THE RELATIONSHIP BETWEEN STRUCTURAL AND FUNCTIONAL POLYMORPHISM OF THE HEMOCYANIN OF THE RED ROCK CRAB *CANCER PRODUCTUS*

by

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Electrophoretic banding patterns (pH 8.9 PAGE) of *Cancer productus* hemocyanin (Hc) indicate phenotypic plasticity within *C. productus*, which is not associated with temperature or salinity changes. Analysis of banding patterns (pH 7.4 PAGE and pH 8.9 PAGE) of *C. productus* Hc over time revealed that neither the Hc oligomer ratio nor subunits changed significantly when crabs were subjected to elevated temperatures (19°C, 32ppt) or decreased salinity (14°C, 16ppt) compared to ambient conditions (14°C, 32ppt). PAGE of pure fractions of 25S Hc and 16S Hc isolated by LPLC revealed only one subunit (subunit A) that was variable between the oligomers. Oxygen binding studies were used to measure differences in oxygen binding of 25S Hc and 16S Hc over a range of pH and lactate concentrations. These results suggest the oxygen affinities of 25S Hc and of 16S Hc are not significantly different in response to changes in pH, although 16S Hc tended to have a higher affinity. Differences in lactate sensitivity between 25S Hc and 16S Hc suggest lactate may be related to phenotypic plasticity observed in *C. productus*.

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INTRODUCTION

Phenotypic plasticity

Phenotypic plasticity is a term that describes the ability of an organism to alter its physiology, morphology or development in response to environmental changes (Callahan et al., 1997). Some changes are reversible, especially physiological ones, whereas other changes, typically morphological, are irreversible. There are two approaches to dissect the genetic and physiological basis of phenotypic reactions to environmental changes according to Pigliucci (1996). One approach is to isolate mutants that interfere with a characterized pattern of phenotypic plasticity. The second approach is to expose plants or animals to various environments and screen for changes in RNA or protein patterns. The second approach will be used throughout this study to determine if *Cancer productus* (Randall) hemocyanin does express phenotypic plasticity. Polymorphism is known to occur in *C. productus* but phenotypic plasticity of *C. productus* hemocyanin has not been studied.

Hemocyanin

Cancer productus (Randall) hemolymph contains the respiratory protein arthropod hemocyanin (Hc). Hemocyanin is the blue copper-protein, which functions as the oxygen carrier in the blood of crustaceans, horseshoe crabs, arachnids and centipedes. Hemocyanin combines reversibly with oxygen and carries it to tissues. It may be a good indicator molecule to reflect phenotypic plasticity in response to developmental or

environmental change. Many arthropod Hcs associate into hexamers whereas others form 12- 24- 36- and 48-mers. Van Holde and Miller (1985) reviewed reasons for differences in Hc subunit heterogeneity among various species. The principal finding was that Hc with subunits that associate primarily into hexamers is composed of one or a small number of subunit types, whereas higher association of Hc requires greater diversity and specialization of subunits. Hemocyanin of *C. productus* is similar to *Cancer magister* in that its subunits associate primarily into 6-mers and 12-mers with the sedimentation coefficients of 16S and 25S, respectively (Ellerton et al., 1970).

The oxygen affinity of arthropod Hc is affected by inorganic ions such as H^+, Ca^{2+} and Mg^{2+} (Zeis et al., 1992). The oxygen binding of some arthropod Hcs also responds to L-lactate and urate (Graham et al., 1983; Zeis et al. 1992). Lactate is a major end product of anaerobic metabolism in crustaceans. The effects of L-lactate binding on the oxygen binding by *Homarus vulgaris* Hc have been thoroughly studied (Zeis et al., 1992). Few studies, however, have focused on the interaction of specific subunits and lactate effects. The allosteric response to lactate appears to be characteristic of only certain subunits. *Panulirus* Hc subunits b and c respond to lactate, but subunit a does not (Johnson et al. 1987).

There is evidence that Hc gene expression may change in response to environmental and developmental changes in arthropods. Studies of *C. magister* Hc demonstrated shifts in subunit composition as the crab changes developmental stages (Terwilliger and Terwilliger, 1982; Brown and Terwilliger, 1992). Similar results have been found in *Cancer productus* (Wache et al. 1988). Mangum (1993) has investigated

the relationship of Hc subunits and oxygen binding in *Uca pugilator*. Results suggested that the observed variation in *Uca pugilator* Hc monomers was responsible for observed variation in oxygen affinity. Extensive studies by Mangum (1997) have demonstrated *Callinectes sapidus* exhibits phenotypic plasticity. Mangum and Rainer (1988) demonstrated *Callinectes sapidus* is capable of altering its intrinsic respiratory properties (i.e., oligomer composition) in response to hypoxia and salinity. Several others have continued the investigation of subunit changes in relationship to environmental factors such as temperature, salinity and hypoxia (Mangum et al., 1988; Mangum, 1994). Little research has been reported that describes the relationship between hemocyanin composition and salinity, temperature and hypoxia in *Cancer productus.*

Life in the intertidal

Cancer productus is a common crustacean, along the North Pacific coast, found from the mid-intertidal to 79m. Several aspects of the biology of the red rock crab, *C. productus,* indicate that it would be of interest in the study of phenotypic plasticity of Hc. First, in the intertidal, *C. productus* experiences considerable variation in temperature, salinity, and hypoxia. Morris and Taylor (1983) conducted a study of diurnal and seasonal variation in physico-chemical conditions within intertidal rock pools. The study demonstrated large diurnal fluctuations in temperature, salinity and partial pressure of oxygen. A study of molluscan and arthropod Hc sensitivity to temperature by Burnett et al. (1988) found that temperature, in a subtidal habitat, is generally less than 15°C and diurnal variation is small, whereas an intertidal habitat can vary more than 30°C. Second,

variations in *C. productus* Hc subunit phenotypes have been observed by Wache et al. (1998). Previous studies indicate differences in the subunit compositions of *C. productus* Hc whereas a more consistent subunit composition is characteristic in *Cancer magister,* a closely related species (Larson et al. 1981; Wache 1985). Studies conducted on *C. magister* and *C. productus* have focused on subunit structure throughout the developmental cycle (Larson et al., 1981; Wache et al., 1988; Durstewitz et al., 1997). Several studies on *C. sapidus* and *U. pugilator*, however, have investigated subunit composition under varying environmental conditions. Finally, respiration in *C. productus* has been well studied and physiological compensations to short term air exposure have been described by Defur and McMahon (1984 a, b).

One of the primary questions this investigation addresses is whether there are different intrinsic oxygen binding properties between *C. productus* Hc oligomers 25S and 16S. These differences may be in response to changes in pH. Secondly, are there differences between 25S Hc and 16S Hc in response to the allosteric effector lactate? The third question addresses whether *C. productus* Hc expresses phenotypic plasticity and can phenotypic plasticity be induced within *C. productus* by altering environmental conditions of temperature and salinity. These experiments will provide insight into the possible role of Hc phenotypic plasticity expression in *C. productus* in response to changes in specific environmental conditions.

