million eggs for C. magister. Certain effects of the laboratory situation may have caused the small egg mass sizes.

An interesting follow-up to this set of experiments would be to determine the size and sex composition of the crab population during different seasons in order to know what percent of the female population is of a size ≥ 146 mm. In 1991 Smith and Jamieson did complete such a study at two different sites. However, data was collected at only one

time point for each site. Censuses taken during different seasons at the same site would be important in reaching a full understanding of the population's composition. Also, because commercial traps, which do not retain smaller crabs, were used a true census of the population was not obtained. Another interesting topic that has received little attention is that of migration patterns. Is there a sex specific movement of crab seasonally or even daily into and out of the bay? Answers to such questions are required if sound management decisions are to be made. The data presented in this preliminary study should serve as a positive stimulus for further investigations.

Summary

Part I examined the viability of egg masses spawned by female crabs ≥ 146 mm, the minimum recreational size limit. Crabs possessing egg masses were collected and held in separate aquaria. All of the crabs ≥ 146 mm had developing egg masses. Egg masses from 8 crabs ≥ 146 mm were observed to have hatched into zoeae. Thus, large female crabs are still capable of producing viable zoeae.

Part II investigated the molting of the female crab. There were 65 crabs that ranged in size from 110 mm to 166 mm in the study. Twelve of these crabs, ranging in premolt size from 116 mm to 141 mm, molted. Thirty-eight of the

crabs died during the experiment without having molted. None of the crabs ≥ 146 mm molted which supports the possibilities of longer molt cycles, skipped molts or terminal molts in large crabs.

Part III asked what proportion of female crabs ≥ 146 mm extruded egg masses, and does this proportion differ from crabs < 146 mm. The sample size was 50 and although 40 of the crabs extruded eggs, only 6 of the egg masses reached full size. The lab environment likely influenced the final egg mass sizes. Only 3 crabs died during this part of the study so that mortality was not an important factor. The proportion of crabs ≥ 146 mm that extruded egg masses was significantly higher than the proportion of crabs < 146 mm that extruded eggs ($p < 0.05$).

The results from Parts I and III demonstrate the reproductive importance of female crabs ≥ 146 mm and therefore support a management policy that disallows recreational or commercial harvesting of female crabs.

CHAPTER III

CONCLUDING SUMMARY

Hemoecdysin, a protein found in the hemolymph of the Dungeness crab, Cancer magister, undergoes extreme concentrational fluctuations in relation to the molt cycle. Hemocyanin, an oxygen-carrying protein, undergoes similar, less dramatic changes in hemolymph concentration. During premolt and early postmolt, when the new exoskeleton is being secreted by the hypodermis, there is a high level of hypodermal hemoecdysin. These results along with immunological evidence suggest that hemoecdysin functions as an integral exoskeletal protein. This is the first major report on the molt-related function of hemoecdysin.

The studies on hemoecdysin in Chapter I are augmented by additional studies on molting and reproduction in adult female Cancer magister. These studies, presented in Chapter II, showed that large females are still capable of producing viable zoeae and that the proportion of crabs that extrude eggs does not decrease with increasing crab size. Thus large female crabs play a significant role in the success of the crab fishery and should not be harvested.

BIBLIOGRAPHY

Chapter I

Bielefeld, M., G. Gellissen and K.D. Spindler (1986). Protein production and the moulting cycle in the crayfish Astacus leptodactylus-I. Stage-specificity of polypeptide patterns in the hypodermis and hepatopancreas. Insect Biochem. 16, 175-180.

Bliss, D.E. (1953). Endocrine control of metabolism in the land crab, Gecarcinus lateralis (Feminville). I. Differences in the respiratory metabolism of sinus glandless and eyestalkless crabs. Biol. Bull. 104, 275-296.

Bliss, D.E. (1963). The pericardial sacs of terrestrial Brachyura. Pages 59-78 in Phylogeny and Evolution of Crustacea H.F. Whittington and W.D.I. Rolfe, editors. Mus. Comp. Zool., Harvard Univ., Cambridge, Massachusetts.

Brouwer, M., M. Wolters and E.F.J. Van Bruggen (1976). Proteolytic fragmentation of Helix pomatia α-hemocyanin: Structural domains in the polypeptide chain. Biochemistry 15, 2618-2623.

