EFFECTS OF FOOD LEVELS AND TEMPERATURE ON GROWTH AND HEMOCYANIN ONTOGENY IN THE JUVENILE DUNGENESS CRAB, *CANCER MAGISTER*

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Title: EFFECTS OF FOOD LEVELS AND TEMPERATURE ON GROWTH AND HEMOCYANIN ONTOGENY IN THE JUVENILE DUNGENESS CRAB, *CANCER MAGISTER*

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Dr. Nora B. Terwilliger

Juveniles of *Cancer magister* were raised from megalopas in warm (21°C) or cold (14°C) seawater and fed either high or low levels of food for 6 months. Carapace width at each molt indicated that crabs reared in cold water and high food attained the largest sizes per molt. Intermolt period was shorter in crabs fed high food levels; within this group crabs raised in warm water had shorter molt cycles than those raised in cold. Hemolymph protein levels and the onset of adult hemocyanin (Hc) were determined using pH 7.4 PAGE and SDS PAGE. Levels of 16S Hc were highest in low food, warm water: cryptocyanin was higher in high food crabs. Onset of adult Hc was found to be neither stage specific nor time dependent. This study suggests that increased food availability has a greater effect on growth of juveniles of *C. magister* than elevated temperature.

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CHAPTER I

INTRODUCTION

The juvenile phase of the life cycle of the Dungeness crab, Cancer magister (Dana) is either spent in shallow estuarine zones or in coastal waters. In the estuary, salinity levels change with the tide, food resources are variable, and large temperature changes may occur seasonally, daily and diurnally (Leffler 1972; Newell 1976). Young crabs must have the ability to compensate for this environmental variability in order to survive in a wide range of habitats. If an organism can tolerate change, the possible benefits derived from an estuarine existence include refuge from larger cannibalistic conspecifics, and accelerated growth as a result of more abundant food supplies and warmer temperatures than are found in offshore waters (Botsford and Wickham 1978; Stevens et al. 1982).

The populations of juvenile C. magister crabs that inhabit nearshore coastal waters experience lower temperatures and presumably decreased food availability (Tasto 1983; Armstrong and Gunderson 1985). These field studies found growth rates of C. magister to be substantially slower in colder nearshore coastal waters than in warmer estuarine waters. Development of C. magister either inside or outside the estuary raises interesting questions regarding the influence of environmental factors on development. It is important to conduct empirical studies of crab physiology and development, to gain an

understanding of the mechanisms that allow juveniles to exploit coastal estuaries or colder nearshore waters.

The primary questions addressed by this investigation are first, how flexible is growth in juveniles of C. magister in response to environmental conditions? And two, is the expression of adult Hc fixed to a specific developmental stage? In the present investigation, juveniles of C. magister were reared from megalopas under controlled conditions in the laboratory to study the effects of temperature and food limitation on growth and development. Growth was measured in terms of intermolt period and size at molt, and development was monitored by observed changes in the subunit composition of the hemocyanin of C. magister as determined by gel electrophoresis. Specifically, I hypothesized that increased temperature and increased food availability would accelerate growth, molting, and the appearance of adult hemocyanin.

Life History

The ability of C. magister to exist in both estuarine and nearshore environments is due in part to its life cycle. Cancer magister shows tremendous diversity in body structure, mode of feeding, and habitat range during its life cycle. Mating usually occurs in coastal locations in late spring to early fall when intermolt males search out premolt females through a pheromonal homing system (Pauley et al. 1986). Several days prior to the female's molt, the male clasps the female in a mating embrace. The male usually stays with the female until after she molts; he deposits spermatophores in her spermatothecae while her exoskeleton is still soft. Fertilization occurs as the female

extrudes the eggs in the autumn. In Oregon, females usually carry embryos (up to 1.5 million) from October to January, when young C. magister zoeae hatch (Rudy and Rudy 1983). The five swimming zoeal stages occur in nearshore waters and progressively move offshore. Larval duration is lengthy, ranging anywhere from 45 to 158 days depending on environmental conditions (Jamieson and Phillips 1988). During its planktonic existence as a zoea and then as a megalopa, C. magister feeds on phytoplankton, is photopositive to moderate light (Jacoby 1982), and can exhibit diel vertical migration (Hobbs and Botsford 1992). The zoeae metamorphose into megalopas and settle to the bottom (Lough 1976) in either bays and estuaries or nearshore waters. Lough (1976) observed that substantial numbers of C. magister megalopas first appeared off Newport, Oregon in mid-April and most zoeae were megalopa by early May.

The C. magister megalopa is a swimming-crawling phase which molts into a first juvenile instar on the benthos. Growth and molting continue and it eventually becomes a benthic adult after 11 successive molts (Gutermuth and Armstrong 1989). The juvenile crabs scavenge for fish, mollusks, and crustaceans (Butler 1954) in shallow coastal waters, and large numbers live in eelgrass (Zostera spp.) beds (Stevens and Armstrong, 1984). Large numbers of juvenile C. magister were found in the upper reaches of the South Slough National Estuarine Research Reserve, Charleston OR, during a distribution study just before the start of this study (Dumler, personal observation, summer 1994).

Effect of Food Levels and Temperature on Crab Physiology

Maintaining an efficient food and oxygen supply, in a habitat where food levels and temperatures fluctuate, is essential to survival. The effects of food and temperature resources on growth of C. magister (Reed 1969; Stevens et al. 1984; Oresanz and Gallucci 1988; McMillan et al. 1995), and respiration of C. magister (McMahon et al. 1978; McMahon 1986; Sulkin 1989; Gutermuth and Armstrong 1989; DeWachter and McMahon 1996) have been widely studied. Most of these studies, however, have concentrated on larval and adult crabs even though it is the juvenile stages that regularly experience the extremes found in intertidal areas and the shallow margins of the oceans.

Indirect effects of temperature changes on salinity tolerance may further stress crabs already coping with the direct effects of temperature on metabolism. One study found juvenile crab growth to be significantly affected by the interaction of temperature and salinity in Menippe mercenaria (Wang and Truesdale 1991). In contrast, another study on the same species found that both factors affected the proportion of juveniles that survived; however, temperature but not salinity significantly affected molt frequency of juveniles (Brown 1991). A third study reported that blue crabs (Callinectes sapidus) acclimated to cold temperature (10°C) consumed more oxygen at low salinity than at high salinity (Laird and Haefner 1976). In the present study, salinity was held constant to simplify the study and isolate the effects of temperature and food availability.

Growth

Laboratory studies explored the physiological effects of temperature and food limitation on molting rate and size in crustaceans (Shen 1935; Démeusy 1958; Bückmann and Adelung 1964; Adelung 1971; Leffler 1972; Klein-Bretler 1975). A general pattern of crab growth can be summarized from these investigations. The intermolt period lengthened as the crab aged. Molting was accelerated at higher temperatures and also with greater food supply. In these studies, increases in crab size per molt was generally found to be less at higher temperatures than at lower temperatures, and food availability had a small but demonstrable effect.

Other studies on free-living crabs have examined the connection between environmental conditions and crab growth. Higher rates of growth have been reported for juvenile crabs of C. magister settling in estuaries and shallow embayments compared to the same year-class found in adjacent coastal waters (Tasto 1983; Collier 1983; Stevens and Armstrong 1984; Armstrong and Gunderson 1985; Warner 1987; Guttermuth and Armstrong 1989; Gunderson et al. 1990). A recent study in Puget Sound, Washington linked post-settlement growth rates to seasonal water temperatures (McMillan, et al. 1995). The authors followed the population density, habitat use and growth of two cohorts of intertidal juvenile Dungeness crabs at five sites over several years. Cohorts settling earlier in the year had an average carapace width of 7.2 mm as juvenile first instars. They grew rapidly at summer water temperatures in excess of 15°C and quickly reached sizes of greater than 30 mm carapace width (CW). These juveniles emigrated

from intertidal to subtidal areas by September. Individuals in the later cohort were smaller (5.3 mm CW) and exhibited little growth during decreasing autumn water temperatures. These crabs overwintered in the intertidal with growth rates increasing in March and subtidal emigration occuring 10 months after settlement in April and May. Post-settlement growth rates corresponded to seasonal water temperatures, being greatest for the coastal cohort that settled in May and June. Variations in food levels or diets were not examined.

Not surprisingly, restricted diets affect growth and development of crustaceans. Studies of the effects of nutrition on larval development (see McConaugha 1985 for review) have found food intake regulates intermolt duration and size-at-molt (Anger 1984). On a cellular level, one study on region-specific growth during segment development in Artemia found that growth processes were dependent on the level of nutrients and were enhanced by diets enriched in polyunsaturated fatty acids (Freeman 1990).

Respiration and Metabolism

Respiration rates have been used to determine the physiological means by which adult C. magister (Prentice and Schneider 1979) and juvenile C. magister (Gutermuth and Armstrong 1989) acclimate to thermal stress. The concentration of O_2, activity level of the organism, season, starvation, sex, and water temperature are determining factors of metabolic rates in crustaceans (Vernberg and Vernberg 1972). Changes in gill structure and oxygen requirements during development of C. magister have been documented

(Gutermuth and Armstrong 1989; Brown 1991). When exposed to a specific temperature (6°C, 10°C, 14°C, or 18°C) for three days, juvenile age Cancer magister had a temperature-dependent metabolic response. The metabolic rate or oxygen consumption (Q_{O2}) of very early instar crabs increased from 6°C to 14°C with a $Q_{10} = 2.3$ but remained constant from 14°C to 18°C ($Q_{10} \approx 1$) (Gutermuth and Armstrong 1989). The authors suggested this was evidence that metabolic costs do not become excessive as small crabs exploit warmer water for faster growth. According to the authors, this pattern of increased metabolic rate with temperature was in agreement with a marked difference in sizes of estuarine (35mm CW) versus offshore developing (12mm CW) crabs of the same year class. They concluded that elevated metabolic rates for crustaceans acclimated to higher temperatures (ie. increased respiration and growth rates) would be advantageous for early instar juveniles in a nutrient-rich habitat such as an estuary.

Hemocyanin Structure

The hemolymph of C. magister contains hemocyanin (Hc), a copper-based respiratory protein which is responsible for delivering oxygen to the tissues. Arthropod hemocyanins are present either as hexamers of 6 polypeptide chains or as multiples of these hexamers. On a finer level of structural resolution, the polypeptide chains or subunits represent a heterogeneous class of similarly sized proteins (reviewed in van Holde and Miller 1995). The hemocyanin of C. magister is composed of 6 subunits (1 hexamer) or 12 subunits (2 hexamer aggregate) with sedimentation coefficients of 16S and 25S respectively (Ellerton et al. 1970; Carpenter and van Holde 1973; Larson et al.

1981). Oxygen transport proteins with multisubunit structures generally have greater cooperativity and allosteric regulation, which are necessary for efficient delivery and uptake of oxygen (Savel-Neimann et al. 1988).

Studies have characterized the function and structure of the hemocyanin molecule of adult C. magister (Ellerton et al. 1970; McMahon 1986; Larson et al. 1981). The 25S two-hexamer and 16S hexamer fractions of the hemocyanin molecule occur throughout the crab's life cycle (Terwilliger and Terwilliger 1982). Recently, a third non-respiratory protein also present during ontogeny has been described in hemolymph of C. magister using gel electrophoresis (Otoshi 1994); however, the function of this protein is not yet known.

Investigations have also centered specifically on the function and structure of hemocyanin of C. magister during development (Terwilliger and Terwilliger 1982; Terwilliger et al. 1986; Brown and Terwilliger 1992; Terwilliger and Brown 1993). Megalopal and juvenile hemocyanin of C. magister have an oxygen affinity 50% lower than that of adult hemocyanin under identical experimental conditions (Terwilliger et al. 1986). In addition to functional changes, the structure of the hemocyanin molecule was found to change during the life cycle of C. magister (Terwilliger and Terwilliger 1982). Megalopa and juvenile hemocyanins differ from adult hemocyanin in that they lack one of the adult polypeptide chain subunits (subunit 6), and the relative concentrations of two of the other chains (subunit 4 and 5) are not the same as in the adult. Under controlled conditions of approximately 10°C and a regulated diet, the onset of adult hemocyanin

synthesis as indicated by the appearance of subunit 6 mRNA was found to occur by the sixth juvenile instar (Durstewitz 1996).

Increases in temperature reduce the solubility of oxygen in the water and reduce the oxygen-carrying capacity of the hemolymph. In cold-blooded animals this is of physiological significance because increased temperature is usually accompanied by an increased metabolic rate (or need for oxygen). It is therefore advantageous for the crab in one respect, that hemocyanin deliver oxygen more readily at higher temperatures. An increase in temperature weakens the bond between hemocyanin and oxygen, and causes an increased dissociation of the bond resulting in the hemocyanin giving up oxygen more readily at the tissue (Schmidt-Nielsen 1990). On the other hand, increased temperature results in decreased loading of oxygen at the gills.

Research has shown that changes in subunit composition of crustacean hemocyanins can occur in response to environmental changes such as season, oxygen level, and salinity (reviewed in van Holde and Miller 1995). For example, sex and temporal (time of year) differences in the subunit composition of the Hc of the lobster Palinuris elephas were observed (Bellelli et al. 1985). Also, Mangum and Rainer (1988) provided more direct evidence of the connection between environmental changes and subunit composition by demonstrating that changes in subunit composition of Callinectes sapidus Hc occurred in response to changes in oxygen and salinity levels. Decreases in the availability of food resources for protein synthesis as well as increased temperatures are two more parameters that would likely alter the structure of the hemocyanin molecule of crustaceans.

CHAPTER II

MATERIALS AND METHODS

Collection of Megalopas

The late megalopa stage of C. magister appear in the surface waters near the mouth of the Coos Bay estuary, Oregon, in spring. Megalopas were collected from the plankton by dipnet in the evening as they swarmed around docklights on the outer floating docks of the Charleston boat basin. Megalopas for this study were collected on June 25, 1995, and all experiments were started within 24 hours after transport in cold seawater to the laboratory at the Oregon Institute of Marine Biology, (OIMB).

Laboratory Culture Conditions

In order to investigate whether temperature and food limitation affect development of juvenile instars, crabs were raised in the laboratory under controlled conditions for 6 months. Two seawater tables that each contained 30 megalopas were maintained at average temperatures of 14°C and 21°C respectively, in the same room. Both seawater tables had a continuous supply of running, unfiltered, aerated seawater at a salinity of 30 ppt-33 ppt, pumped on an incoming tide from near the mouth of Coos Bay. These seawater tables had been used exclusively for many years, as holding tanks for live marine invertebrates, and were "well-seasoned" with running seawater before the

introduction of the experimental megalopas. All developmental stages of C. magister were exposed to natural light/dark cycles in these same holding facilities.

Seawater for the cold seawater table (C) was piped directly into the table at an average ambient temperature of 14°C. Seawater for the 21°C seawater tables was first piped into a glass aquarium, heated with four Hartz immersion heaters and allowed to overflow into the water table. This warm seawater table (W) was slowly brought up to experimental temperature over a period of two days after megalopas were added to the seawater table. Temperature was measured with a field thermometer (Fisher Scientific) twice daily in each of the tables, once in the morning and once in the evening.

In the seawater table, each crab was housed individually in a plastic container (8cm x 8cm x 12cm) with screens on 2 opposing sides. The containers were weighted down with small glass specimen dishes and were positioned so that the screens were aligned perpendicular to the direction of the water flow. Each crab was allowed freedom of movement within its container, and all of the containers were maintained in the same physical position in their respective water tables for the duration of the study.