MATERIALS AND METHODS

Collection of *Cancer productus*

Male *Cancer productus* (86-120 mm) were collected subtidally from the Charleston outer boat basin in Coos Bay, Oregon, approximately 1500 meters from the mouth of the bay (Figure 1). *C. productus* were collected using crab pots during slack low and slack high tides. Crabs were maintained in flowing natural seawater at ambient temperature and salinity (10-11°C, 32ppt) until use in oxygen binding experiments and the temperature and salinity experiment. Crabs were maintained at the Oregon Institute of Marine Biology and fed half a mussel every other day.

Temperature and Salinity Experiments

Crabs were maintained under controlled laboratory conditions for seven weeks to investigate whether temperature and salinity affect hemolymph subunit composition. *C. productus* were transferred into 37.85 L aquaria, each consisting of a closed recirculating seawater system at a predetermined temperature and salinity. Aquaria were conditioned two weeks prior to the start of the experiment by placing several centimeters of gravel on the bottom off the aquaria. Crabs were placed in each aquaria for several days to promote the growth of bacterial colonies which would filter out organic waste produced by the crabs throughout the experiment. There were three replicate tanks for each treatment with four crabs per tank. Three control aquaria with four crabs per aquarium were maintained at an average temperature of 14°C and average salinity of 32ppt. Three

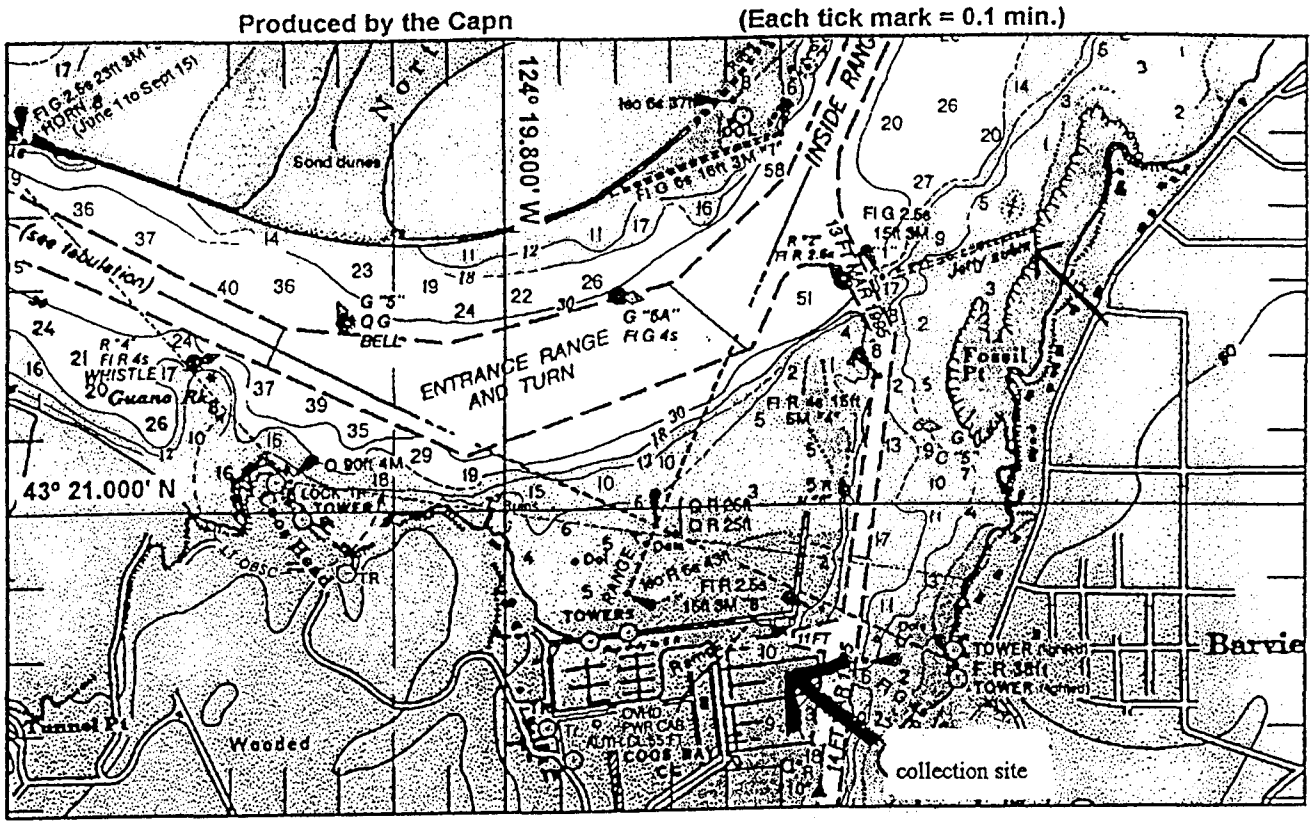

Figure 1. Map of Charleston, OR. boat basin area showing sample collection site.

low salinity treatment aquaria were maintained at an average temperature of 14°C and a salinity of 16ppt. Three warm temperature treatment aquaria were maintained at an average temperature and salinity of 19°C and 32ppt. Low salinity treatments and control aquaria were placed in the same seawater table and surrounded by running seawater at an average temperature of 14°C, pumped on an incoming tide from near the mouth of Coos Bay. Warm temperature treatment aquaria were placed in a nearby seawater table left dry.

Several parameters such as temperature, salinity and nutrition were controlled throughout the experiment. Temperature and salinity of each aquarium were monitored daily so that fluctuations could be controlled. Salinity was measured in parts per thousand (ppt) with a Reichart-Jung refractometer. Each crab was maintained individually in a cylindrical container approximately 9.5 cm in diameter and 29.5 cm long and fed half a fresh mussel every other day. The entire container was composed of plastic mesh material with $1cm^2$ holes. Two containers were placed on the gravel filter system in each aquarium with the remaining two containers on top of the bottom two. All containers were submerged and rotated from top to bottom every two to three days. All *C. productus* were exposed to natural light/dark cycles.

Approximately 19 L of water in each aquarium were siphoned out every two to three days. The 19 L were replaced in the following manner for each treatment. 19 L of fresh seawater, for the low temperature and salinity aquaria, were piped directly into the aquaria at an ambient temperature of 14°C and salinity of 32ppt. Seawater for low salinity treatments was piped first into a 53 L Nalgene container and diluted to 16ppt with

filtered tap water. The diluted seawater was then piped into the salinity treatment aquaria. Seawater for the 19°C, 32ppt aquaria was collected in 2000 L glass beakers and slowly brought up to experimental temperature over a period of six to eight hours using a heating plate. The warmed seawater was then added to the warm temperature aquaria. Crabs were slowly adapted to experimental temperatures and salinity at the onset of the experiment by exposing them to gradual increases in temperature or decreases in salinity.

Oxygen levels were measured weekly, before the water was changed, using an SI model 781/781b Oxygen meter by Strathkelvin Instruments. The partial pressure of oxygen (PO_2) was measured for each tank using a Tucker cell attached to a water bath set at the temperature of the system being measured. The percent saturation of oxygen was then converted to μmol of oxygen.

Ammonia Analysis

Ammonia, the nitrogenous excretory product of *C. productus*, was measured weekly in each aquarium. Total ammonia-nitrogen (NH_4-N) was measured spectrophotometrically according to Bower and Holm-Hansen (1980). Triplicate samples of water from each aquarium plus standards were prepared at ambient temperature. One hour after mixing water samples and reagents, absorbances were measured in 1 cm quartz cuvettes at 640 nm using a Beckman DU70 spectrophotometer.