Brusca, R.C. and G.J. Brusca (1990). Invertebrates. Sunderland, Sinauer Associates, p. 464.

Carlisle, D.B., and P.F.R. Dohrn (1953). Studies on Lysmata seticaudata Risso (Crustacea Decapoda). II. Experimental evidence for a growth- and moult-accelerating factor obtainable from eyestalks. Pubbl. Staz. Zool. Napoli 24, 69-83.

Charniaux-Cotton, H. (1985). Vitellogenesis and its control in malacostracan Crustacea. Amer. Zool. 25, 197-206.

Churchill, E.P., Jr. (1918). Life history of the blue crab. Fish. Bull. 36, 91-128.

Davis, B.J. (1964). Disc gel electrophoreses - II. Method and application to human serum protein. Ann. N.Y. Acad. Sci. 121, 404-427.

Dennell, R. (1947) The occurrence and significance of phenolic hardening in the newly formed cuticle of Crustacea Decapoda. Proc. R. Soc. B 134, 485-503.

Drach, P. (1939). Mue et cycle d'intermue chez les Crustaces Decapodes. Ann. Inst. Oceanogr. 19, 103-391.

Edwards, G.A. (1950). The influence of eyestalk removal on the metabolism of the fiddler crab. Physiol. Comp. Oecologie 2, 34-50.

Edwards, G.A. (1953). Respiratory Metabolism. Pages 96-146 in Insect Physiology, K.D. Roeder, editor. Wiley, New York.

Enderle, U., G. Kauser, K. Reum, K. Scheller, and J. Koolman (1983). Ecdysteroids in the hemolymph of blowfly larvae are bound to calliphorin. Pages 40-49 in The Larval Serum Proteins of Insects, K. Scheller, editor. New York, Thieme-Stratton.

Engel, D.W. (1987). Metal regulation and molting in the blue crab, Callinectes sapidus: copper, zinc, and metallothionein. Biol. Bull. 172, 69-82.

Engel, D.W. and M. Brouwer (1991). Short-Term Metallothionein and Copper Changes in Blue Crabs at Ecdysis. Biol. Bull. 180, 447-452.

Eriksson-Quensel, I.B. and T. Svedberg (1936). The molecular weights and pH stability regions of the hemocyanins. Biol. Bull. 71, 498-547.

Fairbanks, G., T.L. Steck, and D.F.H. Wallach (1971). Electrophoretic analysis of the human erythrocyte membrane. Biochemistry 10, 2606-2616.

Ghiretti-Magaldi, A., G. Tamino and B. Salvato (1975). The monophyletic origin of hemocyanins on the basis of the amino acid composition. Structural implications. Boll. Zool. 42, 167-179.

Grun, L., and M.G. Peter (1983). Selective crosslinking of tyrosine-rich larval serum proteins and of soluble Manduca sexta cuticle proteins by nascent N-acetyldopamine quinone and N-ß-alanyldopamine quinone. Pages 102-115 in The Larval Serum Proteins of Insects, K. Scheller, editor. Stuttgart, Thieme-Stratton.

Grun, L., and M.G. Peter (1984). Incorporation of radiolabeled tyrosine, N-acetyldopamine, N-ß-alanyldopamine, and the arylphorin manducin into sclerotized cuticle of tobacco hornworm (Manduca sexta) pupae. Z. Naturforsch. 39C, 1066-1071.

Hackman, R.H. (1972). Gel electrophoresis and Sephadex thin-layer studies of proteins from an insect cuticle, Agrianome spinicollis (Coleoptera). Insect Biochem. 2, 235-242.

Haunerland, N., and W. Bowers (1986). Binding of insecticides to lipophorin and arylphorin, two hemolymph proteins of Heliothis zea. Arch. Insect Biochem. Physiol. 3, 87-96.

Hopkins, T.L. and K.J. Kramer (1992). Insect cuticle sclerotization. Annu. Rev. Entomol. 37, 273-302.

Hopkins, P.M. (1983). Patterns of serum ecdysteroids during induced and uninduced proecdysis in the fiddler crab, Uca pugilator. Gen. Comp. Endocrinol. 52, 350-356.

Kaliafas, A.D., V.J. Marmares, and C. Christodoulou (1984). Immunocytochemical and electrophoretic studies on the localization of the major haemolymph proteins (ceratitins) in Ceratitis capitata during development. Roux's Arch. Dev. Biol. 194, 37-43.