The low and high temperatures selected for this study were based on data obtained the previous summer (1994) from field stations at the head (ie. riverine input) of the estuary, South Slough National Estuarine Research Reserve (SSNERR), Charleston, OR and at the mouth Coos Bay, OR. (Figure 1). Temperatures were recorded throughout the study at these two sites for comparison with the laboratory temperatures. A Yellow Springs Instruments (YSI 6000) meter measured bottom temperatures at Sengstacken

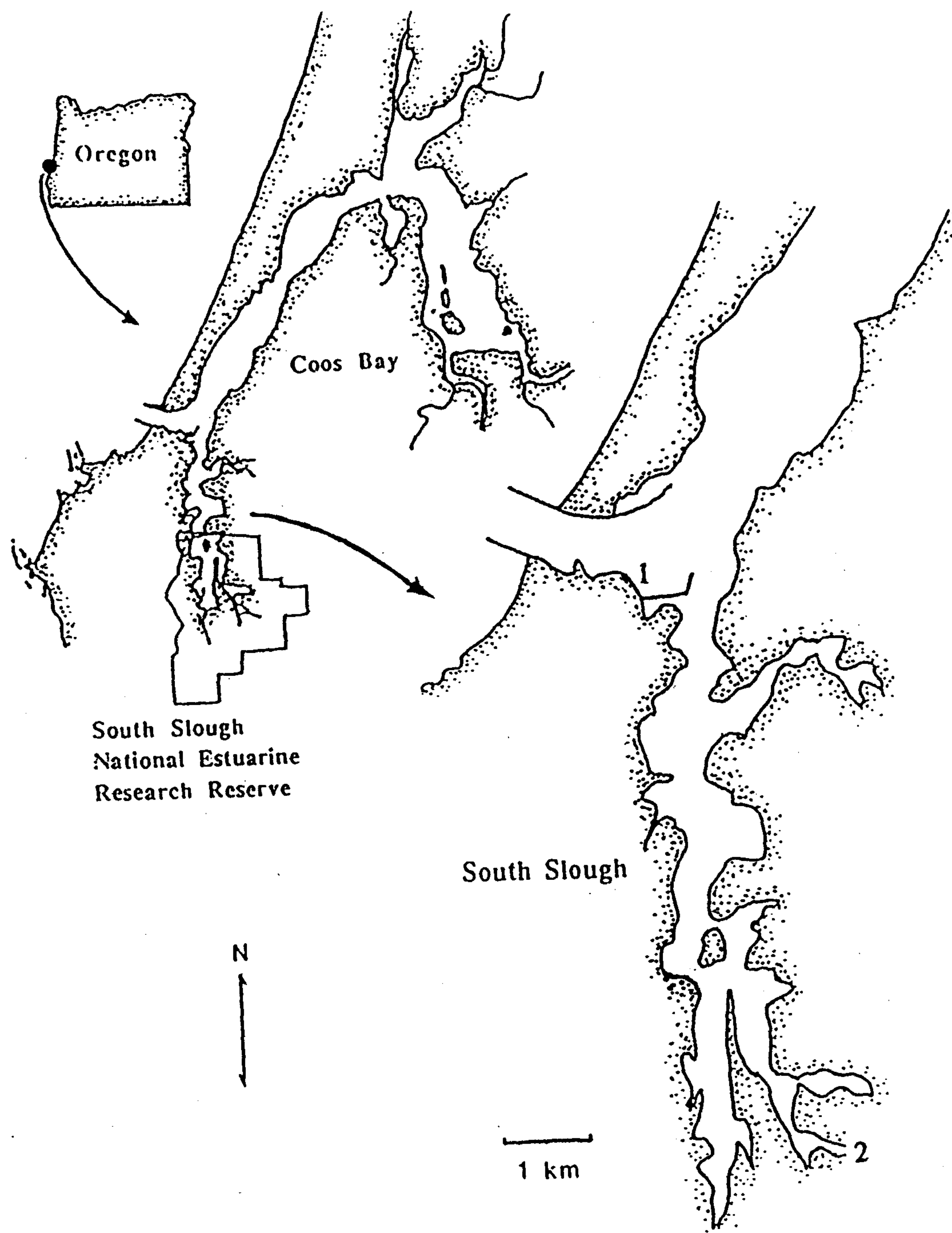
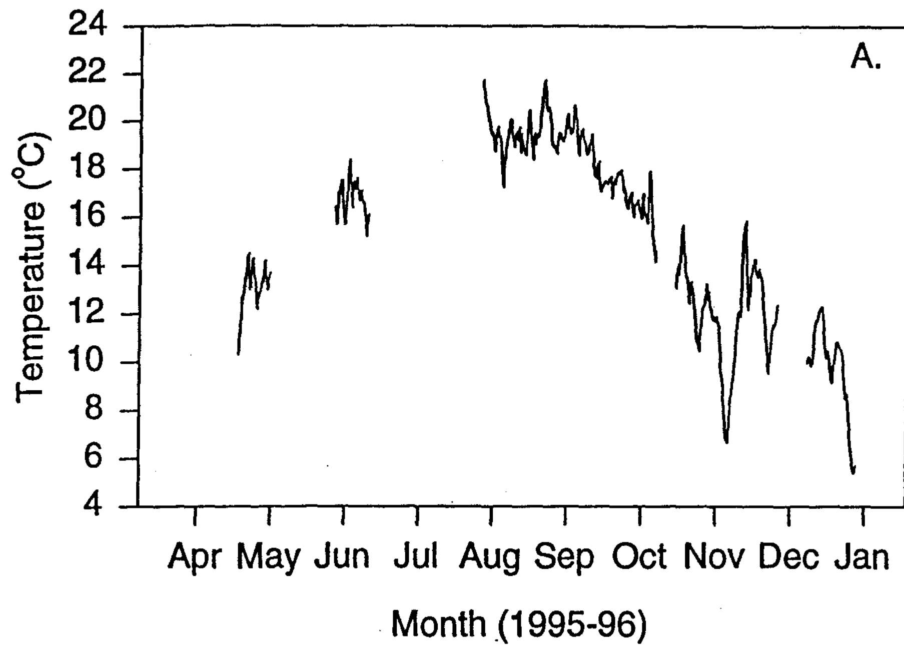


Figure 1. Study sites for temperature monitoring within Coos Bay and South Slough National Estuarine Research Reserve (SSNERR), Charleston, OR. 1-Boathouse Dock, Oregon Institute of Marine Biology; 2- Sengstaken Arm, SSNERR

Arm (SSNERR) (Figure 2). Another YSI meter was used to record surface temperatures during slack tide at the Boathouse Dock, Oregon Institute of Marine Biology (OIMB) (Figure 3). Due to variability of open-flow systems, the laboratory water temperatures ranged from 10°C to 17°C in the cold water table and 17°C to 25°C in the warm water table but maintained an average difference of 7°C throughout the study period (Figure 4).

Crabs in each of these temperature regimes were randomly subdivided into two groups which received either high levels of food (HF) or low levels of food (LF); 15 crabs were assigned at random to each group. Sex was externally indeterminate at the start of the study. When sex of juvenile crabs could be discerned later during the study, numbers of males and females were approximately equal. The treatment groups were designated as: cold, high food (CHF), cold, low food (CLF), warm, high food (WHF), and warm, low food (WLF). Juveniles in the high food groups (CHF and WHF) were fed an unlimited diet of fresh mussel (Mytilus edulis) (bits of mantle approximately 3mm x 3mm) daily, for the duration of the study. Those in the low food groups (CLF and WLF) were every fourth day from the start of the study on the same diet as above but were allowed to feed for only four hours during the designated feeding day. Brachyuran crabs are known predators on mollusks (Elner 1978), therefore Mytilus edulis was used. Little evidence exists for the reingestion of cast exuviae post-molt in crustaceans (Wheatley and Gannon 1995), and no information is available for C. magister. Assuming the cast exuviae in crustaceans is a possible source of dietary calcium, only the dorsal carapace was removed from the containers in the present study so that crabs were allowed access to



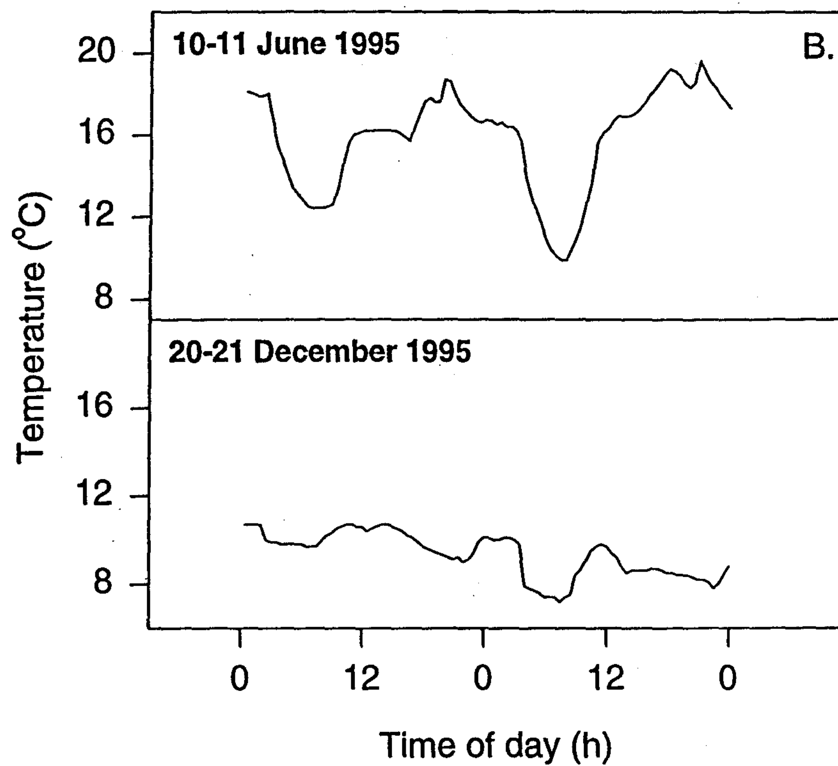


Figure 2. Bottom seawater temperatures (°C) at SSNERR, Charleston, Oregon recorded throughout the six month study (A). Gaps in the data reflect periods when data was downloaded. Examples of diurnal temperature cycling (B) over two day periods during the spring and winter 1995 (lower graph).

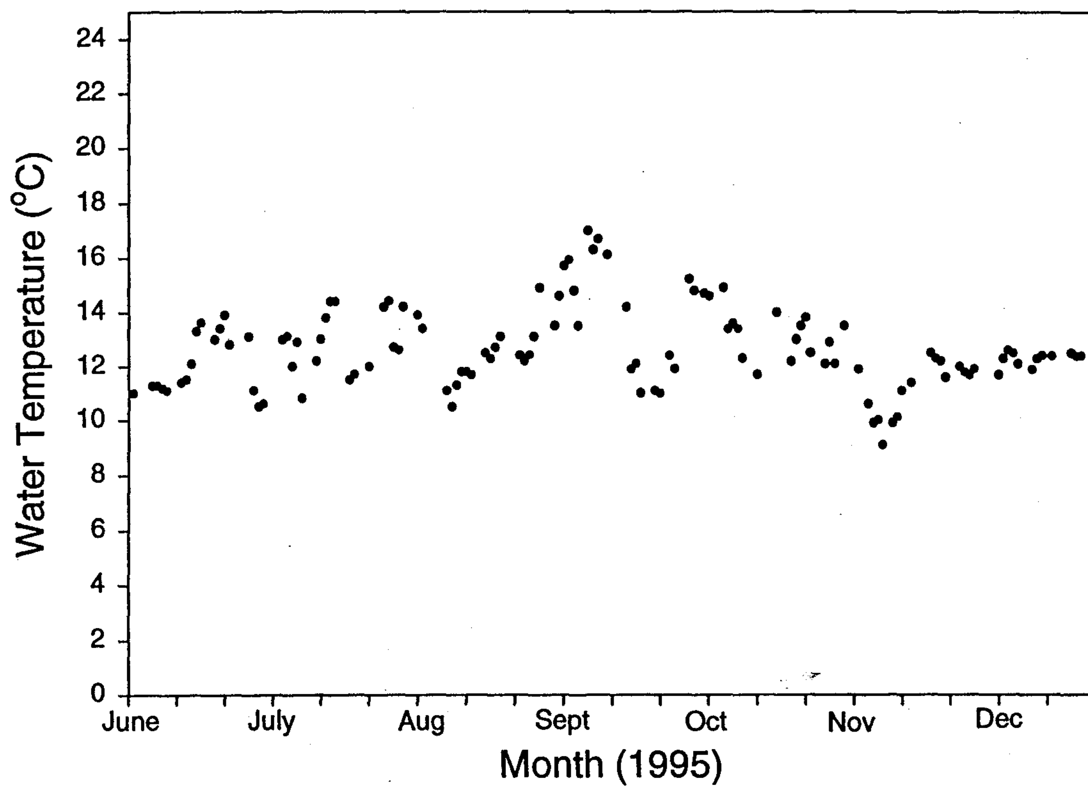


Figure 3. Surface seawater temperatures (°C) at high tide, Boathouse Dock, Oregon Institute of Marine Biology, Charleston, OR.

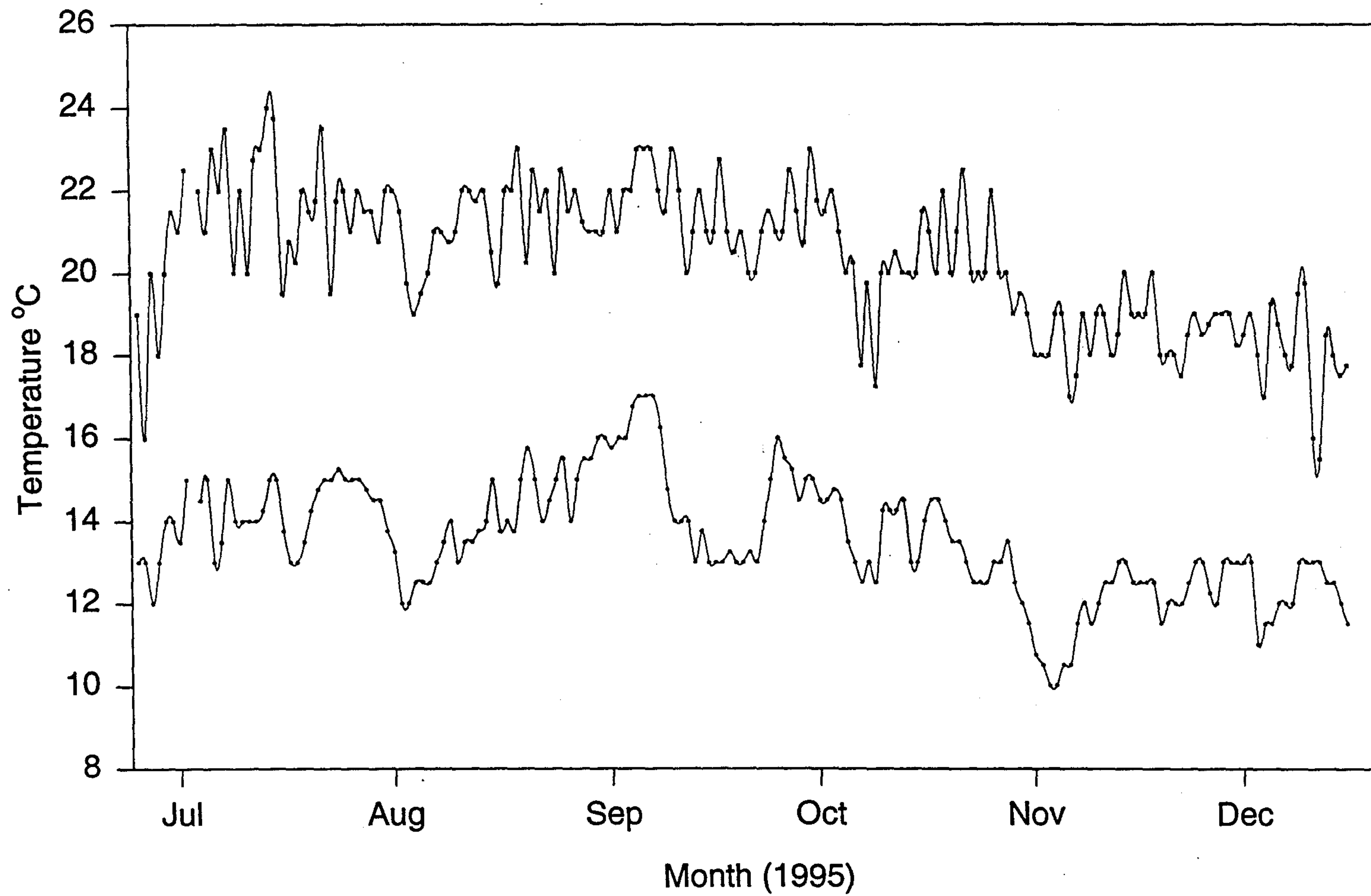


Figure 4. Mean seawater temperatures (°C) recorded in the laboratory: warm seawater table (above) and cold seawater table (below).

the exoskeleton as a possible nutritional source. Most exoskeletons from crabs in the present study disappeared within a few days and may have been ingested.