Ammonia Analysis Reagents

Reagents were prepared volumetrically according to Bower and Holm-Hansen (1980) using dionized water no earlier than seven weeks prior to use.

Seawater-100% seawater prepared by filtering fresh seawater through 11.0 cm filter paper and 50% seawater prepared by diluting seawater with dionized water down to 16ppt and filtered through 11.0 cm filter paper.

Ammonium sulfate standard solutions- Stock solution (100mg NH_4-N/L). 0.472g of ammonum sulfate/L Stored in a brown glass bottle. *Working standard solution* (10mg NH_4-N/L). Diluted stock solution 1:10 with dionized water and stored in brown glass bottle.

Ammonium sulfate working standard solution was diluted with 100% seawater to create 1µg/l, 0.5µg/l and 0.1µg/l standards. A second set of standards for the 50% seawater aquaria were made using 50% seawater to dilute the working standard. 100% and 50% standards were used because absorbances increase with decreasing salinity (Bower and Holm-Hansen, 1980).

Samples consisted of 1 ml of water from each aquarium with 120µl salicylate catalyst solution and 200µl alkaline-hypochlorite solution. Absorbances at 640nm were read against dionized water after one hour but before three hours. Sample absorbances were converted to concentrations of total ammonia as in Bower and Holm-Hansen (1980). Standard values were derived from the average absorbance of an ammonium sulfate working standard solution with 0.5 µg/l of ammonia.

Hemolymph Sample Preparation

Initial hemolymph samples were obtained and prepared within 24 hours of crab collection. Approximately 0.3-0.4 mls of hemolymph were withdrawn by needle and syringe from the sinus at the base of a walking leg and allowed to coagulate on ice for at least 30 minutes according to Terwilliger and Brown (1993). Samples were then centrifuged at 10°C for five minutes at 13,200 rpm, and the supernatant containing Hc was stored at 10°C. Hemolymph samples were collected weekly from each crab throughout the seven-week experiment and prepared in the same manner as initial samples.

Polyacrylamide Gel Electrophoresis

All hemolymph samples collected were analyzed according to a protocol described by Terwilliger and Terwilliger (1982). Gel electrophoresis was used to analyze relative amounts of 25S Hc and 16S Hc and for identifying hemocyanin subunit composition and concentrations.

pH 7.4 PAGE

Non-dissociating, non-denaturing polyacrylamide gel electrophoresis at pH 7.4 with 5% acrylamide gels (pH 7.4 PAGE) was used to analyze the amounts of 16S and 25S oligomers (Terwilliger and Terwilliger, 1982). Hemolymph sample preparation for pH 7.4 PAGE consisted of diluting 5µl hemolymph supernatant with 20µl of pH 7.4 dilution buffer and 5µl of bromophenol blue/glycerol 3:1 (Fairbanks et al. 1971). pH 7.4

dilution buffer consisted of 0.1M HCl, 0.1M NaCl, 0.01M $CaCl_2$ and 0.01M $MgCl_2$ titrated with 1M Tris to pH 7.4. 4µl of each sample were loaded in a well of the gel. The hemolymph was electrophoresed at pH 7.4 for two hours at 35 mamps. Buffers for pH 7.4 PAGE electrophoresis included: lower gel buffer, 0.05 M Tris-HCL (pH 6.8); upper gel buffer, 0.05M Maleic Acid, 0.05M NaOH and 0.05M Tris (pH 7.4). Gels were stained with Coomassie Blue and destained with 10% acetic acid.

pH 8.9 PAGE

Aliquots of (the remaining) hemolymph were electrophoresed at pH 8.9 according to Davis (1964) for comparison of monomeric subunits among individual crabs. Hemolymph was dialyzed for 24 hours against pH 8.9 upper gel buffer, containing 0.052M Tris, 0.052M Glycine and 0.499mM EDTA. 5µl of dialyzed hemolymph were diluted with 20µl of pH 8.9 upper gel buffer and mixed with 5µl bromophenol blue/glycerol (3:1) (Fairbanks et al. 1971). Samples were then electrophoresed at pH 8.9 with 7.5% acrylamide gels for approximately 90 minutes at 35 mamps. Buffers for pH 8.9 electrophoresis included: lower gel buffer, 0.049M Tris, 0.05M HCl and 0.499mM EDTA pH 8.1, upper gel buffer is the same as pH 8.9 upper gel buffer used to dialyze samples.

Gel-Pro Analyzer 3.0 Protein Quantification

Relative amounts of 16S Hc and 25S Hc fractions and monomeric subunits within crabs were compared via gel analysis of weekly samples with imaging software, Gel-Pro Analyzer 3.0. The relative amounts of 25S Hc versus 16S Hc and the relative amounts of each subunit present within an individual crab's hemolymph were quantified. The percent of 25S Hc and 16S Hc equaled 100% for analysis purposes. Umax Binuscan PhotoPerfect (scanning software) was used to capture images of the gels using identical parameters for light/dark and contrast.

The protein bands, for all pH 8.9 and pH 7.4 PAGE samples, were identified for each lane of a gel with Gel-Pro. The background was subtracted and the absolute integrated optical density (IOD) was measured for each band and the whole hemolymph. IOD measured the volume of the band in each lane profile. The IOD for each band was then converted to percent of protein per lane (sample) by dividing the band IOD by the sum IOD for all the bands identified within a lane. Prior to statistical analysis, the precent of each subunit or oligomer within the hemolymph was converted using an Arcsine transformation.

PAGE pH 7.4 and pH 8.9 Data Analysis

The effects of salinity and temperature on oligomer concentrations and whole hemolymph levels were tested by 1 way analysis of variance with repeated measures (ANOVA). The subunit concentrations were tested by 2-way analysis of variance with repeated measures. Changes in ammonia levels and oxygen levels within the aquaria

were also tested by 2-way ANOVA. Statistical significance was accepted at $p<0.05$. Statistical analyses were done using SYSTAT® 9 (SYSTAT, Inc. 1999). In all cases data met the assumptions of normality and homogeneity of variance except where noted.

Hemocyanin Oligomer Purification

Low pressure liquid chromatography (LPLC) was used to purify the 25S Hc fraction from 16S Hc fraction in *C. productus* hemolymph based on size differences of the oligomers. Approximately 8ml of hemolymph were collected from *C. productus* and concentrated down to 3ml using Centriplus centrifugal filter devices and a Beckman J2-HS centrifuge. The crabs sampled were collected in the Charleston boat basin and maintained in running seawater. Crabs used for oxygen binding studies were not experimentally manipulated prior to use. Concentrated hemolymph was run through an agarose column, Biogel A 1.5M, equilibrated against 0.1 ionic strength Tris-HCl, pH 7.5 buffer containing 0.1M NaCl, 0.01M $MgCl_2$ and 0.01M $CaCl_2$, titrated with 1M Tris to pH 7.5. The purity of the 16S Hc and 25S Hc fractions were then analyzed on 5% acrylamide gels at pH 7.4.