Kanost, M.R., J.K. Kawooya, J. Law, R. Ryan, M. VanHeusden, R. Ziegler (1990). Insect haemolymph proteins. Advances in Insect Physiology 22, 299-396.

King, T., J. Jukes (1969). Nondarwinian evolution. Science 164, 788-798.

Koeppe, J.K., and L.I. Gilbert (1973). Immunochemical evidence for the transport of haemolymph protein into the cuticle of Manduca sexta. J. Insect Physiol. 19, 615-624.

Konig, M., O.P. Agrawal, H. Schenkel, and K. Scheller (1986). Incorporation of calliphorin into the cuticle of the developing blowfly, Calliphora vicina. Rou'x Arch. Dev. Biol. 195, 296-301.

Kozloff, E. (1990). Invertebrates. Orlando, Holt, Rinehart and Winston, p. 506.

Kumari, S.S., D.M. Skinner (1993). Proteins of crustacean exoskeleton II: Immunological evidence for their relatedness to cuticular proteins of two insects. J. Exp. Zool. 265, 195-210.

Laemmle, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227, 680-685.

Levenbook, L. (1985). Insect storage proteins. Pages 307-346 in Comprehensive Insect Physiology, Biochemistry, and Pharmacology, Vol. 10, G. Kerkut, and L. Gilbert, editors. Oxford, Pergamon.

Linzen, B., N. Soeter, A. Riggs, H.J. Schneider, and W. Schartau, et al. (1985). The structure of arthropod hemocyanins. Science 229, 519-524.

Lontie, R., M. DeLey, H. Robberecht and R. Witters (1973). Isolation of small functional subunits of Helix pomatia haemocyanin after subtilisin treatment. Nature New Biol. 242, 180-182.

Mangum, C.P. (1985). Molting in the blue crab Callinectes sapidus: a collaborative study of intermediary metabolism, respiration and cardiovasculr function, and ion transport. Preface. J. Crustacean Biol. 5, 185-187.

Mangum, C.P., B.R. McMahon, P.L. Defur, and M.G. Wheatly (1985). Gas exchange, acid-base balance, and the oxygen supply to the tissues during a molt of the blue crab, Callinectes sapidus. J. Crustacean Biol. 5, 188-206.

Markl, J., A. Markl, W. Schartau, and B. Linzen (1979). Subunit heterogeneity in arthropod hemocyanins: I. Chelicerata. J. Comp. Physiol. 130, 283-292.

Munn, E., G. Greville (1969). The soluble proteins of developing Calliphora erythrocephala, particularly calliphorin, and similar proteins in other insects. J. Insect Physiol. 15, 1935-1950.

Mykles, D.L. (1980). The mechanism of fluid absorption at ecdysis in the American lobster, Homarus americanus. J. Exp. Biol. 84, 89-101.

Nickerson, K.W. and K.E. Van Holde (1971). A comparison of molluscan and arthropod hemocyanin-I. Circular dichroism and absorption spectra. Comp. Biochem. Physiol. 39B, 855-872.

Nyst, R.H. (1941). Contribution a l'etude de l'hormone nymphogene. Am. Soc. Zool. Belg. 72, 74-104.

O'Brien, J.J., S.S. Kumari, and D.M. Skinner (1991). Proteins of crustacean exoskeletons: I. Similarities and differences among proteins of the four exoskeletal layers of four brachyurans. Biol. Bull. 181, 427-441.

Phillips, D.R., and B.G. Loughton (1976). Cuticle protein in Locusta migratoria. Comp. Biochem. Physiol. 55B, 129-135.

Poulson, D.F. (1935). Oxygen consumption of Drosophila pupae. I. Drosophila melanogaster. Zeitschr. vergl. Physiol. 22, 466-472.

Rao, K.R. (1968). The pericardial sacs of Ocypode in relation to the conservation of water, molting, and behavior. Am. Zool. 8, 561-567.

Scheller, K., H.P. Zimmerman, and C.E. Sekeris (1980). Calliphorin, a protein involved in the cuticle formation of the blowfly, Calliphora vicina. Z. Naturforsch 35C, 387-389.

Schneiderman, H.A. (1952). Variations in dehydrogenase activity during the metamorphosis of the Cecropia silkworm. Ph.D. Thesis, Harvard University.