Morphological Data

Crabs were surveyed daily, and data was collected for every stage (instar) of growth. The date of molt (ecdysis) was noted, and the size of the individual was recorded on the day of each molt and 2 days post-molt. Carapace width (CW) was measured to the nearest 0.1mm with calipers across the carapace, between the notches just anterior to the tenth anterolateral spines.

Hemolymph Samples

Hemolymph samples were collected once a week from juvenile crabs until fifth instar, when samples were collected twice a week. Hemolymph samples (approximately 2ul) from second instar juveniles and older were withdrawn by micro-capillary pipets from the sinus at the base of a walking leg. Hemolymph was allowed to coagulate for 30 minutes on ice and then centrifuged in an Eppendorf microcentrifuge (4°C) at 8000 rpm for 2 minutes. The supernatant was divided into an aliquot for SDS PAGE and an aliquot for pH 7.4 PAGE.

Electrophoresis

All hemolymph samples collected over the course of the study were analyzed using a modified protocol described by Terwilliger and Terwilliger (1982). Hemolymph

aliquots were separated by pH 7.4, 5% polyacrylamide gel electrophoresis (7.4 PAGE) (Davis 1964) and 7.5% discontinuous sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS PAGE) (Laemmli 1970). The pH 7.4 PAGE is a non-denaturing gel run at physiological pH.

pH 7.4 PAGE

Polyacrylamide gel electrophoresis (pH 7.4 PAGE) was used to analyze the structure of the intact hemocyanin molecule. Whole hemolymph aliquots (approximately 2 uL) were diluted 1:1 with 0.1 M Tris/HCL (pH 7.5), 0.1 M in NaCl, 10 mM/L in $CaCl_2$, and 10 mMol/L in $MgCl_2$; half of the diluted aliquot was used for the pH 7.4 PAGE and the other half for SDS. pH 7.4 samples included approximately 20% glycerin to prevent convection anomalies in the wells, and 6ul of sample was added to each well. Hemolymph was electrophoresed on 5% polyacrylamide slab gels (37:1 acrylamide: bisacrylamide) at 35 mAmps for 3 hours. Electrophoresis buffer systems included: lower gel buffer, 0.05 M Tris-HCL (pH 6.8); upper gel buffer, 1M Tris-maleate (pH 7.4). Whole hemolymph of C. magister was used as a calibrant. Gels were stained with Coomassie brilliant blue R (Fairbanks et al. 1971) and destained with 10% acetic acid. Gels were dried between cellulose sheets and stored at room temperature.

JAVA Protein Quantification

The relative amounts of 25S hemocyanin (Hc), cryptocyanin (Cc) and 16S hemocyanin (Hc) protein present in each hemolymph sample was quantified using video

image analysis software (JAVA, Jandel Scientific). Images of pH 7.4 non-denaturing gels were captured using identical camera settings and magnification. Each protein band was outlined, the background subtracted, and the area and average intensity measured. Area intensity units (AIU) were calculated using the equation: ((255-average intensity) X area)/ 10,000. This equation results in a workable number which inverts the grayscale so that larger numbers equal high intensities (ie. normally the maximum value 255 equals white, and zero equals black). Values of zero were considered visually undetectable. To compare between treatment groups at any given point in time, sampling dates for individuals were assigned to the nearest 20% of the molt cycle. Values clustering around 10% (0-20% molt cycle) were placed in the first interval, values clustering around 30% (21-40% molt cycle) were placed in the second interval and so on. These intervals were then listed chronologically within each instar.

SDS PAGE

Hemolymph aliquots, diluted as above, were used for the SDS procedure (Laemmli 1970). The aliquots were mixed 1:1 with an SDS incubation buffer to yield final concentrations of 2% SDS, 0.05 M dithiothreitol (DTT), 10% glycerin, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 62.5 mM Tris-HCL (pH 6.8), and 0.01% bromophenol blue. All samples were heated in a boiling water bath for 1.5 minutes and then stored at -20°C until they were analyzed by SDS PAGE the next day. Four ul samples were loaded in each gel lane. The running buffer (pH 8.3) contained 0.025M Tris-OH, 0.192 M glycine, 0.1% SDS, and 0.1M EDTA. Acrylamide slab gels (7.5%)

were electrophoresed at 100V for about 4 hours and stained with Coomassie brilliant blue R (Fairbanks et al. 1971). Gels were destained with 10% acetic acid. All protein bands present were recorded immediately after the destaining procedure to avoid the possibility of bands fading with time. Gels were dried between cellulose sheets and stored at room temperature.

Statistical Analyses

Data were examined for normality using Kolmogorov-Smirnov (Lilliefors) one sample test. Homogeneity of variances was tested using the Dunn-Sidak method. The K-S Lilliefors test for normality within temperature groups and between food groups indicated that measurements of carapace width were normally distributed. Intermolt period was, in most cases, normally distributed with the exception of 3 cases: cold water/high food group at 4th instar, warm water/low food group at 3rd instar, and warm water/high food group at 4th instar. Comparisons of carapace width measurements and intermolt period were reported using a two-sample t-test if they were normally distributed or using a Mann-Whitney U-test statistic in each of the groups above which did not meet the assumptions for a normal distribution (Zar 1984). Separate variance t-tests were conducted because variances were unequal. The Dunn-Sidak method, employed because multiple t-tests were conducted, requires an adjusted significance level for each individual test to reduce the experiment-wise error rate for k number of comparisons (Zar 1984). Few individuals molted beyond 5th instar in the low food group therefore sample sizes beyond this instar were too small for a comparison between food groups.

Apparent differences between cold and warm treatment groups are discussed but statistical analyses were not performed because of potential pseudoreplication resulting from the necessary separation of temperature treatments into different seawater tables. Even though both seawater tables were well-seasoned, it remains a possibility that differences unrelated to temperature may exist between groups exposed to warm and cold conditions.

The Kolmogorov-Smirnov one sample t-test (Lilliefors) determined that the data for onset of adult Hc were not normally distributed with the exception of number of weeks to onset of adult Hc for high and low food groups in cold water. Between food-group comparisons of adult hemocyanin onset, measured by instar and number of elapsed weeks, were performed using the Mann-Whitney U test statistic for non-parametic analyses (Zar 1984).

CHAPTER III

RESULTS

Size-at-Instar

Effect of Food Levels

Data on individual carapace width at each instar for each treatment (CHF, CLF, WHF, and WLF) are shown in Figure 5. Mean CW for each treatment group by instar is shown in Table 1 and Figure 6. The effect of food on size within each temperature group was determined for each instar (two sample t-test or Mann-Whitney U statistic). In both the cold and warm seawater tables, crabs in the high food group were significantly larger than those in the low food group for 3rd instar through 5th instar (Table 2 and Figure 7). Sizes were not statistically different in 1st and 2nd instar juveniles. Carapace width became progressively larger with each instar within a treatment group.

Effect of Temperature

When compared between similar feeding regimes, crabs reared in the cold seawater table were consistently larger at a given instar than those reared in the warm seawater table (Figure 8). The difference in carapace width measurements between temperature groups increases dramatically after 4th instar.

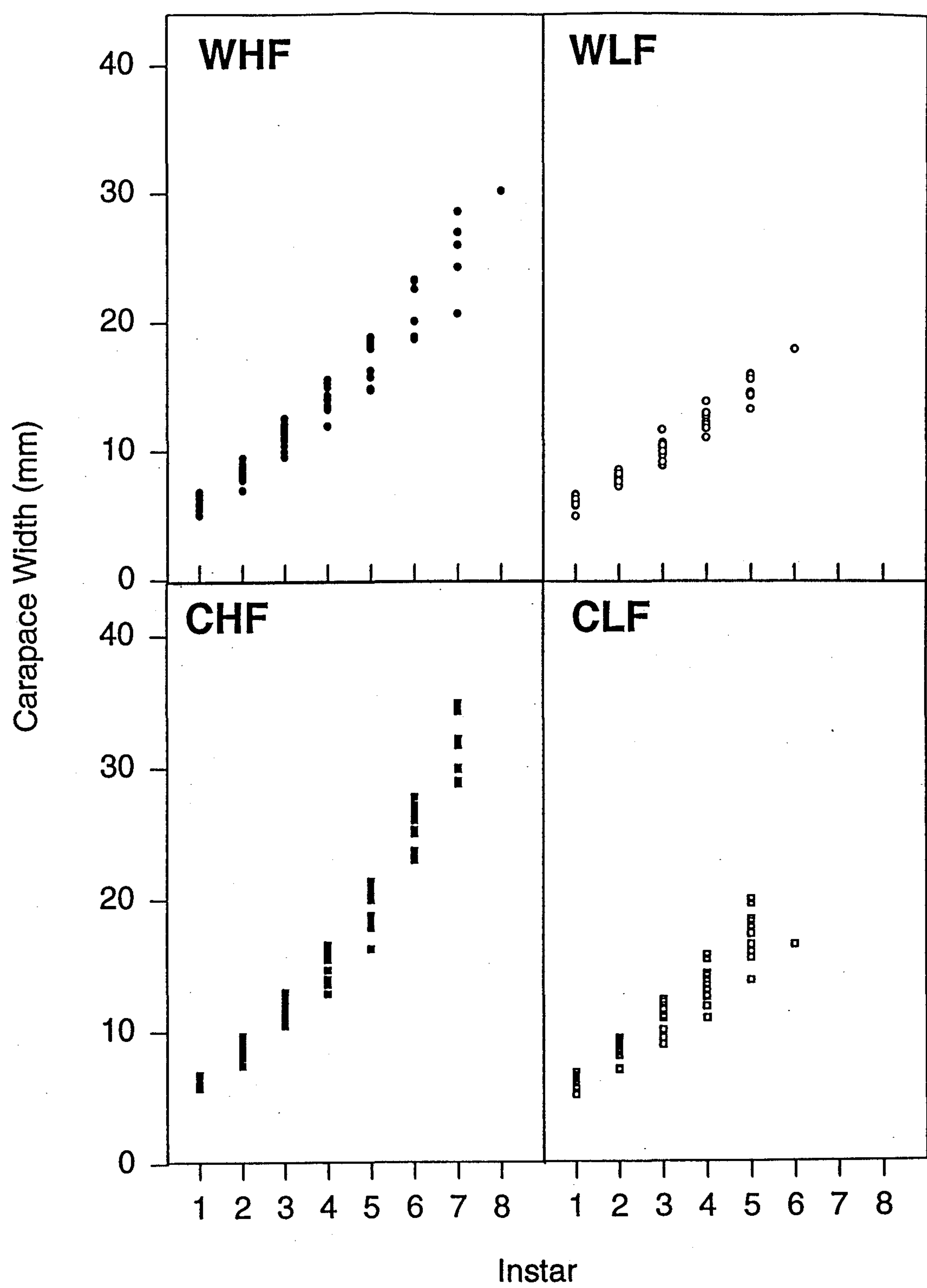


Figure 5. Effects of food levels and temperature on size (CW, 0.1mm) of individual C. magister juveniles (CHF- cold, high food; CLF- cold, low food; WHF- warm, high food; WLF- warm, low food). Each crab is represented by a symbol.

Table 1. Mean (± S.E.M) intermolt duration (days) and mean size (CW, 0.1mm) of juvenile Cancer magister at each instar for each treatment group (CHF-cold, high food: CLF-cold, low food: WHF-warm, high food:WLF-warm, low food)

Group	1st Instar		2nd Instar		3rd Instar		4th Instar		5th Instar		6th Instar		7th Instar	
	Days	Size	Days	Size	Days	Size	Days	Size	Days	Size	Days	Size	Days	Size
CHF	14.9	6.3	16.8	9.0	20.8	12.1	22.6	15.5	34.9	20.1	46.4	25.3		31.8
± s.e.m.	0.5	0.1	0.9	0.2	0.4	0.2	1.7	0.2	2.0	0.3	1.9	0.4		0.7
CLF	24.8	6.3	32.5	8.6	34.1	10.8	39.8	13.5	45.0	17.1		16.5		
± s.e.m.	0.8	0.1	1.4	0.2	0.9	0.3	1.2	0.4	n=1	0.5		n=1		
WHF	10.3	6.1	14.5	8.5	16.1	11.4	20.9	14.0	30.0	17.4	51.6	21.2	34.0	25.2
± s.e.m.	0.5	0.1	0.6	0.2	0.9	0.3	2.4	0.4	5.6	0.6	5.7	0.8	n=1	1. 4
WLF	36.1	6.1	30.7	8.0	37.2	10.1	36.0	12.4	45.0	14.9		17.9		
± s.e.m.	1.9	0.1	1.5	0.1	2.5	0.2	1.9	0.2	n=1	0.3		n=1		

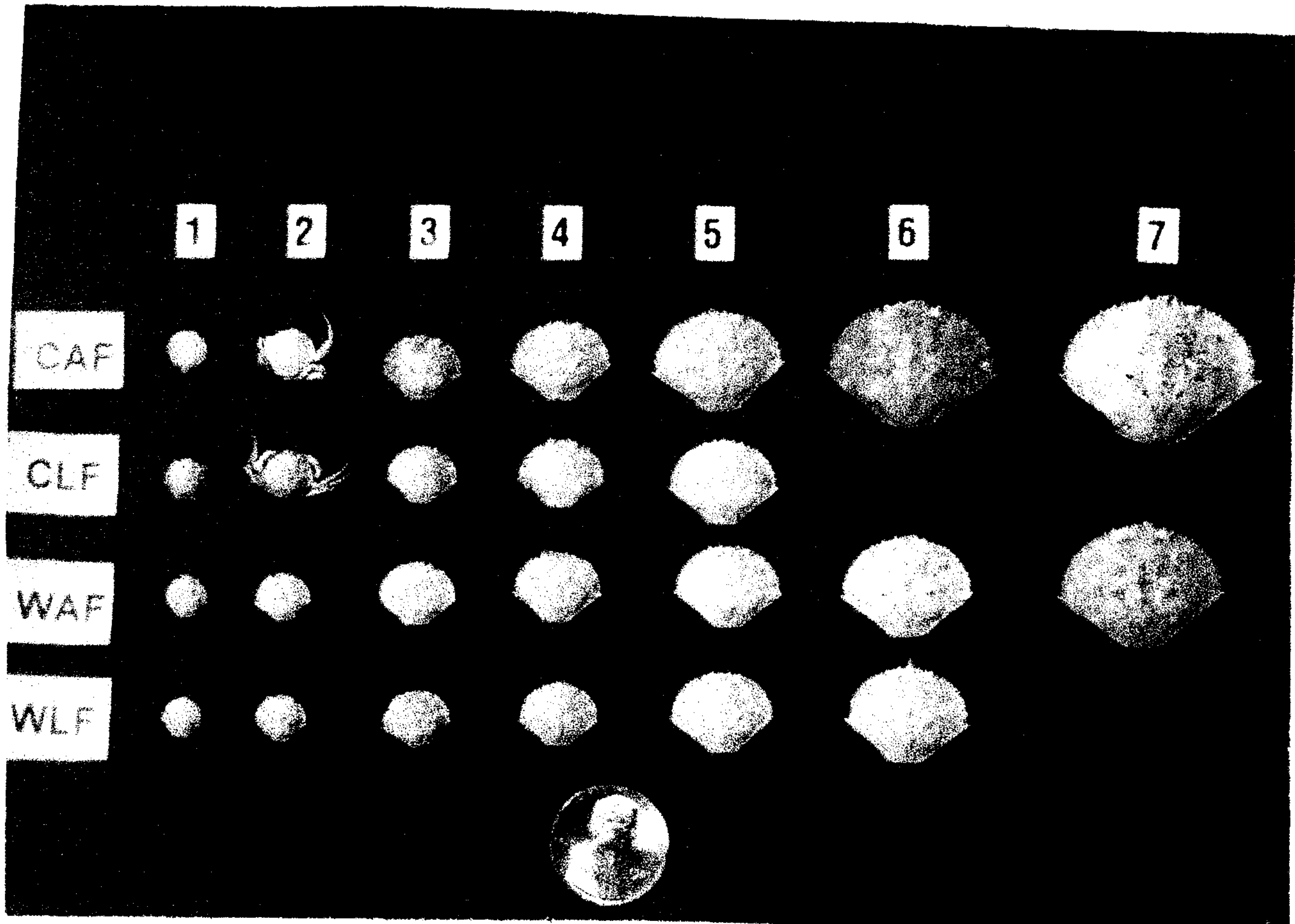


Figure 6. Mean size (CW, 0.1mm) of C. magister juveniles at each instar for each of the four treatment groups (CHF- cold, high food; CLF- cold, low food; WHF- warm, high food; WLF- warm, low food). CAF and WAF were later renamed CHF and WHF, respectively.