Oxygen Equilibria of *C. productus* Hc

Oxygen equilibrium properties of purified 25S Hc and 16S Hc fractions were measured to provide information as to the way respiratory proteins of *C. productus* combine with oxygen. Oxygen binding was conducted tonometrically at 10°C according to a protocol described by Benesch et al. (1965). Oxygen equilibrium properties for

duplicate samples of each oligomer fraction were measured. First an aliquot of Hc was deoxygenated by alternately connecting the tonometer to a vacuum pump briefly and then allowing the sample to equilibrate on ice. This process was repeated until bubbles ceased to develop. The process was then repeated at room temperature until there was no change measured by a spectrophotometer at 340 nm indicating the hemocyanin was in the deoxygenated form. The sample in the tonometer was equilibrated in a 10°C water bath for 10 minutes and the deoxygenated spectrum was measured. A known amount of air was then introduced into the tonometer. The volume of air added into the tonometer was recorded and the protein and oxygen were allowed to equilibrate ten minutes at experimental temperature (10°C). Absorbances were then measured from 250 nm to 450 nm using the Beckman DU70 spectrophotometer. Succeeding volumes of air were introduced in a similar fashion. After a series of at least six points had been determined, the tonometer was opened to the atmosphere and air was introduced to oxygenate the protein. The absorbance was then measured for 100% oxygenation. Finally, the protein was exposed to pure oxygen to record any further change in absorbance. The pH of the sample was measured at 10°C immediately after the oxygen equilibrium curve was completed. Temperature of the room and barometric pressure were also recorded.

This experiment was repeated at different pHs to determine the Bohr effect. Duplicate samples were measured at each pH. The pH of the hemolymph was changed by dialyzing purified 16S Hc and 25S Hc fractions collected through LPLC in 1x Tris buffer of 0.1M HCl, 0.1M NaCl, 0.01M $CaCl_2$ and 0.01M $MgCl_2$ titrated to the desired pH. 16S and 25S fractions were dialyzed for 24 hours at 10°C against Tris buffers at pHs

7.3, 7.5, 7.6, 7.7, 7.9, 8.1 and 8.2. After dialysis, oxygen binding curves were measured for each sample in duplicate according to the procedure used to create the initial curve.

Effects of the allosteric effector, lactate, on the oxygen affinity of *C. productus* Hc were also measured . The 25S Hc and 16S Hc fractions were dialyzed for 24 hours at 10°C against pH 8.0 buffer 0.1M Tris-HCl, 0.1M NaCl, 0.01M $MgCl_2$ and 0.01M $CaCl_2$, titrated with 1M Tris to pH 8.0 to increase the pH of the oligomer fractions from pH 7.5 to pH 8.0. Lactate and NaCl were added to each tonometer for a total volume of 2.5 mls of hemolymph sample at a specified lactate concentration. Lactate concentrations measured were 0, 2.5, 5 and 10 mM. Dialysis was used to remove added lactate and NaCl between measurements of oxygen binding at each lactate concentration. Hemolymph samples were dialyzed for 24 hours against pH 8.0 buffer.

The percent Hc which was saturated with oxygen (y) was calculated using the following formula: $y/100=A_r-A/A_r-A_o$. In this equation A is the absorbance measured and A_r and A_o are the absorbances of the deoxygenated and oxygenated protein respectively. The partial pressure of oxygen was determined by the following relationship PO_2 =ml air injected x 0.21 (B-Hp) T/V T_o. B is the barometric pressure, H is the relative humidity, p equals the vapor pressure of water, T is temperature in the tonometer in °Kelvin, T_o is room temperature in °Kelvin and V is the volume of air in the tonometer. A Hill plot was constructed to analyze the oxygen binding data. A Hill plot is the logarithm of the quantity y/100-y (where y is the percent of the respiratory protein saturated with oxygen) versus the logarithm of the partial pressure of oxygen (PO_2). The Hill plot was then used to determine the $logP_{50}$.

The log P_{50} versus pH were plotted to show the Bohr effect. This is useful in analyzing the effect of pH on O_2 affinity, i.e. the percent of Hc bound to oxygen versus the pH of the Hc. The log P_{50} versus log [lactate] was also plotted.

Oxygen Binding Data Analysis

Results are expressed as Log P_{50} versus pH and Log P_{50} vs Log [lactate]. Analysis of co-variance (ANCOVA) was used to test for significance of treatment (pH or lactate concentration) and significance of oligomer. Statistical significance was accepted at $p<0.05$. Statistical analyses were done using SYSTAT® 9 (SYSTAT, Inc. 1999).

RESULTS

Temperature and Salinity

Cancer productus hemolymph consistently appeared as two broad bands representing 25S Hc and 16S Hc fractions when electrophoresed on pH 7.4 PAGE (Figure 2). A third band electrophoreses between 25S and 16S bands. This band corresponds to the cryptocyanin band characterized in *C. magister* by Terwilliger et al. (1999).

The low salinity (14°C, 16ppt) and high temperature, h(19°C, 32ppt) treatments had varying effects on the percent of 25S and 16S fractions of *C. productus* hemocyanin compared to crabs in control conditions (14°C, 32ppt) (Figure 3). In the temperature study, initially the average amount of 25S Hc for 14°C, 32ppt and 19°C, 32ppt were similar at 77%. The percent of 25S Hc for 19°C, 32ppt increased up to 79% throughout weeks 1 and 2 compared to 14°C, 32ppt levels which decreased to 72%. Throughout the rest of the experiment, the percent of 25S Hc for high and low temperature treatment crabs (14°C, 32ppt and 19°C, 32ppt) increased steadily. 25S Hc levels for 19°C, 32ppt crabs were consistently higher than 14°C, 32ppt levels.

There were changes in oligomer concentrations throughout the experiment for both temperature treatments. The percent of 25S Hc in *C. productus* placed in the high temperature treatment (19°C, 32ppt), although consistently higher than the other two treatments (14°C, 32ppt and 14°C,16ppt) , was not significantly different; $p=0.59$ (table 1). There was a significant change in the percent 25S Hc concentration over time for low

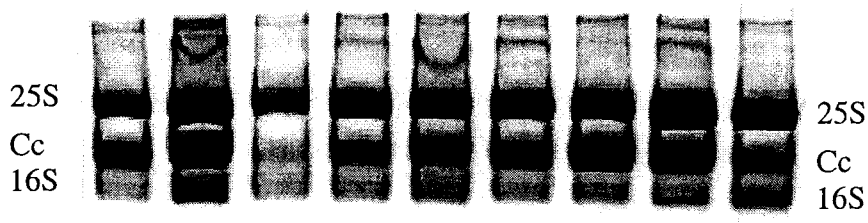

Figure 2. The 25S Hc and 16S Hc banding pattern characteristic of *C. productus* hemocyanin. Hemolymph samples from 9 individual crabs electrophoresed on a 5% polyacrylamide gel, pH 7.4. Total sample size: n=36. Upper band in each lane represents 25S Hc, midband corresponds to cryptocyanin band identified in *C. magister* and lower band represents 16S Hc.