Schneiderman, H.A., and C.M. Williams (1953). The physiology of insect diapause. VII. The respiratory metabolism of the Cecropia silkworm during diapause and development. Biol. Bull. 105, 320-334.

Scudamore, H.H. (1947). The influence of the sinus gland upon molting and associated changes in the crayfish. Physiol. Zool. 20, 187-208.

Skinner, D.M. (1962). The structure and metabolism of a crustacean integumentary tissue during a molt cycle. Biol. Bull. (Woods Hole, Mass.) 123, 635-647.

Skinner, D.M. (1985). Molting and Regeneration. Pages 43-146 in The Biology of Crustacea, Vol. 9, D.E. Bliss and L.H. Mantel, editors. Academic Press, New York.

Skinner, D.M., S.S. Kumari, and J.J. O'Brien (1992). Proteins of the crustacean exoskeleton. Am. Zool. 32, 470-484.

Spindler, K.D., Keller R. and O'Connor, J.D. (1980). On the mode of action of ecdysteroids in crustaceans. Pages 255-264 in Biosynthesis, Metabolism and Mode of Action of Invertebrate Hormones, J. Hoffmann and M. Porchet, editors. Springer-Verlag, Berlin.

Stevenson, J.R. (1985). Dynamics of the integument. Pages 1-42 in The Biology of Crustacea, Vol. 9, D.E. Bliss and L.H. Mantel, editors. Academic Press, New York.

Stringfellow, L.A., and D.M. Skinner (1988). Molt-cycle correlated patterns of synthesis of integumentary proteins in the land crab Gecarcinus lateralis. Dev. Bio. 128, 97-110.

Telfer W.H., P. Keim, and J. Law (1983). Arylphorin, a new protein from Hyalophora cecropia: Comparisons with calliphorin and manducin. Insect Biochem. 13, 601-613.

Telfer, W.H., and H. Massey (1987). A storage hexamer from Hyalophora that binds riboflavin and resembles the apoprotein of hemocyanin. Pages 305-314 in Molecular Entomolgy, J. Law, editor. New York, Alan R. Liss.

Telfer, W.H., and J.G. Kunkel (1991). The Function and Evolution of Insect Storage Hexamers. Annu. Rev. Entomol. 36, 205-228.

Terwilliger, N.B. and R.C. Terwilliger (1982). Changes in the subunit structure of Cancer magister hemocyanin during larval development. J. Exp. Biol. 221, 181-191.

Terwilliger, N.B. (1991). Hemocyanins in crustacean oocytes and embryos. Pages 31-39 in Crustacean Egg Production, A. Wenner and A. Kuris, editors. Rotterdam, A.A. Balkema Publishers.

Terwilliger, N.B. and K. O'Brien (1992). Cancer magister "non-respiratory protein" is a molt related hemocyanin. Amer. Zool. 32, 44A.

Travis, D.F. (1955). The molting cycle of the spiny lobster, Panulirus argus Latreille. II. Pre-ecdysial histological and histochemical changes in the hepatopancreas and integumental tissues. Biol. Bull. 108, 88-112.

Travis, D.F. (1960). Matrix and mineral deposition in skeletal structures of the decapod Crustacea. Pages 57-116 in Calcification in Biological Systems, R.F. Sognnaes, editor. AAAS, Washington, DC.

Truchot, J.P. (1992). Respiratory function of arthropod hemocyanins. Pages 377-404 in Advances in Comparative and Environmental Physiology, Berlin, Springer-Verlag.

Vacca, L.L., and M. Fingerman (1975a). The mechanism of tanning in the fiddler crab, Uca pugilator-I. Tanning agents and protein carriers in the blood during ecdysis. Comp. Biochem. Physiol. 51B, 475-481.

Vacca, L.L., and M. Fingerman (1975b). The mechanism of tanning in the fiddler crab, Uca pugilator-II. The cyclic appearance of tanning agents and attached carrier proteins in the blood during the molting cycle. Comp. Biochem. Physiol. 51B, 483-487.

Van Holde, K. and E.F.J. Van Bruggen (1971). The hemocyanins. Pages 1-55 in Biological Macromolecules, Vol. 5, Pt A., S.N. Timasheff and G.D. Fasman, editors. New York, Marcel Dekker.