Table 2. Effects of food levels on size at a specified instar and intervals between molts. Mann-Whitney U-test (U) or t-tests (t) are used where appropriate. Probabilities (p) are calculated for individual t-tests; * indicates those p that are less than the adjusted Dunn-Sidak α' (i.e., $p < 0.05$)

	Cold Water Group				Warm Water Group			
	Size, $\alpha' < 0.010$		Intermolt, $\alpha' < 0.013$		Size, $\alpha' < 0.010$		Intermolt, $\alpha' < 0.013$	
Instar	t	U	t	U	t	U	t	U
1	0.09 ($p = 0.928$)		10.34* ($p < 0.001$)		0.226 ($p = 0.823$)		12.96* ($p < 0.001$)	
2	1.17 ($p = 0.252$)		9.28* ($p < 0.001$)		2.679 ($p = 0.901$)		10.17* ($p < 0.001$)	
3	3.54* ($p = 0.002$)		13.41* ($p < 0.001$)		3.953* ($p = 0.001$)			100.0* ($p < 0.001$)
4	4.57* ($p < 0.001$)			211.0* ($p < 0.001$)	3.698* ($p = 0.002$)			66.0* ($p = 0.002$)
5	4.98* ($p < 0.001$)				3.863* ($p = 0.003$)			

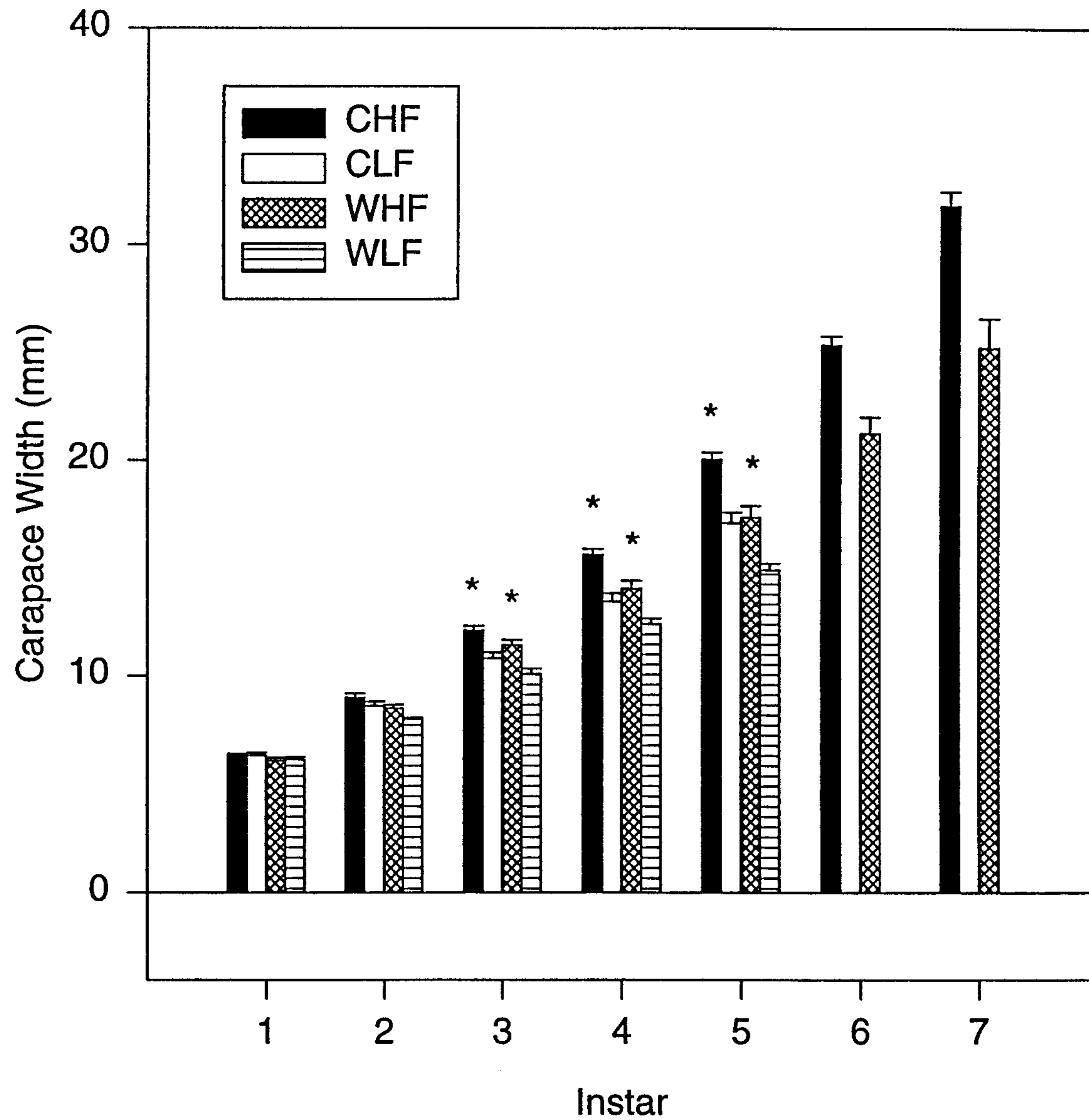


Figure 7. Bar graph of mean size (CW, 0.1mm) (± S.E.M.) of C. magister juveniles in each treatment group (CHF- cold, high food; CLF- cold, low food; WHF- warm, high food; WLF- warm, low food). Numbers of surviving crabs at 4th instar are: CHF (15), CLF (13), WHF (9), and WLF (11).

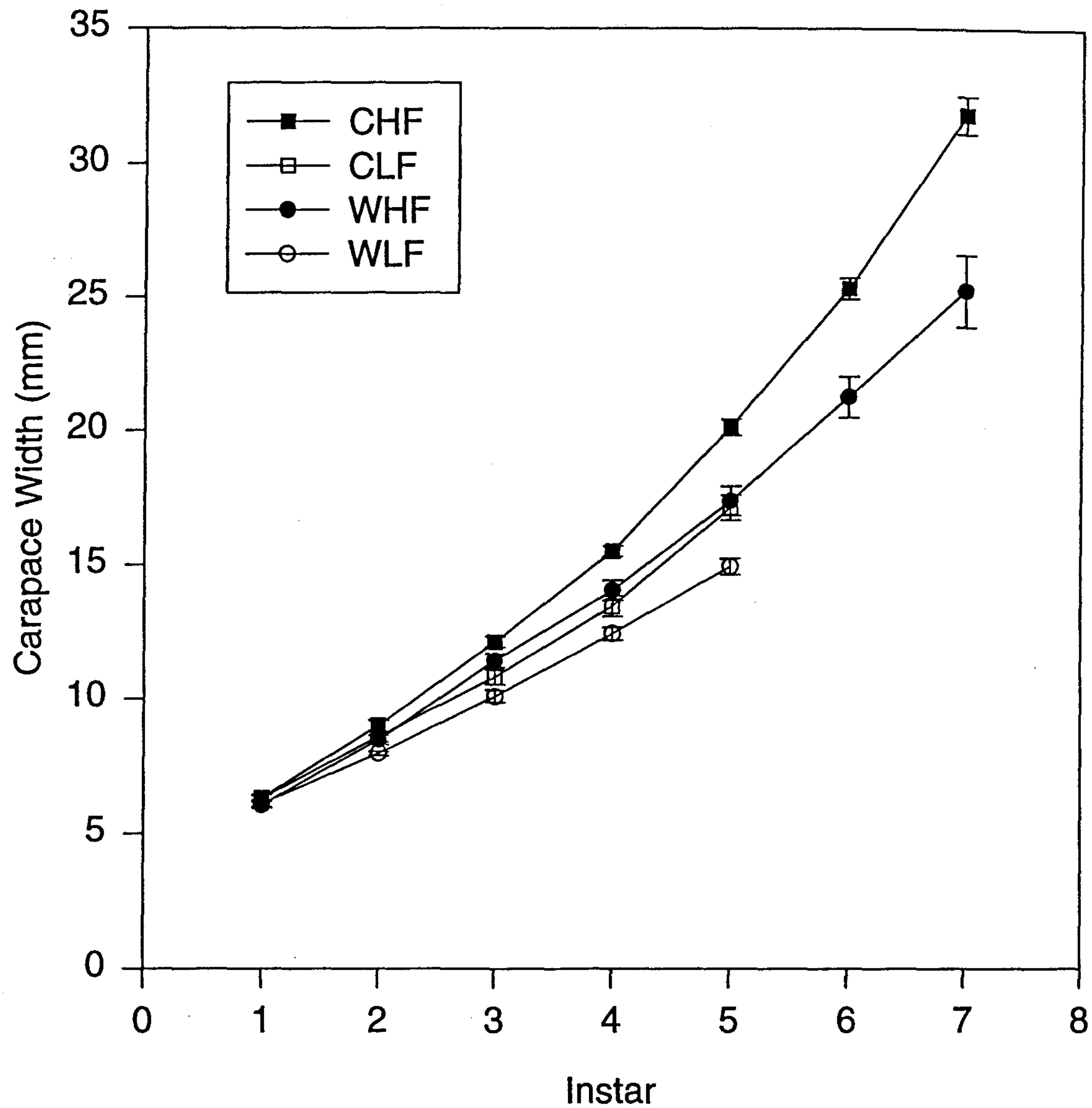


Figure 8. Line graph of mean size (CW, 0.1mm) (± S.E.M.) of C. magister juveniles in each treatment group (CHF- cold, high food; CLF- cold, low food; WHF- warm, high food; WLF- warm, low food). Numbers of surviving crabs at 4th instar are: CHF (15), CLF (13), WHF (9), and WLF (11).

Molting Rate

Effect of Food Levels

Growth is described not only by size-at-instar but also by molting rate. Here, molting rate is defined in terms of the intermolt period (i.e., days between molts). Data is absent for the later instars in the CLF and WLF groups because the experiment was stopped before they reached the next instar, not because mortality rates were high for these groups. Data on individual intermolt length at each instar for each treatment (CHF, CLF, WHF, and WLF) is shown in Figure 9. One outlier, present in the CHF group, was excluded from analyses due to an extremely protracted 4th instar (approximately four times longer than average). Mean intermolt lengths for each treatment group by instar are shown in Table 1 and graphed in Figure 10. The effect of food on intermolt length within each temperature group was determined for each instar (two sample t-test or Mann-Whitney U statistic). Comparisons of intermolt length were significant between food groups within each temperature for 1st through 4th instars (Table 2). In both cold and warm groups, crabs fed abundant amounts of food had significantly shorter intermolt periods than crabs whose diet were limited. Intermolt period became progressively longer with each instar within a treatment group.

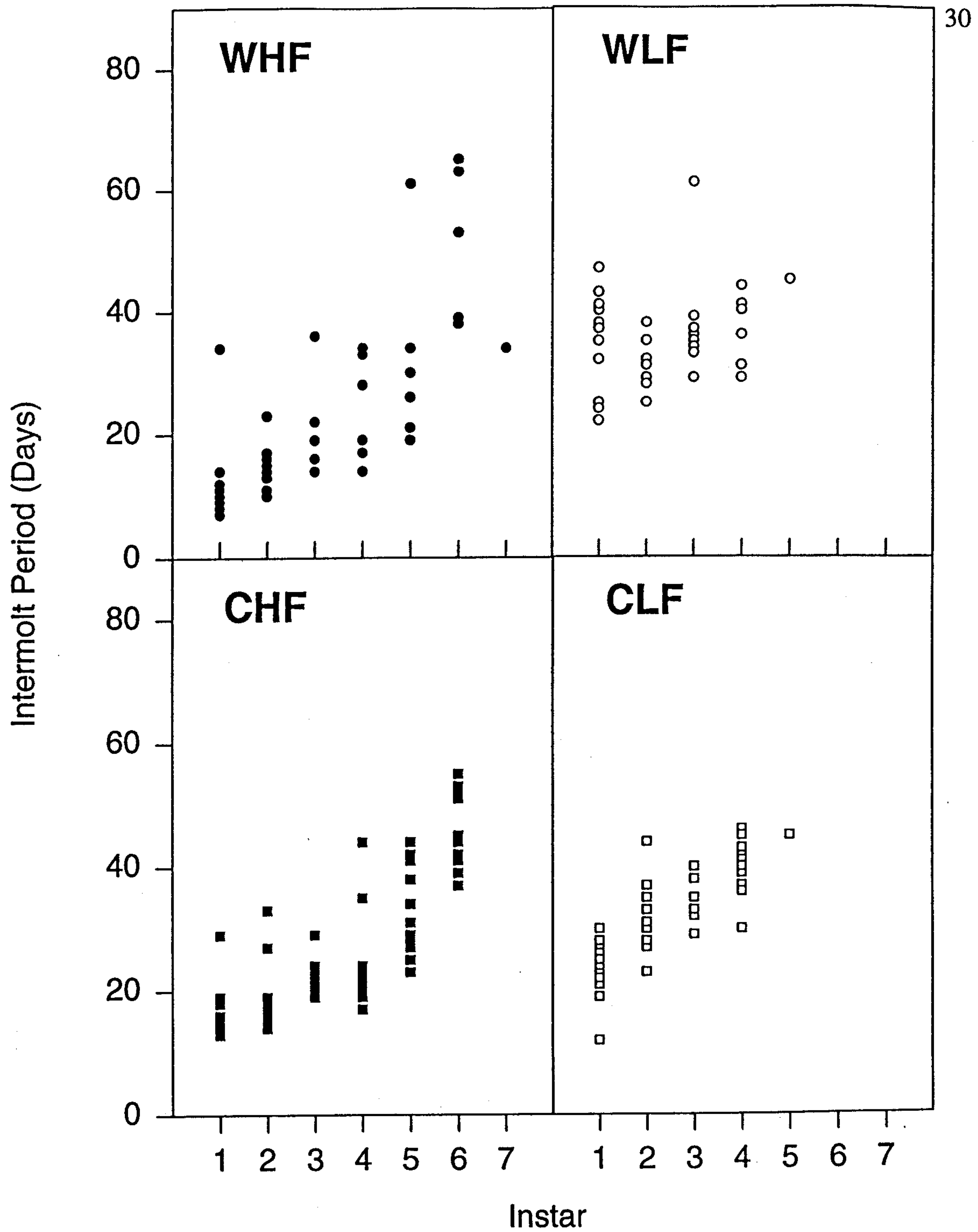


Figure 9. Intermolt duration (days) of individual C. magister juveniles in each treatment group (CHF- cold, high food; CLF- cold, low food; WHF- warm, high food; WLF- warm, low food). Each symbol represents an individual crab.