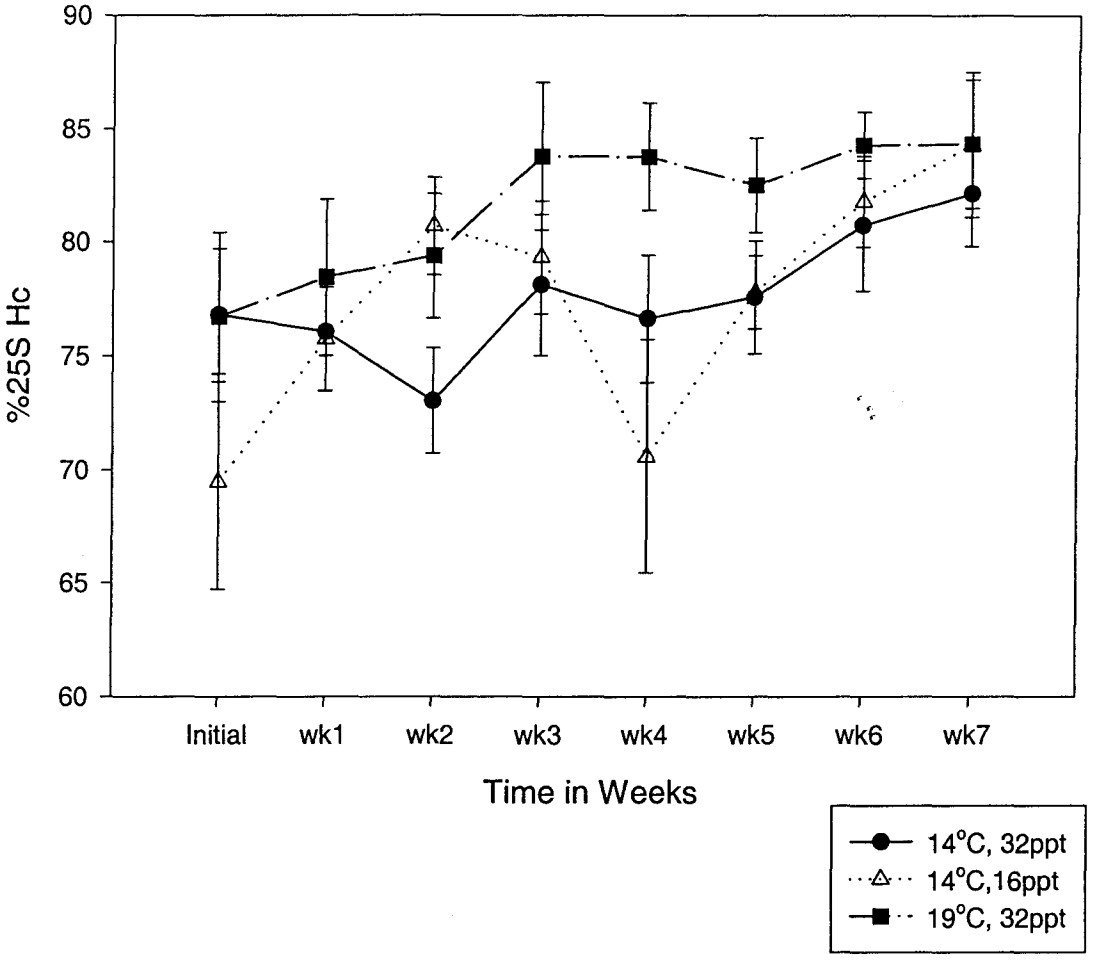

Figure 3. %25S Hc oligomer composition in Hc from *C.productus* vs time in weeks. Standard error mean bars are shown for the averages of each treatment ($14^{o}C$,32ppt; $14^{o}C$,16ppt and $19^{o}C$,32ppt). Samples analyzed on pH 7.4 PAGE and Gel Pro Analyzer 3.0. %25S Hc and %16S Hc equal 100% for analysis purposes.

Table 1
Repeated-measures, 2-way ANOVA on the effect of temperature and salinity on oligomer concentrations in *Cancer productus* hemocyanin. Treatments used include 14°C,32ppt; 14°C,16ppt and 19°C, 32ppt. Measurements of oligomers include initial and weeks 1 through 7 (see Fig. 3). Univariate and multivariate repeated measures analysis.

Source	sum-of squares	df	Mean -square	F-ratio	P
Between subjects					
Treatment	117.27	2	58.63	0.55	0.59
Error	1284.78	12	107.07		
Within subjects					
Time	352.8	7	50.4	3.38	0.003
time x treatment	228.86	14	16.35	1.1	0.37
Error	1251.39	84	14.9		

multivariate repeated-measures analysis

Effect	Wilk's lambda	Hypoth. df	Error df	F-ratio	P
Time	0.21	7	6	3.31	0.08
time x treatement	0.23	14	12	0.94	0.55

salinity (14°C, 16ppt), high temperature (19°C, 32ppt) and control (14°C, 32ppt) treatment crabs; p=0.003.

The percent 25S Hc for low salinity crabs (14°C, 16ppt) fluctuated throughout the experiment from 70% to 85% (Figure 3). The percent 25S Hc in the hemolymph of low salinity treatment (14°C, 16ppt) crabs on day 0 was lower than levels in crabs placed in the high salinity treatment (14°C, 32ppt). Levels of 25S in 14°C, 16ppt crabs then increased over weeks 1 and 2 to levels higher than 25S levels in 14°C, 32ppt crabs. 14°C, 16ppt 25S levels decreased from week 2 to week 4 then increased over the remainder of the experiment.

The percent 25S Hc present in low salinity treatment crabs (14°C, 16ppt) did not vary significantly from the other two treatments (14°C, 32ppt and 19°C, 16ppt) with p=0.59. There was however, a significant interaction between time and treatments for control, low and high salinities (p=0.003). The change in oligomers over time is not great enough to be detected by post hoc-tests due to the small sample size. Increases in the percent 25S Hc were observed from week 1 to week 7 for the high salinity treatment (14°C, 32ppt) (Figure 3).

Levels of whole hemolymph for all treatments were similar throughout the experiment (Figure 4). The levels of whole hemolymph in 14°C, 32ppt *C. productus* decreased over the first three weeks and leveled out throughout the remainder of the experiment. Levels of whole hemolymph for 14°C, 16ppt and 19°C, 32ppt treatment crabs remained constant throughout the experiment and were similar to 14°C, 32ppt hemolymph levels after week 1. There was no significant difference in whole