Van Holde, K., K. Miller (1982). Hemocyanins. Q. Rev. Biophys. 15, 1-12.

Welinder, B.S. (1975b). The crustacean cuticle. III. Composition of the individual layers in Cancer pagurus cuticle. Comp. Biochem. Physiol. 52A, 659-663.

Willott, E., X. Wang and M.A. Wells (1989). cDNA and gene sequence of Manduca sexta arylphorin, an aromatic amino acid-rich larval serum protein. J. Biol. Chem. 264, 19052-19059.

Zuckerkandl, E. (1960). Hemocyanine et cuivre chez un Crustace Decapode dans leurs rapports avec le cycle d'intermue. Ann. Inst. Oceanogr. Monaco 38, 1-122.

Chapter II

Butler, T.H. (1960). Maturity and breeding of the Pacific edible crab, Cancer magister Dana. Journal of the Fisheries Research Board of Canada 17, 641-646.

Couch, J. (1837). Observations on some circumstances attending the process of Exuviation in shrimp and lobsters. Mag. Zool. Bot. 1, 170-173.

Freese, L., and C.E. O'Clair (1985). Condition of Dungeness crabs, Cancer magister, at a benthic deposit of decomposing bark; Physical trauma and reduced reproductive success. Pages 223-225 In B.R. Melteff, symposium coordinator. Proceedings of the symposium on Dungeness crab biology and management, October 9-11, 1984, Alaska Sea Grant Report 85-3, Anchorage.

Hankin, D. G., N. Diamond, M. Mohr, and J. Ianelli (1985). Molt increments, annual molting probabilities, fecundity and survival rates of adult female Dungeness crabs in northern California. Pages 189-206 In B.R. Melteff, editor. Proceedings of the symposium on Dungeness crab biology and management. University of Alaska, Alaska Sea Grant Report 85-3, Fairbanks.

Hankin, D.G., N. Diamond, M.S. Mohr, and J. Ianelli (1989). Growth and reproductive dynamics of adult female Dungeness crabs (Cancer magister) in northern California. Journal du Conseil, Conseil International pour l'Exploration de la Mer 46, 94-108.

Mayer, D. L. (1972). Synopsis of biological data on the Dungeness crab Cancer magister (Dana, 1852) with emphasis on the gulf of Alaska. Pages 287-312 In D.H. Rosenberg, editor. A review of the oceanography and renewable resources of the northern Gulf of Alaska. Alaska Sea Grant Report 73-3, Fairbanks.

Miller, R. J., and P. G. O'Keefe (1981). Seasonal and depth distribution, size, and molt cycle of the spider crabs. Chionecetes opilio, Hyas coarctatus in a Newfoundland Bay. Can. Tech. Rep. Fish. Aquat. Sci. 1003, 4 + 18p.

Olson, B. and R. Childers (1993). A bad taste. Oregon's Agricultural Progress Summer/Fall, 19-23.

Shields, J.D., R.K. Okazaki and A.M. Kuris (1991). Fecundity and the reproductive potential of the Yellow Rock crab Cancer anthonyi. Fishery Bulletin, U.S. 89, 299-305.

Skinner, D.M. (1985). Molting and Regeneration. Pages 43-146 In The Biology of Crustacea, Vol. 9, D.E. Bliss and L.H. Mantel, editors. Academic Press, New York.

Smith, B.D., and G.S. Jamieson (1991). Possible consequences of intensive fishing for males on the mating opportunities of Dungeness crabs. Transactions of the American Fisheries Society 120, 650-653.

Snow, C.D., and J.R. Neilsen (1966). Premating and mating behavior of the Dungeness crab (Cancer magister Dana). Journal of the Fisheries Research Board of Canada 23, 1319-1323.

Wild, P.W. (1980). Effects of seawater temperature on spawning, egg development, hatching success, and population fluctuations of the Dungeness crab, Cancer magister. CalCOFI Rep. 21, 115-120.

Wild, P.W. (1983). Comparisons of ovary development in Dungeness crabs, Cancer magister, in central and northern California. Pages 189-196 In P.W. Wild and R.N. Tasto, editors. Life history, environment, and mariculture studies of the Dungeness crab, Cancer magister, with emphasis on the central California fishery resource. California Department of Fish and Game, Fish Bulletin 172.