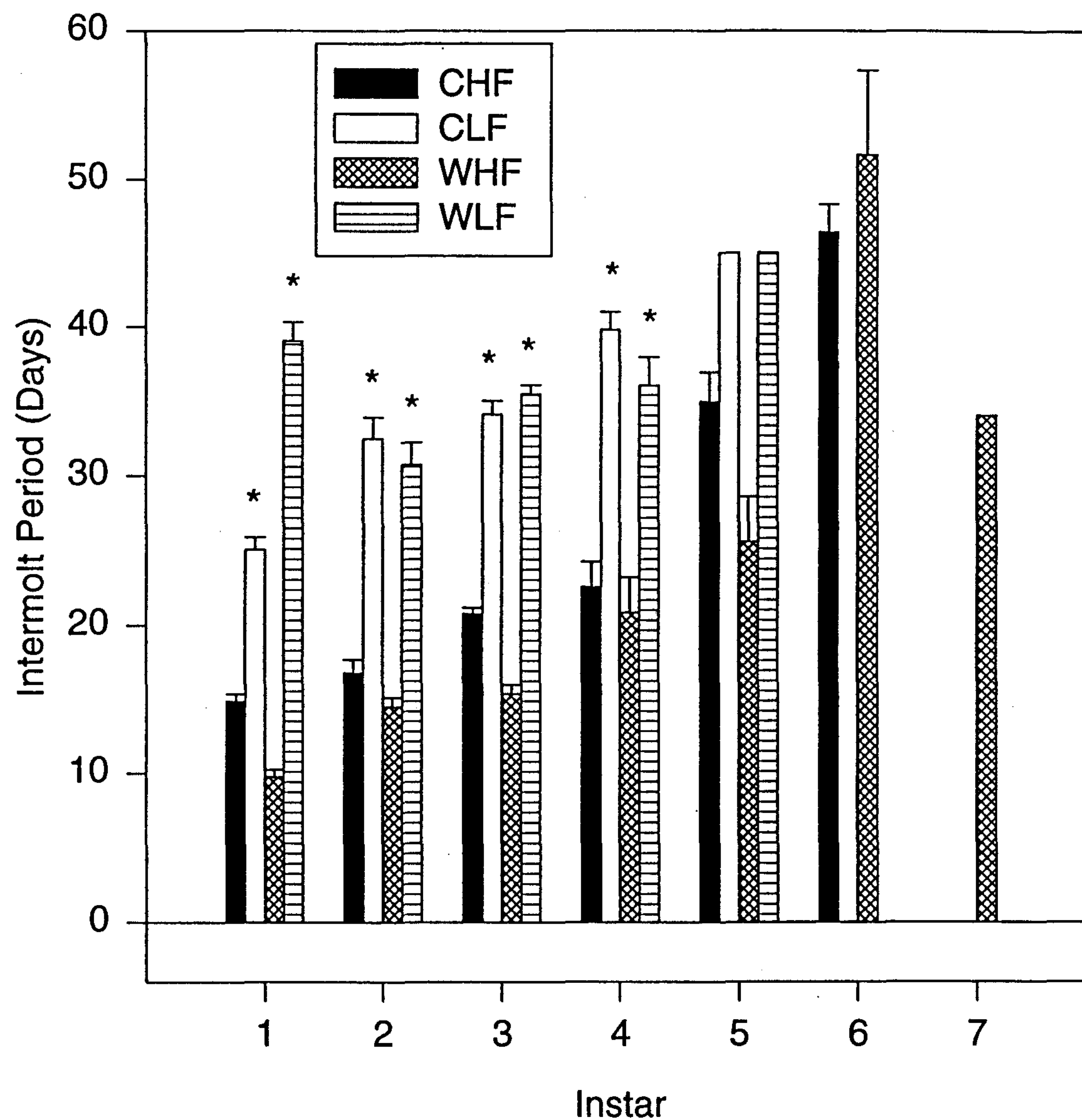


Figure 10. Mean intermolt duration (days) (± S.E.M.) of C. magister juveniles by instar for each treatment group (CHF- cold, high food; CLF- cold, low food; WHF- warm, high food; WLF- warm, low food). Numbers of surviving crabs at 4th instar are: CHF (15), CLF (13), WHF (9), and WLF (11).

Effect of Temperature

Within the high food level group, crabs reared in the warm seawater table had consistently shorter intermolt lengths at a given instar than those reared in the cold seawater table (Table 1, Figure 10). Within the low food level group, no difference in intermolt was evident.

Hemolymph Proteins

The major proteins of the hemolymph of C. magister are resolved into three bands using pH 7.4 PAGE (Terwilliger and Terwilliger 1982) (Figure 11). This electrophoretic technique separates high molecular weight oligomers (e.g. hemocyanin (Hc) and cryptocyanin (Cc)) on the basis of size and charge. Megalopa, juvenile and adult hemolymph have one slowly migrating band corresponding to 25S Hc molecules, one fast band corresponding to 16S Hc, and depending on the molt phase a band of Cc midway between the two (Terwilliger and Terwilliger 1982).

When hemolymph samples were taken through multiple sequential molt cycles for six months in this study, patterns of change in protein concentrations over time could be seen. Levels of 25S Hc, 16S Hc, and Cc found in the hemolymph of C. magister using pH 7.4 PAGE varied with molt cycle as has been shown by previous investigations (Terwilliger and Terwilliger 1982; Otoshi 1994).

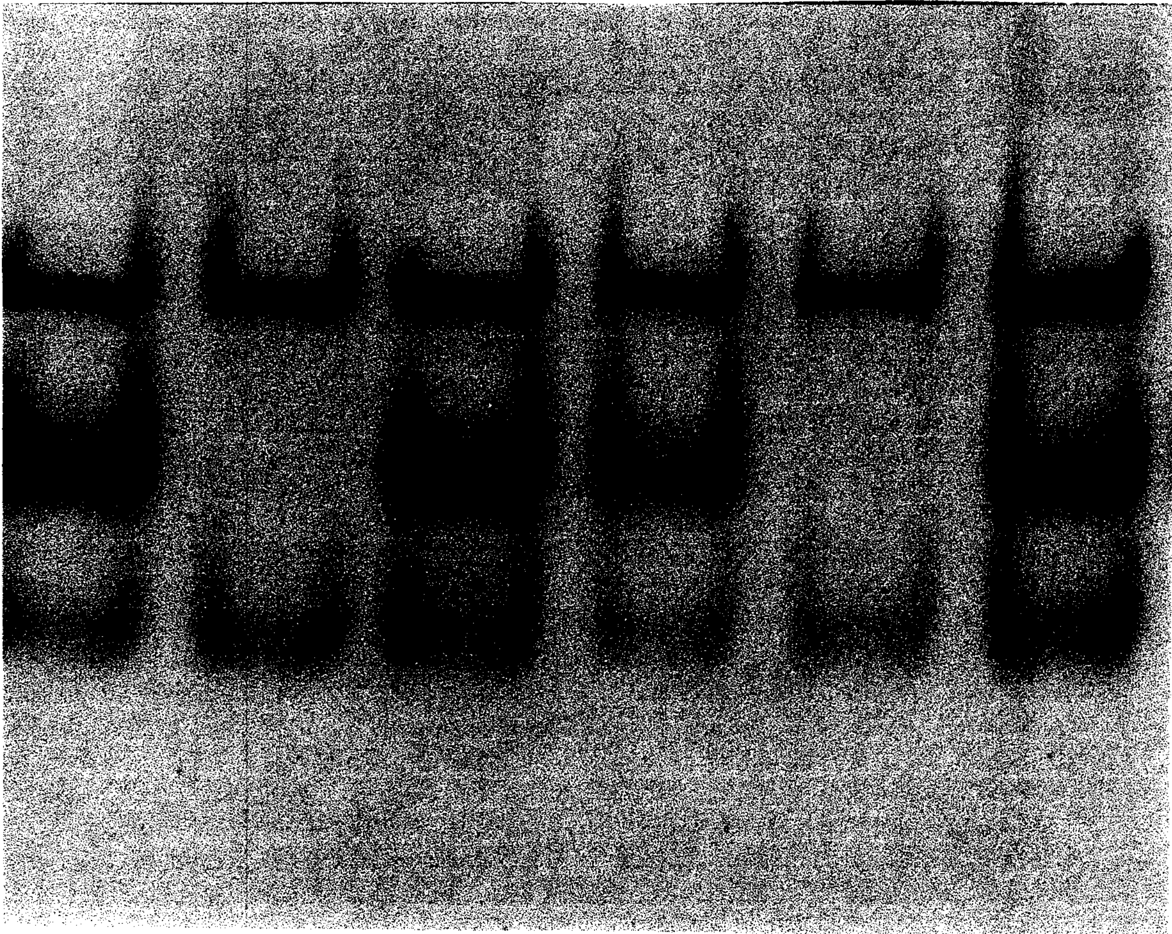


Figure 11. pH 7.4 PAGE of juvenile C. magister hemolymph. The top band in each lane is 25S Hc, the middle lane (if present) is Cc, and the bottom band is 16S Hc. From the left of the photo, lanes 2 and 5 are examples of hemolymph from crabs that lack Cc.

25S Hemocyanin

Hemolymph protein levels rose during premolt, started falling just before ecdysis, and continued falling postmolt. After each molt the postmolt levels of 25S Hc quickly returned to levels found in the previous instars (Figure 12). Mean average intensity units ± S.E.M. of 25S Hc levels are shown separately for each of the four treatment groups in Figure 13, as an example of the variability in data of the hemolymph protein studies. At maximal levels of 25S Hc, a double peak was seen. Levels of 25S Hc in the low food groups were similar to levels in the cold, high food group. The levels of 25S Hc in the warm, high food group were much higher relative to the other treatments at 2nd instar but steadily decreased, dropping to low levels by 5th instar. Within low food groups, differences in temperature groups were negligible.

16S Hemocyanin

The warm water treatment groups had slightly higher 16S Hc levels than the cold water groups (Figure 14). 16S Hc protein levels, like the 25S Hc levels, quickly returned to levels seen in previous postmolt instars. A double peak also was seen midway through the molt cycle. Within each temperature group, crabs in the low food groups had higher levels of 16S Hc than those in the high food groups. The warm, low food group had noticably higher 16 Hc levels than all three other groups.

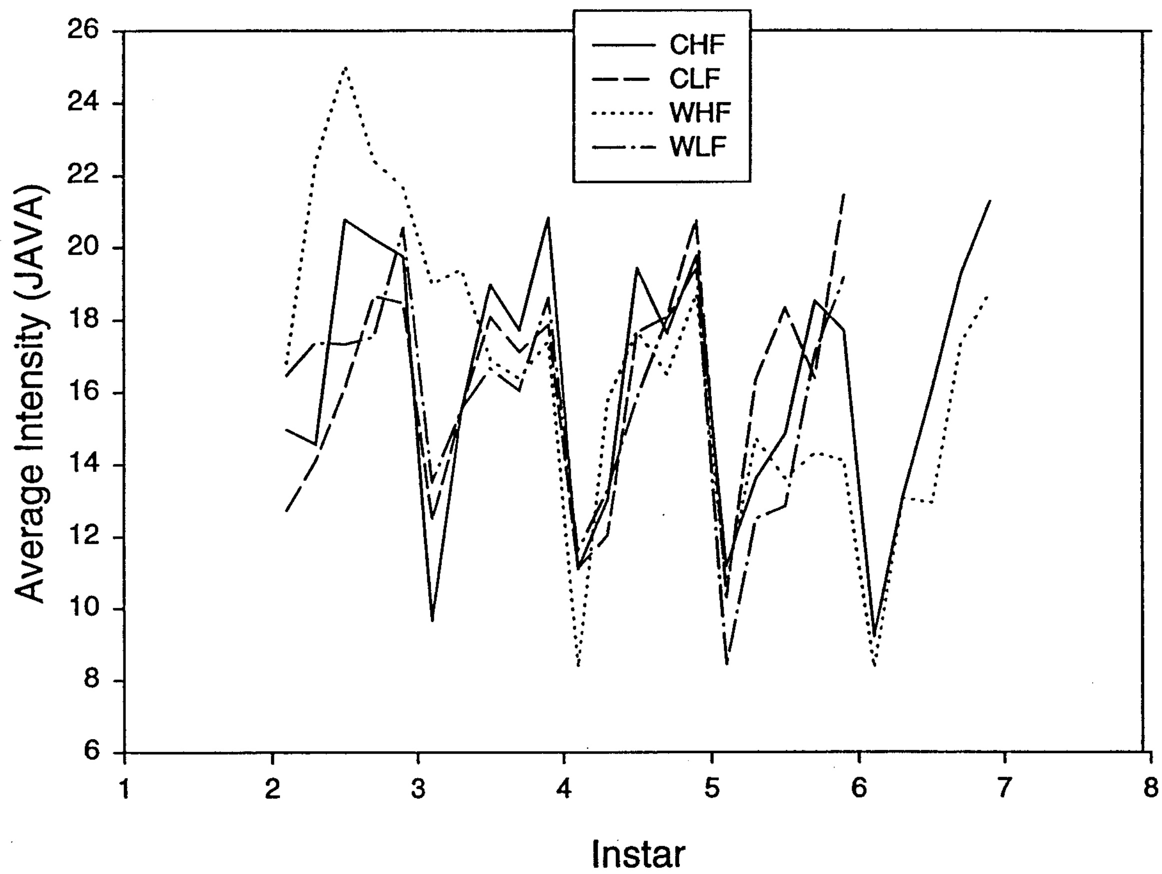


Figure 12. Mean average intensity units (JAVA) for 25S Hc at each instar for each treatment group (CHF- cold, high food; CLF- cold, low food; WHF- warm, high food; WLF- warm, low food).

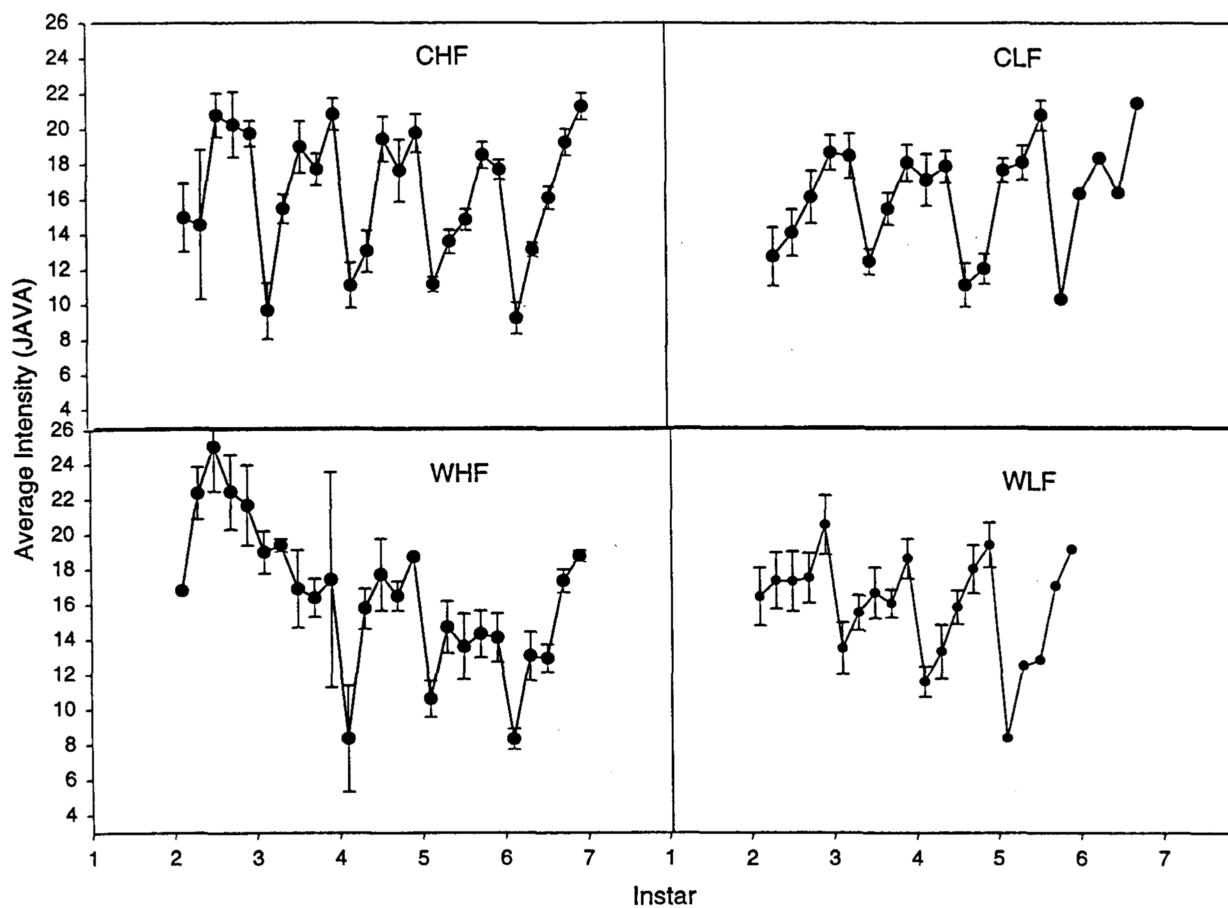


Figure 13. An example of the mean average intensity units (JAVA) ± S.E.M. for 25S Hc at each instar for each treatment group (CHF- cold, high food; CLF- cold, low food; WHF- warm, high food; WLF- warm, low food).