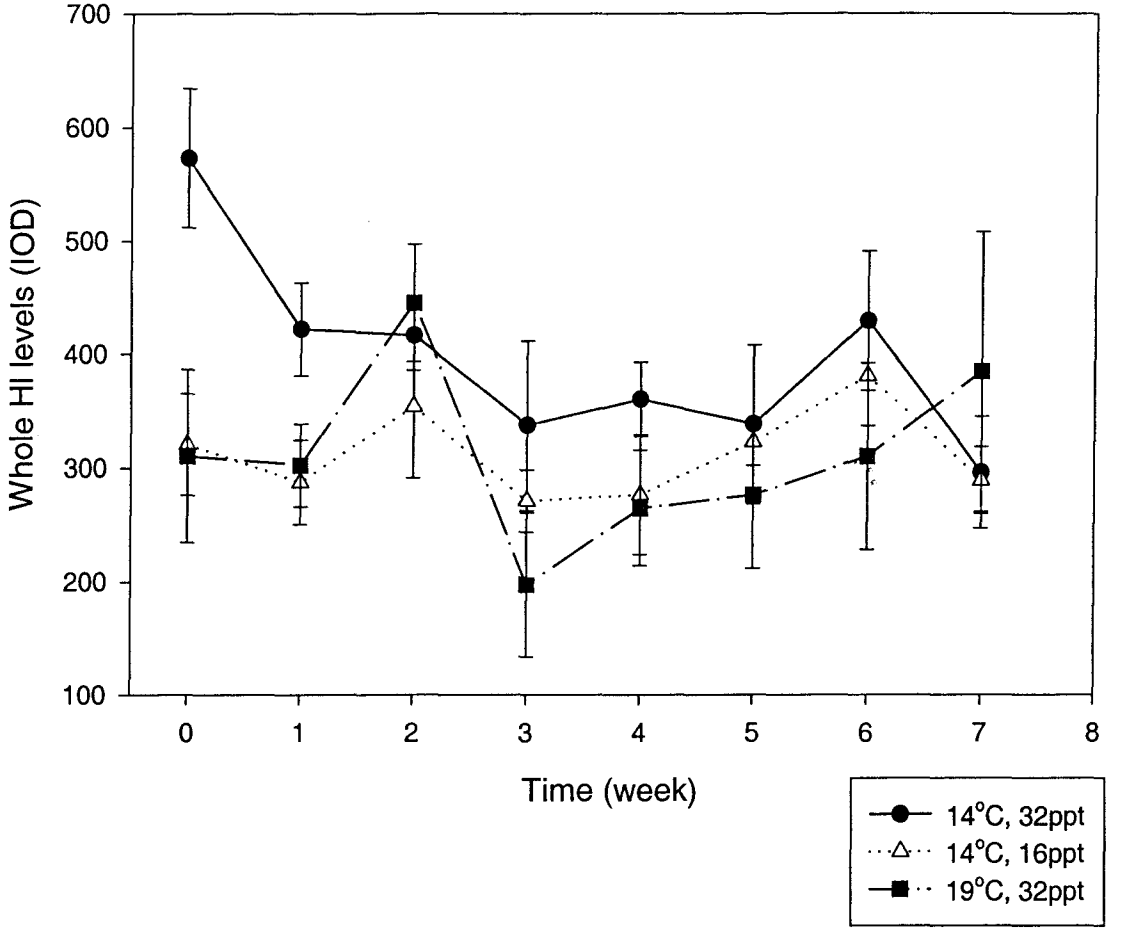

Figure 4. Effects of varying temperature and salinity on whole HI levels in *Cancer productus.* Hemolymph levels expressed in IOD (integrated optical density). Treatments included 14°C, 32ppt; 14°C, 16ppt and 19°C,32ppt. Standard error bars are shown for the average HI levels for crabs in each treatment. Sample size=5 for each treatment.

hemolymph levels between any of the treatments with p=0.13, nor was there a significant change in whole hemolymph levels over time (p=0.05).

The average partial pressure of oxygen (PO_2) for all three treatments varied slightly over time (Figure 5). 14°C, 16ppt treatment PO_2 levels ranged from 148 mmHg to 157 mmHg. The PO_2 of 14°C, 32ppt treatments ranged from 154 to 161 mmHg. High temperature treatments' (19°C, 32ppt) PO_2 levels fluctuated from 152 to 159 mmHg. There was not a significant difference in PO_2 between any of the treatments; p=0.09. There also was not a significant change in PO_2 over time for the treatments; p=0.29.

Oxygen concentrations within tanks varied throughout the experiment with a decrease in oxygen in all three treatments (Figure 6). Average oxygen concentrations in the low salinity treatment (14°C, 16ppt) were consistently higher than in the high salinity treatments (14°C, 32ppt). Throughout the experiment, the average oxygen concentrations for the high temperature treatments (19°C, 32ppt) were lower than the other two treatments. Differences in oxygen levels of the three treatments were significant; p<0.001. Each treatment varied significantly from the other two.

Average ammonia levels, within all treatments, fluctuated throughout the experiment (Figure 7). Ammonia levels for 14°C, 32ppt and 19°C, 32ppt treatments increased within the first week then remained constant throughout the rest of the experiment. Average ammonia levels for 14°C, 16ppt treatments were higher than the other treatments at the beginning of the experiment, but decreased to levels similar to 14°C, 32ppt and 19°C, 32ppt ammonia levels for the remainder of the experiment.

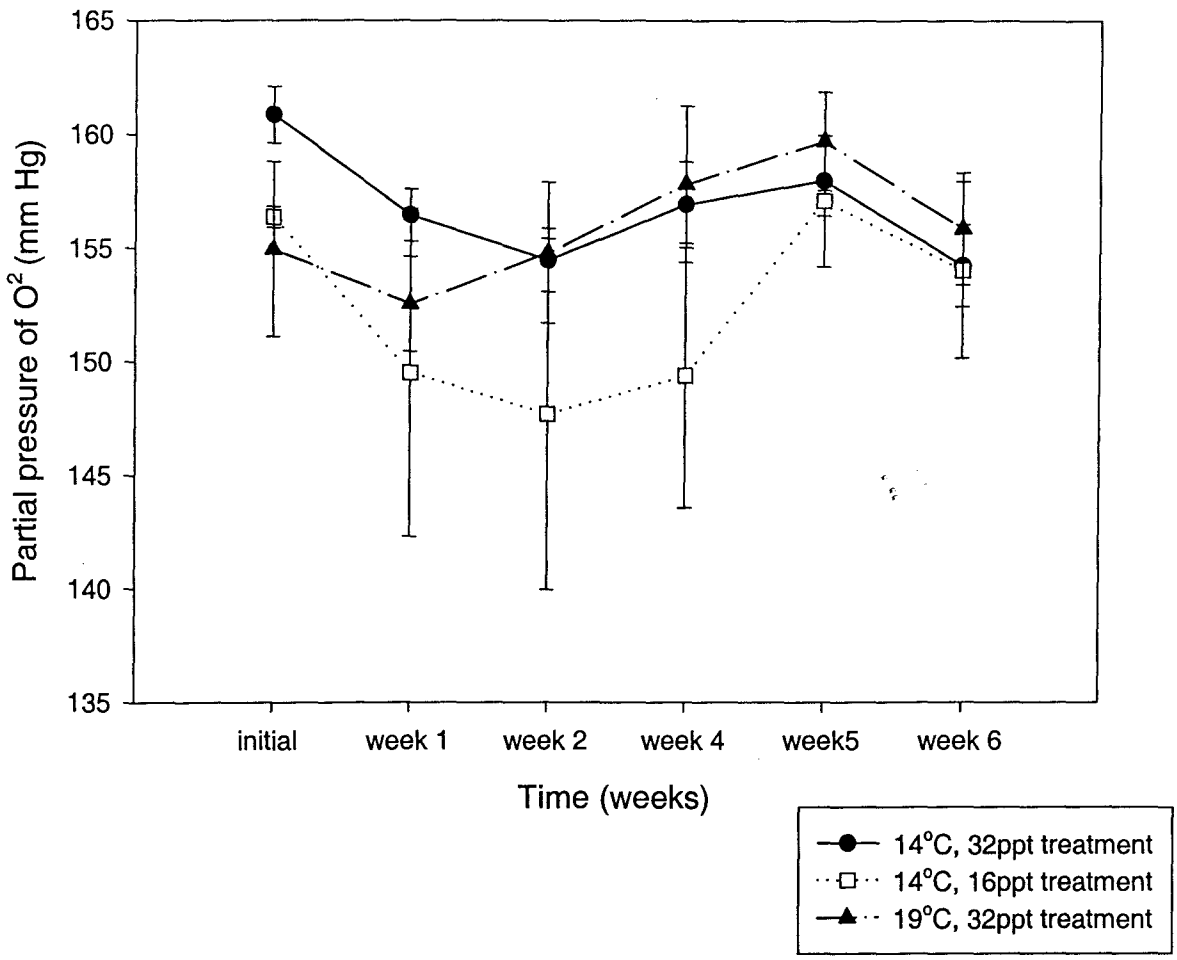

Figure 5. Average PO_2 for each treatment vs time in weeks. PO_2 measured in mm Hg. Treatments include 14°C,32ppt; 14°C, 16ppt and 19°C, 32ppt. PO_2 levels are above normal levels for seawater at 150 mm Hg except weeks 1,2 and 4 for 14°C,16ppt treatment. Standard error bars are shown for the average partial pressure for crabs in each treatment.