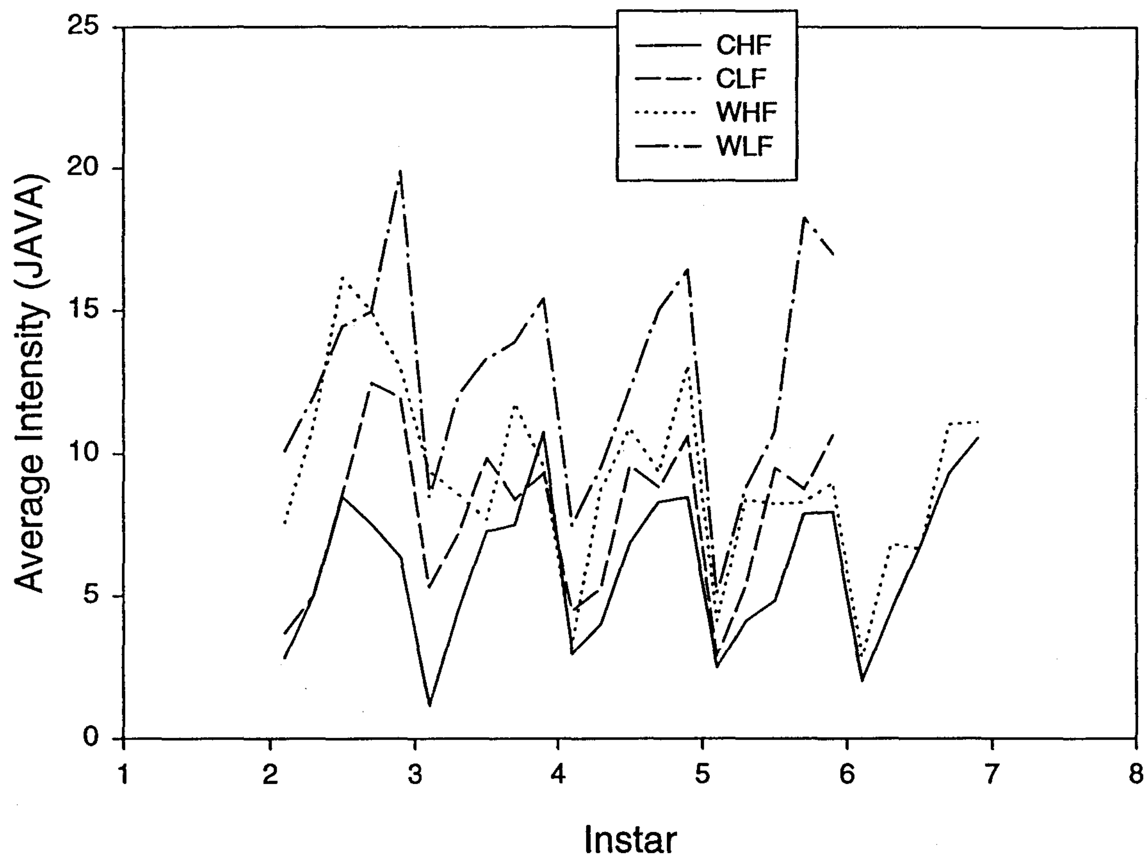


Figure 14. Mean average intensity units (JAVA) for 16S Hc at each instar for each treatment group (CHF- cold, high food; CLF- cold, low food; WHF- warm, high food; WLF- warm, low food).

Cryptocyanin

Cryptocyanin levels remained in higher concentration in the high food groups (CHF and WHF) than the limited food groups throughout the study, especially in the early stages of instars 2-5 (Figure 15). Temperature did not seem to have a profound effect on protein levels of Cc. The levels of Cc for all groups except WLF increased with time and did not return to levels found in previous instars. The double peak seen in the respiratory protein levels was also present in Cc.

Levels of Cc, a related non-respiratory protein (Terwilliger and Otoshi 1995), showed greater decreases post-molt and increases pre-molt than hemocyanin (ie., the magnitude of change was greater for cryptocyanin) (Figure 15). In general, the longer the intermolt duration, the longer it took cryptocyanin levels to return to their pre-molt state or vice versa, unlike Hc levels. For example in the WHF group, beginning with 6th instar, crabs grew for longer periods of time without cryptocyanin than in earlier instars and by 7th instar could go up to 3 weeks without cryptocyanin (data not shown). Younger crabs died after such prolonged periods without Cc. Young crabs that perished had low levels of 25S Hc, almost non-existent 16S Hc, and undetectable Cc 3-3 1/2 weeks before death. In the majority of crabs cryptocyanin never entirely disappeared (i.e., became visually undetectable) at ecdysis until after crabs reached 6th instar. It is not known whether the temporary disappearance of Cc in older instars was due to the natural lengthening of the intermolt period with age or was a result of resouce allocation toward growth rather than storage. Dilution of the hemolymph due to water uptake during

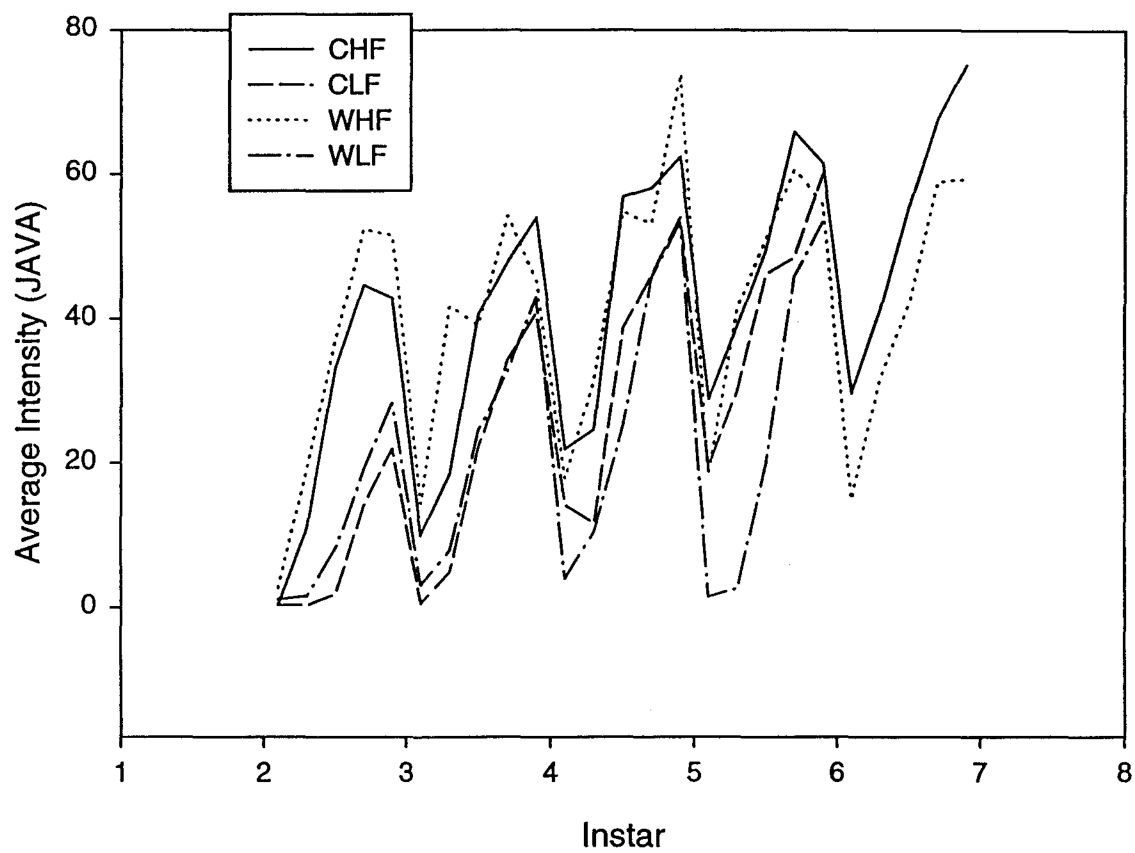


Figure 15. Mean average intensity units (JAVA) for Cc at each instar for each treatment group (CHF- cold, high food; CLF- cold, low food; WHF- warm, high food; WLF- warm, low food).

molting could not account for decreasing levels of hemolymph proteins because the pattern of flucuations in Cc protein levels was different from 25S Hc and 16S Hc.

Onset of Adult Hemocyanin by Instar and Age

SDS PAGE is an effective technique for analyzing the subunit composition of Hc and Cc. The reducing agent, dithiothreitol, breaks both intra- and intermolecular disulfide bonds. Sodium dodecylsulfate denatures and dissociates multisubunit proteins like hemocyanin and cryptocyanin into subunits (Figure 16). Adult C. magister 25S Hc has 6 subunits by SDS PAGE which appear as electrophoretically distinct bands ranging in size from 67,300 daltons to 81,800 daltons (Larson et al. 1981). Early stage juvenile 25S Hc contains only 5 of the 6 subunits (Terwilliger and Terwilliger 1982).

Effect of Food Levels

SDS-PAGE results were analyzed to determine the first appearance of adult hemocyanin for individuals in each treatment group. The timing of the appearance of subunit 6, measured both in terms of instar and the number of weeks from the initiation of the study, were compared using Mann-Whitney U test (Zar 1984). For three of the four treatment groups (CHF, CLF, WHF), Hc of C. magister showed a stage-specific switch around 5th instar from a juvenile form lacking subunit 6 to an adult form that contains subunit 6 (Figure 17). Those reared in warm water under limited food conditions (WLF) exhibited adult Hc at a significantly more immature instar than those given abundant food

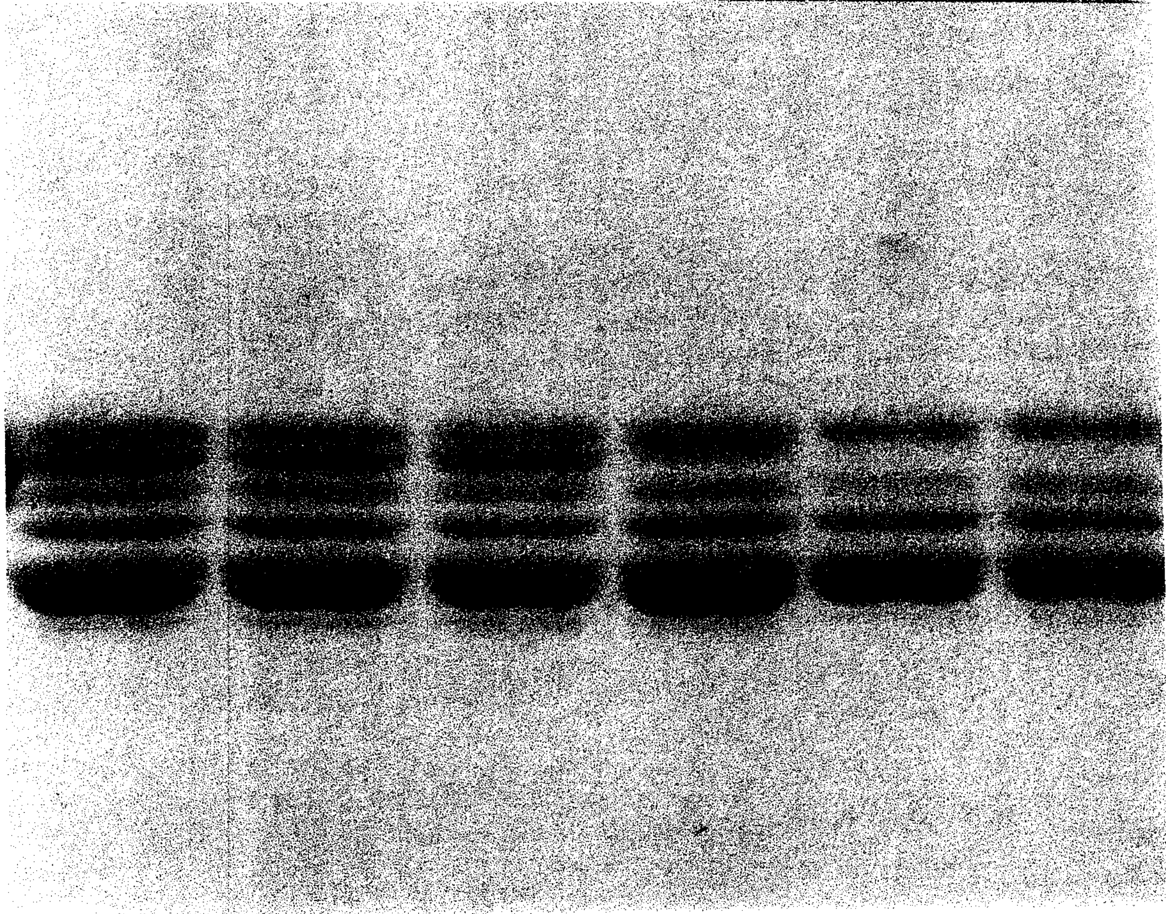


Figure 16. SDS PAGE of juvenile C. magister hemolymph. Subunits are designated as 1 through 6 from top to bottom. From the left of the photo, lane 2 is an example of hemolymph from a crab that contains Cc and adult Hc. Lane 5 is hemolymph from a crab that lacks Cc and has only a trace of subunit 6.

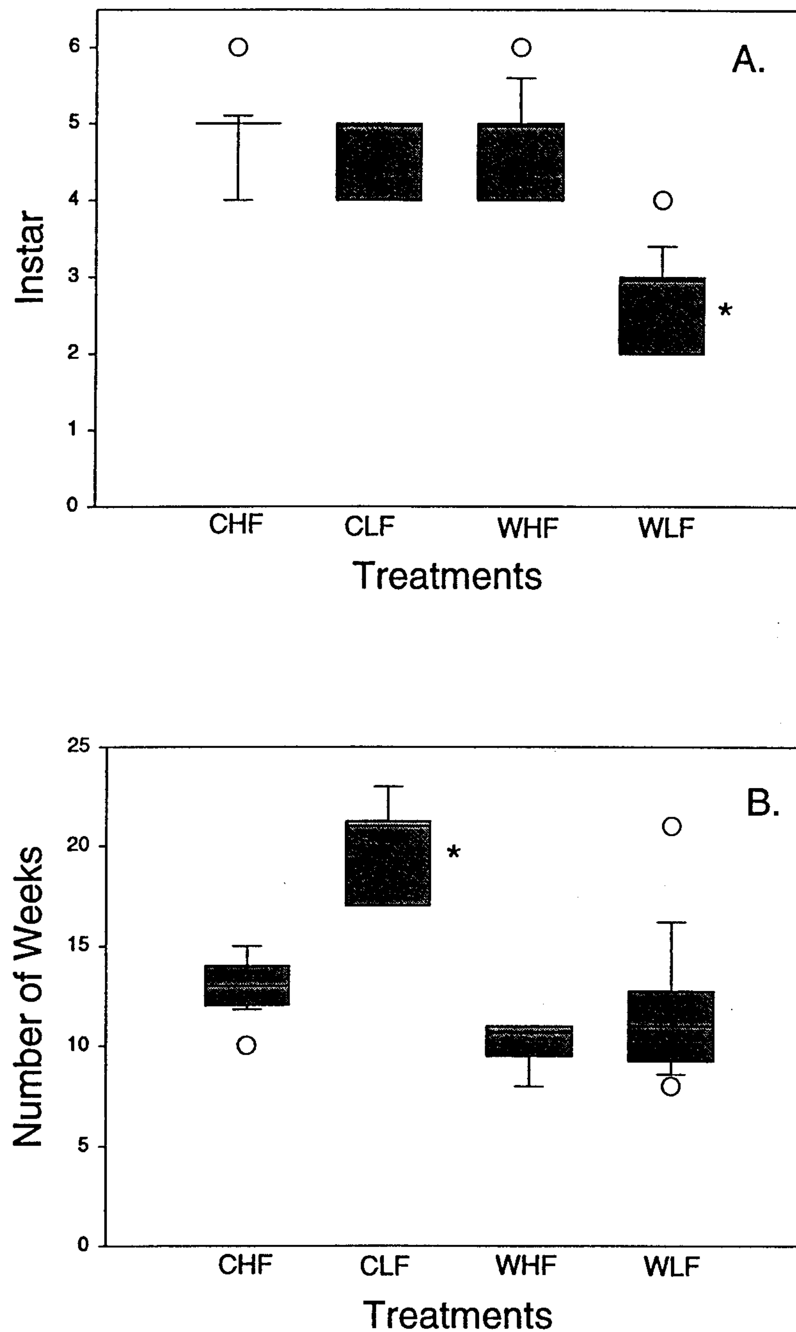


Figure 17. Median onset of adult Hc (dark bars on boxplots) in juvenile C. magister by instar (A) and number of weeks from the initation of the study (B) by treatment group (CHF- cold, high food; CLF- cold, low food; WHF- warm, high food; WLF- warm, low food). Boxes encompass the 25th to the 75th percentile and open circles denote single outliers. Asterisks indicate significance at $p<0.05$.

(Mann-Whitney U= 97.0, df = 1, $p < 0.001$). For crabs reared in cold water, adult Hc appeared at a similar instar regardless of feeding regime (U= 117.0, df = 1, p = 0.135).

Similarly, there were significant differences in the absolute timing of the onset of adult Hc in the hemolymph in terms of number of weeks from the beginning of the study. For three of the four treatment groups (CHF, WHF, and WLF) onset of adult Hc occurred approximately during the same number of weeks from the beginning of the study (Figure 17). In this case, both the high food level and low food level crabs in the warm water group exhibited adult Hc after similar periods of time (U= 34.5, df = 1, p = 0.238) even though crabs fed abundant food had developed through 2-3 more instars. In contrast, the high and low food crabs in cold water showed marked differences from one another in the timing of adult Hc depending on food levels; crabs with a restricted diet exhibited adult Hc approximately 7 weeks later than crabs fed high food levels (t = 9.3, df = 19.2, $p < 0.001$).

Effect of Temperature

The timing of the appearance of the 6th subunit, measured both in terms of instar and the number of weeks from the initiation of the study was qualitatively compared between temperature groups. For crabs fed high levels of food the onset of adult Hc by instar was very similar for the cold and warm treatments. For those given low levels of food, the warm water crabs had a precocious onset of adult Hc. Differences were also apparent in the absolute timing of the onset of Hc in terms of number of weeks from the beginning of the study. Crabs in the warm water treatments for both high and low food

levels had earlier onset of adult Hc. Figure 18 summarizes the trends in size, molting rate, and onset of adult Hc of the four treatment groups.

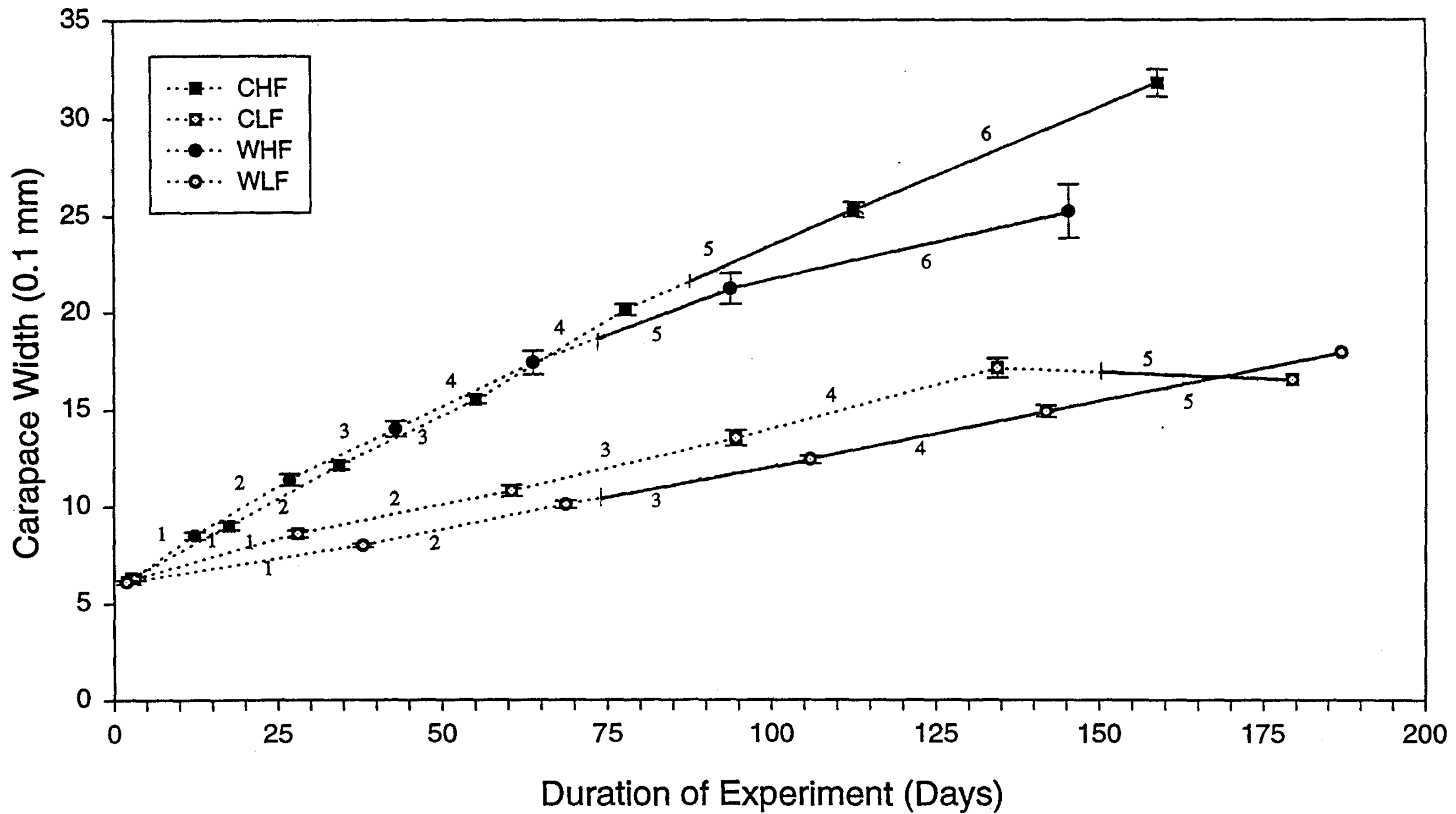


Figure 18. Mean size (CW, 0.1mm) ± S.E.M. at mean cumulative number of days to each instar from the initation of the sudy for each C. magister treatment group (CHF- cold, high food; CLF- cold, low food; WHF- warm, high food; WLF- warm, low food) by instar. Instars are denoted by numbers between data points. The dotted line indicates juvenile type Hc and the solid line indicates mean onset of adult Hc.

CHAPTER IV

DISCUSSION

In response to changes in temperature and food availability, juveniles of C. magister show a substantial flexibility in morphology, growth rates and hemolymph proteins. This flexibility enhances C. magister's ability to inhabit estuarine and nearshore waters. Juveniles that develop within estuaries experience seasonal, diurnal, and tidal changes in water temperature, and they probably experience higher prey abundance than in nearshore habitats. Those crabs that develop in nearshore waters experience more constant environmental conditions and likely more sparse food resources. Growth and molting results in the present study suggest that while temperature is important, food availability plays an even greater role than temperature in the growth of juveniles of C. magister.

Size and Growth Rate

In the present study, juveniles of C. magister fed high levels of food grew larger and faster than those fed low levels of food. Juveniles of C. magister raised in cold water reached larger sizes but grew more slowly than crabs raised in warm water within the high food groups. Data within the low food groups was variable. These findings are interesting in light of previous observations on growth in C. magister. Investigations of growth in postsettlement C. magister in Washington, found growth to be strongly

correlated to temperature, with considerably higher rates measured in warmer estuarine waters compared to oceanic waters (Tasto 1983; Armstrong and Gunderson 1985; Gutermuth and Armstrong 1989; Gunderson et al. 1990). Larvae settled at the same time, but estuarine recruits were exposed to water temperatures around 16°C while coastal recruits experienced 10°C or less and grew more slowly. By September, estuarine postsettlement crabs in Grays Harbor ranged between 30 mm and 50 mm CW, but coastal recuits of the same age class were 10 mm to 18 mm CW. Similar size ranges were found in the present study; however, differences in size and growth rate were strongly correlated with differences in food levels. Increased food availability rather than elevated temperature may confer a greater competitive advantage to estuarine juveniles of C. magister over their coastal conspecifics.

Stevens (1984) had hypothesized that colder bottom-water temperatures off the Washington coast would cause a reduced metabolic rate, slower growth, and general energetic depression in juvenile C. magister. Results from the present study found that growth was not depressed at colder temperatures, but in fact cold water crabs attained larger sizes at a particular instar than warm water crabs. Limited food availability was particularly detrimental for crabs raised in warmer temperature water. While metabolic rates were not measured, warm water crabs were more active. The combination of temperature-induced activity and increased metabolic rate could severely deplete available reserves in those crabs fed a restricted diet. The molt cycle was also faster in crabs raised in warmer water. At first glance, the increased molting rate would suggest a fster overall growth rate for crabs in warm water, but data from this study proves

otherwise. Therefore, a tradeoff seems to exist between gaining a competitive advantage (i.e., faster molting) during optimal environmental conditions, and coping within the crabs physiological limits during stressful conditions. A highly accelerated molt cycle becomes compromised at some point with reduction in carapace sizes.

Although the diet composition of C. magister has been studied (Butler 1954) further investigations are necessary to address nutritional requirements during development. The hierarchial partitioning of nutrients in crustaceans has been investigated (Freeman 1990 and references therein) but no studies have explored this in the juvenile stages of C. magister. The present study demonstraes that clearly food levels affect growth and intermolt duration; future studies might include further determination of the specific stages at which tissues grow, and the key regulating factors. Comparison of this data with seasonal variation in food availability, feeding behavior and activity levels will provide a more comprehensive picture of juvenile C. magister growth and habitat use.

Hemolymph Protein Levels

Proteins dissolved in the hemolymph of a crab are an important part of a highly coordinated molecular system which regulates respiration and molting. Molting in crustaceans is a physiologically stressful process and can cause an increased metabolic rate (Leffler 1972). One of these proteins, Hc, functions as an oxygen transporter and is important during premolt 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). Levels of 25S hemocyanin (Hc), 16S Hc, and cryptocyanin (Cc) varied with the molt cycle, increasing in pre-molt and decreasing slightly in post-molt. In the present study both elevated temperature and food limitation affected hemolymph protein levels during the molt cycle of juvenile C. magister. The most dramatic differences in protein levels were seen between food groups in early instars 2,3, and 4 which may suggest that younger instars are more sensitive to environmental conditions than later stages.

The 16S one-hexamer fraction of Hc of juveniles of C. magister fluctuated not only with the molt cycle but among treatment groups as well. Levels of 25S Hc, the two-hexamer fraction, were similar for each of the four treatment groups; however levels of 16S Hc were higher in juveniles fed low food levels and also in those raised in warm water. The increase in 16S Hc is significant for two reasons. First, crustacean hemocyanins are highly sensitive to temperature so a very efficient O_2 transport system is necessary (Mangum 1980). The temperature dependence of oxygen transport in the blood has been proposed as a mechanism explaining both large and small thermal responses of O_2 uptake (Mangum 1977). For example, total oxygen uptake in the blue crab Callinectes sapidus doubled with a rise in ambient temperature in the range of 15-25°C (Mauro and Mangum 1982). Second, the changes in the ratio of C. magister 16S Hc to 25S Hc may affect the oxygen affinity in the crabs. A study of the blue crab, Callinectes sapidus, found the ratio of 1-hexamer to 2-hexamer Hc molecules varied in natural populations and that isolated 2-hexamers have a lower O_2 affinity and greater cooperativity than isolated hexamers (Mangum et al. 1991). This difference in affinity was detectable in

both isolated fractions and native mixtures of different proportions of the two. In the present study, if the 16S Hc fraction of C. magister hemolymph has a higher oxygen affinity than the 25S Hc fraction, than the higher levels of 16S Hc found in C. magister raised in warm water would result in an increase in the oxygen affinity of C. magister hemolymph in response to low oxygen conditions.

The functional and structural role of respiratory proteins during thermal stress have been documented (Mangum 1977; Mauro and Mangum 1982; Brown 1991). The role of Cc, a non-respiratory protein found in the hemolymph of C. magister remains to be elucidated. Juvenile C. magister Cc (earlier termed hemoecdysin) decreases in concentration by approximately five-fold after the molt and remains low for twice as many days of the molt cycle as do the hemocyanins (Otoshi 1994). Transmission electron microscopy has revealed that cryptocyanin has a sedimentation coefficient of 16S, a molecular weight of 450,000 daltons and a hexameric shape (Terwilliger and Terwilliger 1982).

The presence of copper-free proteins similar in size to hexameric Hc have been demonstrated in crustaceans (Markl et al. 1979; Otoshi 1994). It is not yet known if they are similar to the insect storage proteins called hexamerins. The storage hexamers are a family of insect proteins that reach extraordinary concentrations in the hemolymph just prior to metamorphosis; their amino acids are incorporated into new tisues and proteins during adult development but they may also be incorporated into cuticle as intact protein and in one case were found to be diverted to a small degree into energy metabolism (as

reviewed in Telfer and Kunkel 1991). Protein comprises 30-50% of the exoskeletons of arthropods (Kumari and Skinner 1993).

Cryptocyanin is structurally related to insect storage hexamers (N. Terwilliger, personal communication.) but the function of this non-respiratory protein is not yet known. In the present study, levels of Cc were higher in juveniles fed high food, and no temperature effect was observed. The hypothesis that Cc acts as a storage protein is supported by the fact that Cc levels increase over successive molts and that juveniles of C. magister fed high levels of food had higher levels of Cc than crabs fed low food. Interestingly, the absence of cryptocyanin for a period of 3 weeks or more was directly related to the incidence of mortality.

Onset of Adult Hemocyanin

The Hc of juveniles of C. magister changes in subunit composition during stages of development (Terwilliger and Terwilliger 1982). In the present study, ontogeny of C. magister Hc showed neither a stage-specific switch (instar) nor a time dependent switch (duration of the study in days). If we disregard the WLF group as the least likely of the four environmental scenarios wild crabs would encounter (ie. prolonged thermal stress and starvation) then the switch to adult Hc would appear to be stage-specific. It is also possible that the ontogenetic shift in adult Hc in crabs in the wild might normally occur at a specific age (ie. the same number of days from metamorphosis) and therefore the lagtime in adult Hc onset in the CLF group was due to depressed metabolic rate and lack of food. The combined effects of food limitation and elevated temperatures in the WLF

group may cause a physiological acceleration of adult Hc appearance. More, likely the molt schedule in these crabs is delayed while Hc ontogeny moves along at a normal pace from the start of metamorphosis.