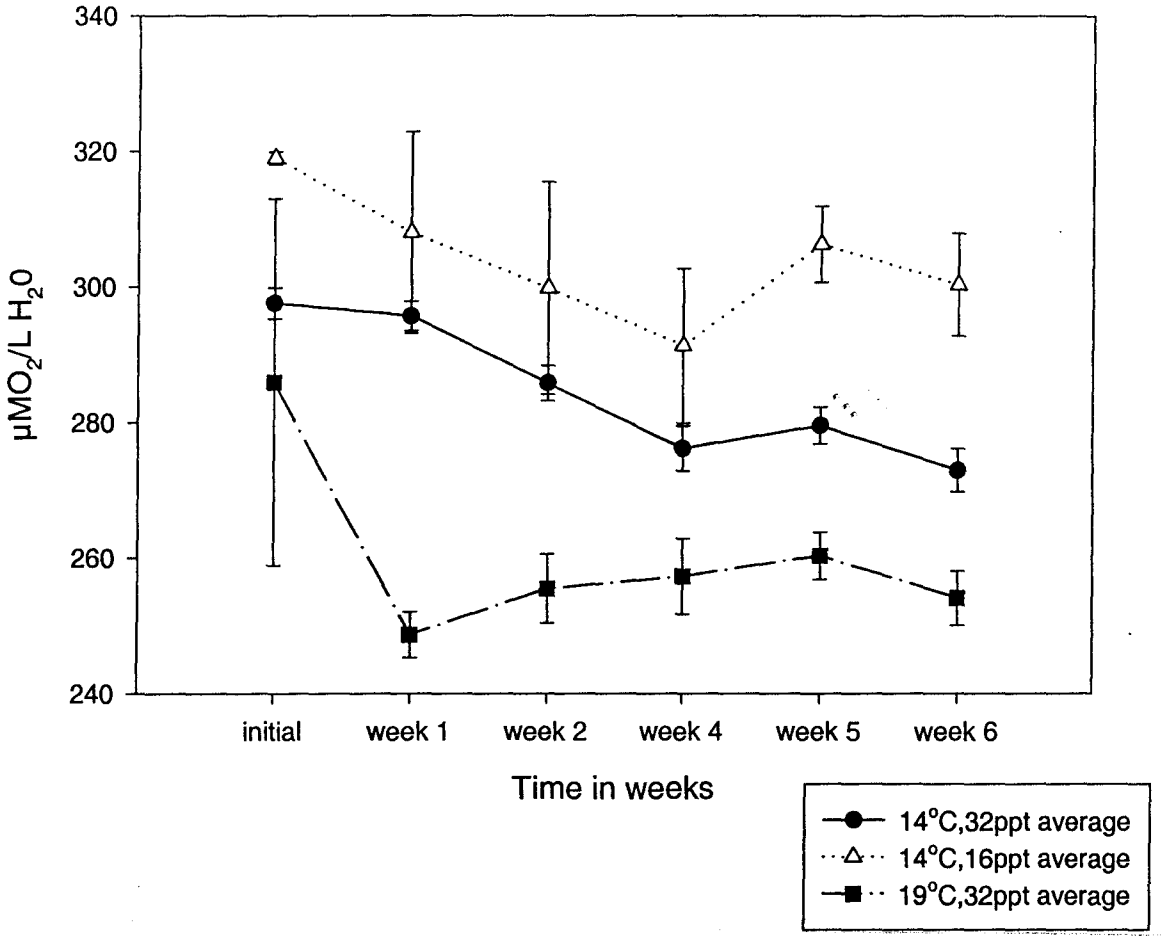

Figure 6. Average oxygen concentration for each treatment over time in weeks. Oxygen levels in $\mu MO_2/L\ H_20$. Treatments include 14°C, 32ppt; 14°C, 16ppt and 19°C, 32ppt. These data correspond to the order of coxygen concentrations expected solely based on temperature and salinity. Standard error means are shown for all values.