The subunit composition of crustacean hemocyanins is highly heterogeneous (Markl, 1986). The role of different subunit compositions on oxygen binding is an important component of the crab's ability to adapt to changing environmental conditions. Crabs with adult Hc have an oxygen affinity 50% higher than juveniles (Terwilliger et al. 1986) and therefore earlier onset of adult Hc may be a significant adaptive factor in increasing the amount of O_2 carried in the hemolymph to the tissues. A recent study demonstrated that none of the juvenile stages of C. magister that were examined showed significant increases in the rate of oxygen consumption in response to changing salinity levels. First instar juveniles, however, were found to be more sensitive to an increase in temperature from 10°C to 20°C (Brown and Terwilliger 1992). One hypothesis that Brown and Terwilliger (1992) suggested to explain the differences between adult and juvenile Hc in C. magister involved divalent cations as hemolymph protein modulators. Ontogenetic changes in hemolymph magnesium regulation, resulting in high hemolymph magnesium in megalopa and juvenile crabs compared to the adults, at 10°C in 100% seawater, may partially compensate for the low intrinsic oxygen affinity of the juvenile type Hc or vice versa (Brown and Terwilliger 1992). Low oxygen conditions might induce accelerated adult Hc onset in conjunction with accelerated adult-form ionic regulation.

Cancer magister is an important commercial species which has a life history sensitive to environmental change. Alteration of estuarine habitat, poor water quality, and dredging in west coast estuaries kills hundreds of thousands of crabs annually (Stevens 1981). Juvenile crabs living in estuaries are thought to benefit from the warmer temperatures, decreased predation, and increased food availability (Bottsford and Wickham 1978; Stevens et al. 1982). This study helps to define the relative importance of estuarine habitat in the life cycle of C. magister by experimentally isolating the effects of temperature and food on crab development. This study has implications for the preservation of estuaries as effective and necessary nursery grounds for the developmental stages of C. magister.

BIBLIOGRAPHY

Adelung, D. 1971. Untersuchungen zur Häutungsphysiologie der Dekapoden Krebse am Beispiel der Strandkrabbe Carcinus maenas. *Helgoländer Wiss. Meersunters* 22: 66-119.

Anger, K. 1984. Influence of starvation on moult cycle and morphogenesis of Hyas araneus larvea (Decapoda, Majidae). *Helogol. Wiss. Meeresuntersuch* 38: 21-33.

Armstrong, D. A., and D. R. Gunderson. 1985. The role of estuaries in Dungeness crab early life history: A case study in Grays Harbor, Washington. In *Proceedings of the Symposium on Dungeness Crab Biology and Management. Alaska Sea Grant Report No. 85-3,* ed. B. R. Melteff, 145-70.

Bückmann, D., and D. Adelung. 1964. Der Einfluss der Umweltfaktoren auf das Wachstum und den Häutungsrhythmus der Strandkrabbe Carcinides maenas. *Helgoländer Wiss. Meeresunters* 10, no. (1-4): 91-103.

Bellelli, A., L. Zolla, B. Giardina, S. Constantini, A. Cau, and M. Brunori. 1985. Hemocyanin from Palinurus elephas: general properties and effects of heavy metals. *Biochim. Biophys. Acta* 830: 325-31.

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: 275-96.

Botsford, L. W., and D. E. Wickham. 1978. Behavior of age-specific, density-dependent models and the northern California Dungeness crab (Cancer magister) fishery. *J. Fish. Res. Board Can.* 35: 833-43.

Brown, A. C. 1991. Effects of temperature and salinity on the respiratory physiology of the Dungeness crab, Cancer magister, during development. Ph.D diss., University of Oregon.

Brown, A. C., and N. B. Terwilliger. 1992. Developmental changes in ionic and osmotic regulation in the Dungeness crab, Cancer magister. *Biol. Bull.* 182: 270-277.

Butler, T. H. 1954. Food of the commercial crab in the Queen Charlotte Islands region. *Fish. Res. Board. Can. Pac. Prog. Report* 99: 3-5.

Carpenter, D. E., and K. E. van Holde. 1973. Amino acid composition, amino terminal analysis and subunit structure of Cancer magister hemocyanin. *Biochemistry* 12: 2231-38.

Collier , P. C. 1983. Movement and growth of post-larval Dungeness crabs, Cancer magister, in the San Francisco area. *Fish Bull. (Cali.)* 172: 125-33.

Démeusey, N. 1958. Recherches sur la mue de puberté du Décapode Brachyoure, Carcinus maenas Linné. *Archs Zool. Exp. Gén.* 95: 253-491.

Davis, B. 1964. Disc gel electrophoresis. II. Method and application to human serum proteins. *Ann. N.Y. Acad. Sci.* 12: 404-27.

De Wachter, B., and B. R. McMahon. 1996. Haemolymph flow distribution, cardiac performance and ventilation during moderate walking activity Cancer magister (Dana) (Decapoda, Crustacea). *J. Exp. Biol.* 199: 627-33.

Durstewitz, G. 1996. Molecular studies of hemocyanin expression in the Dungeness crab. Ph.D diss., University of Oregon.

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

———. 1953. Respiratory metabolism. In *Insect Physiology,* ed. K. D. Roeder, 96-146. New York: Wiley.

Ellerton, H. D., D. E. Carpenter, and K. E. van Holde. 1970. Physical studies of hemocyanins. V. Characterization and subunit structure of the hemocyanin of Cancer magister. *Biochemistry* 9: 2225-32.

Elner, R. W. 1978. The mechanisms of predation by the shore crab, Carcinus maenas (L.) on the edible mussel, Mytilus edulis. *L. Oecologia* 36: 333-44.

Fairbanks, G., T. L. Steck, and D. F. H. Wallach. 1971. Electrophoretic analysis of human erythrocyte membrane. *Biochem.* 10: 2606-16.

Freeman, J. A. 1995. Epidermal cell cycle and region-specific growth during segment development in Artemia. *J. Exp. Zool.* 271: 285-95.

———. 1990. Regulation of tissue growth in crustacean larvae by feeding regime. *Biol. Bull.* 178: 217-21.

Gunderson, D. R., D. A. Armstrong, Y. Shi, and R. A. McConnaughey. 1990. Patterns of estuarine use by the juvenile English sole (Parophrys vetulus) and Dungeness crab (Cancer magister). *Estuaries* 13, no. 1: 59-71.

Gutermuth, F. B., and D. A. Armstrong. 1989. Temperature-dependent metabolic response of juvenile Dungeness crab Cancer magister Dana: ecological implications for estuarine and coastal populations . *J. Exp. Mar. Biol. Ecol.* 126: 135-44.

Hobbs, R. C., and L. W. Botsford. 1992. Diel vertical migration and timing of metamorphosis of larvae of the Dungeness crab Cancer magister. *Mar. Biol.* 112: 417-28.

Jacoby, C. 1982. Behavior responses of the larvae of Cancer magister Dana (1852) to light, pressure, and gravity. *Mar. Behav. Physiol.* 8: 267-83.

Jamieson, G. S., and A. C. Phillips. 1988. Occurrence of Cancer crab (C. magister and C. oregonensis) megalopae off the west coast of Vancouver Island, British Columbia. *Fish. Bull.* 86: 525-42.

Klein-Breteler, W. C. M. 1975. Laboratory experiments on the influence of environmental factors on the frequency of moulting and the increase in size at moulting of juvenile shore crabs, Carcinus maenas. *Neth. J. of Sea Res.* 9, no. 1: 100-120.

Kumari, S. S., and 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.

Laemmli, H. K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* 227: 680-685.

Laird, C. E., and P. A. Haefner Jr. 1976. Effects of intrinsic and environmental factors on oxygen consumption in the blue crab, Callinectes sapidus Rathbun. *J. Exp. Mar. Biol. Ecol.* 22: 171-78.

Larson, B. A., N. B. Terwilliger, and R. C. Terwilliger. 1981. Subunit heterogeneity of Cancer magister hemocyanin. *Biochim. Et Biophys. Acta.* 667: 294-302.

Leffler, C. W. 1972. Some effects of temperature on the growth and metabolic rate of juvenile blue crabs, Callinectes sapidus, in the laboratory. *Mar. Biol.* 14: 104-10.

Lough, R. G. 1976. Larval dynamics of the Dungeness crab, Cancer magister, off the central Oregon coast, 1970-1971. *Fish. Bull* 74: 353-75.

Mangum, C. P. 1980. Respiratory function of the hemocyanins. *Am. Zool.* 20: 19-38.

———. 1977. Temperature adaptation. In *Physiology of Annelids,* ed. P. J. Mill, 447-78. New York: Academic Press, Inc.

Mangum, C. P., J. Greaves, and J. S. Rainer. 1991. Oligomer composition and oxygen binding of the hemocyanin of the blue crab Callinectes sapidus. *Biol. Bull.* 181: 453-58.

Mangum, C. P., and J. S. Rainer. 1988. The relationship between subunit composition and O2 binding of the blue crab hemocyanin. *Biol. Bull.* 174: 77-82.

Markl, J. 1986. Evolution and function of structurally diverse subunits in the repiratory protein hemocyanin from arthropods. *Biol. Bull.* 171: 90-115.

Markl, J., A. Hofer, G. Bauer, B. Markl, M. Kempter, M. Brenzinger, and B. Linzen. 1979. Subunit heterogeneity in arthropod hemocyanins. II. Crustacea. *J. Comp. Physiol.* 133: 167-75.

Mauro, N. A., and C. P. Mangum. 1982. The role of the blood in the temperature dependence of oxidative metabolism in decapod crustaceans. I. Intraspecific responses to seasonal differences in temperature. *J. Exp. Zool.* 219: 179-88.

McConaugha, J. R. 1985. Nutrition and larval growth. In *Crustacean Issues 2. Larval growth,* ed. A. M. Wenner, 127-54. Boston: Balkema Press.

McMahon, B. 1986. Oxygen binding by hemocyanin: compensation during activity and environmental change. In *Invertebrate Oxygen Carriers,* ed. B. Linzen, 299-319. Berlin: Springer-Verlag.

McMahon, B., F. Sinclair, C. D. Hassal, P. L. deFur, and P. R. H. Wilkes. 1978. Ventilation and control of acid-base status during temperature acclimation in the crab, Cancer magister. *J. Comp. Physiol.* 128: 109-16.

McMillan, R. O., D. A. Armstrong, and P. A. Dinnel. 1995. Comparison of intertidal habitat use and growth rates of two Northern Puget Sound cohorts of 0+ age Dungeness crab, Cancer magister. *Estuaries* 18, no. 2: 390-398.

Newell, R. C. 1976. Adaptation to intertidal life. In *Adaptation to Environment: essays on the physiology of marine animals,* ed. R. C. Newell, 1-82. London: Butterworths.

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

Oresanz, J. M., and V. F. Gallucci. 1988. Comparative study of postlarval life-history schedules in four sympatric species of Cancer (Decapoda: Brachyura: Cancridae). *J. Crust. Biol.* 8, no. (2): 187-220.

Otoshi, C. A. 1994. 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. Master's thesis, University of Oregon.

Pauley, G. B., D. A. Armstrong, and T. W. Heun. 1986. Dungeness crab. Species profiles: Life histories and environmental requirements of coastal fishes and invertebrates (Pacific Northwest). *Biol. Report* 82, no. 11.63, TR EL-82-4.

Poulson, D. F. 1935. Oxygen consumption of Drosophila melanogaster. *Zeitschr. Vergl. Physiol.* 22: 466-72.

Prentice, E. F., and D. E. Schneider. 1979. Respiration and thermal tolerance of the Dungeness crab, Cancer magister Dana. *Comp. Biochem. Physiol.* 63A: 591-97.

Reed, P. H. 1969. Culture methods and effects of temperature and salinity on survival and growth of the Dungeness crab (Cancer magister) larvae in the laboratory. *J. Fish Res. Board Can.* 26: 389-97.

Rudy Jr., P., and L. H. Rudy. 1983. *Oregon estuarine invertebrates: An illustrated guide to the common and important invertebrate animals*. Oregon: Biological Service Program/FWS.

Savel-Niemann, A., J. Markl, and B. Linzen. 1988. *J. Mol. Biol.* 204: 385-95.

Schmidt-Nielsen, K. 1990. *Animal physiology:adaptation and environment*. New York: Cambridge University Press.

Schneiderman, H. A. 1952. Variations in dehdrogenase activity during the metamorphosis of the Cecropia silkworm. Ph.D. diss., 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.

Shen, C. J. 1935. An investigation of the post-larval developmnet of the shorecrab Carcinus maenas, with special reference to the external secondary sexual characters. *Proceed. Zool. Soc. Lond.*: 1-33.

Stevens, B. G. 1981. Dredging-related mortality of Dungeness crabs associated with four dredges operating in Grays Harbor, Washington. *Final Report to Washington Department of Fisheries and U.S. Army Corps of Engineers, Seattle District*: 1-148.

Stevens, B. G., and D. A. Armstrong. 1984. Distribution, abundance, and growth of juvenile Dungeness crabs, Cancer magister, in Grays Harbor Estuary, Washington. *Fish. Bull.* 82, no. 3: 469-83.

Stevens, B. G., D. A. Armstrong, and R. Cusimano. 1982. Feeding habits of the Dungeness crab Cancer magister as determined by the index of relative importance. *Mar. Biol.* 72: 135-45.

Stevens, B. G., D. A. Armstrong, and J. C. Hoeman. 1984. Diel activity of an estuarine population of Dungeness crabs, Cancer magister, in relation to feeding and environmental factors. *J. Crust. Biol.* 4, no. 3: 390-403.

Sulkin, S. D., and G. L. McKeen. 1989. Laboratory study of survival and duration of individual zoeal stages as a function of temperature in the brachyuran crab, Cancer magister . *Mar. Biol.* 103: 31-37.

Tasto, R. N. 1983. Juvenile Dungeness Crab, Cancer magister, studies in the San Francisco Bay area . *California Department of Fish and Game Fish Bulletin* 172: 135-54.

Telfer, W. H., and J. G. Kunkel. 1991. The function and evolution of insect storage hexamers. *Annu. Rev. Entomol.* 36: 205-28.

Terwilliger, N. B., and A. C. Brown. 1993. Ontogeny of hemocyanin function in the Dungeness crab Cancer magister: the interactive effects of developmental stage and divalent cations on hemocyanin oxygenation properties. *J. Exp. Biol.* 183: 1-13.

Terwilliger, N.B., and C.A. Otoshi. 1994. Cryptocyanin and hemocyanin: Fluctuations and functions of crab hemolymph proteins during molting. *The Physiologist* 37, no. 5: A-67.

Terwilliger, N. B., and R. C. Terwilliger. 1982. Changes in the subunit structure of Cancer magister hemocyanin during larval development. *J. Exp. Zool.* 221: 181-91.

Terwilliger, N. B., R. C. Terwilliger, and R. Graham. 1986. Crab hemocyanin function changes during development. In *Invertebrate Oxygen Carriers,* ed. B. Linzen, 333-35. Berlin: Springer-Verlag.

van Holde, K. E., and K. I. Miller. 1995. Hemocyanins. In *Advances in Protein Chemistry,* ed.Vol. 47. Academic Press, Inc.

Vernberg, W. B., and F. J. Vernberg. 1972. *Environmental physiology of marine animals*. New York: Springer-Verlag.

Wang, S. Y., and F. M. Truesdale. 1991. Temperature and salinity effects on early juvenile development of the Stone crab, Menippe mercenaria. *Annual Meeting of American Society of Zoologists* 31, no. 5: 91A #454.

Warner, R. W. 1987. Age and growth of male Dungeness crabs, Cancer magister, in northern California. *Calfornia Department of Fish and Game Bulletin* 73: 4-20.

Wheatley, M. G., and A. T. Gannon. 1995. Ion regulation in crayfish: freshwater adaptations and the problem of molting. *Amer. Zool.* 35: 49-59.

Zar, J. H. 1984. *Biostatistical analysis*. New Jersey: Prentice Hall.