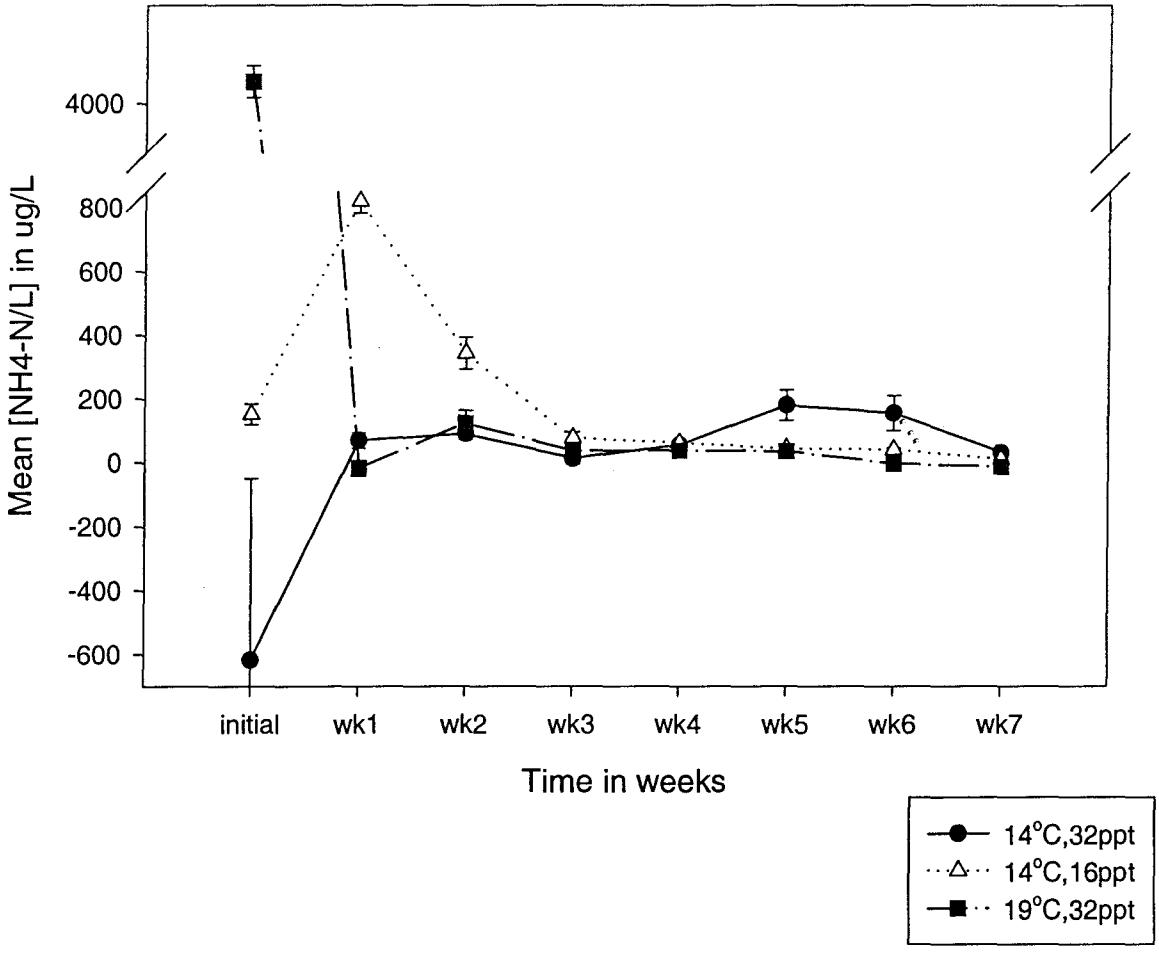

Figure 7. Mean [NH4-N/L] over time in weeks for all three treatments ($14^{o}C$,32ppt; $14^{o}C$,16ppt and $19^{o}C$,32ppt). Standard error means shown as error bars.

Ammonia levels among all treatments varied significantly ($p<0.001$). Ammonia levels also varied significantly over time ($p< 0.001$).

PAGE pH 8.9

The Hc of *C. productus* separates into a total of 12 bands in the presence of EDTA at pH 8.9 (Figure 8). Not all of the 12 subunits are found in an individual and not all occur in a constant amount (Figure 9). As few as 4 bands and as many as 9 were found within an individual crab. The most common number of bands was 6. 44% of the crabs sampled contained hemocyanin composed of 6 of the 12 identified subunits. 30.6% of the crabs were characterized by hemocyanin composed of 5 subunits. Although not all 12 subunits occurred in each individual, subunits A and C were always present (Figure 10). Several other bands, F, G, H, J, K and L were frequent while others L, B, D, E and I were infrequent.

Examples of subunit patterns and variations in band densities are illustrated in Figure 11. The photograph demonstrates several of the Hc phenotypes of *C. productus*. The bands at the top half of the gel represent slower moving non-hemocyanin components of the hemolymph. The qualitatively invariant subunits (A and C) are present in both hemolymph samples labeled.

A large number of phenotypes were observed in the male *C. productus* sampled (Figure 12). Twenty four phenotypes were observed in the 36 crabs sampled. The most common phenotype consisted of the six subunits A, C, F, H, J and L. This phenotype

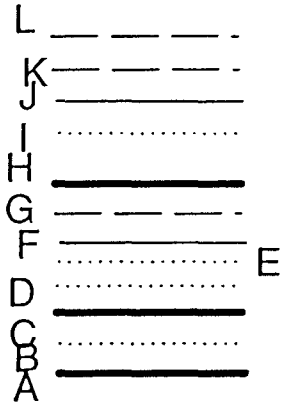

Figure 8. Positions of subunits composing hemolymph of male *Cancer productus* analyzed on pH 8.9 PAGE, 7.5% acrylamide. Figure represents all possible bands identified throughout the experiment within all crabs sampled, n=36. The highest density of each band is represented as follows: thick solid line=maximal, thin solid line- high, long dashes =medium and dotted line=low.

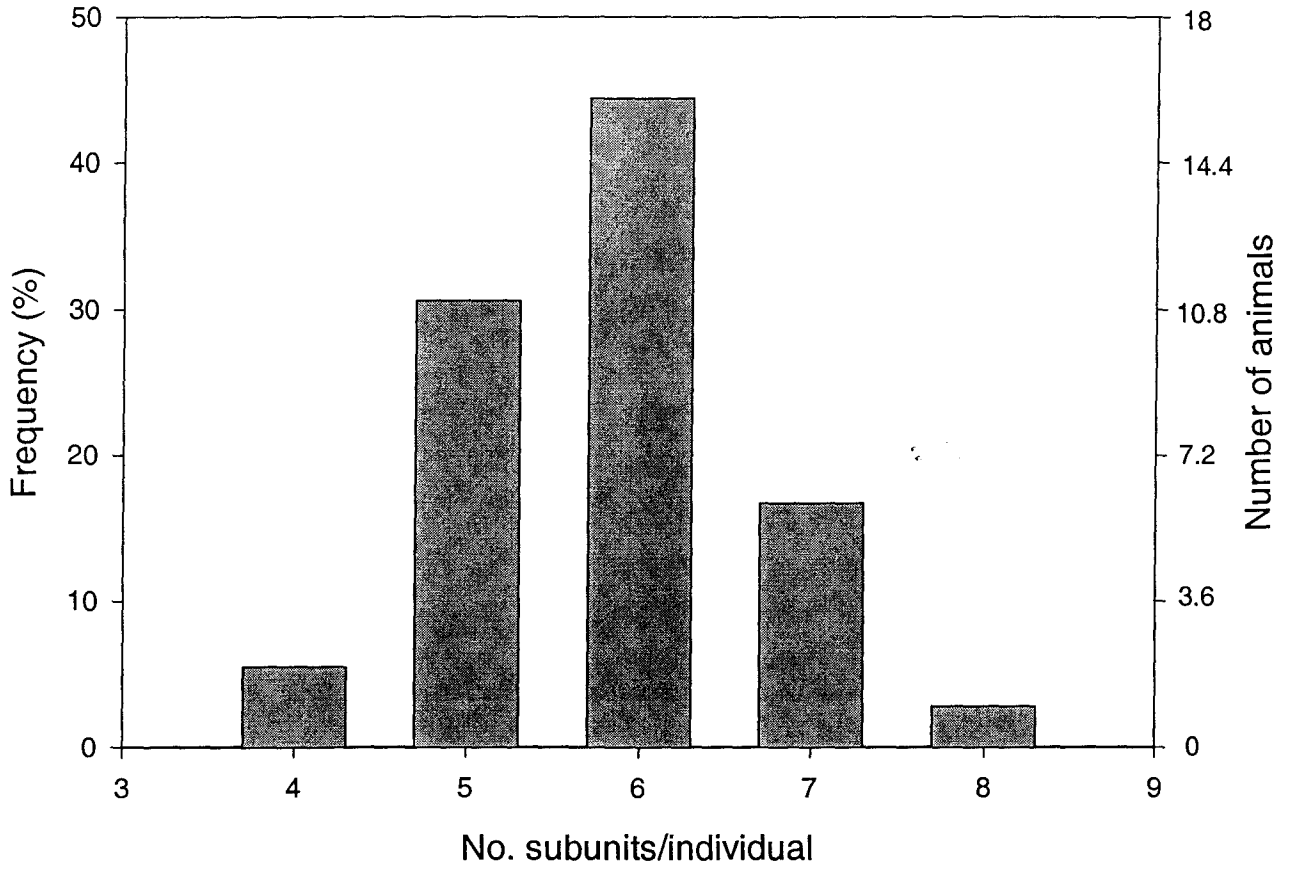

Figure 9. Frequencies of the total number of subunits found in individual *Cancer productus* (n=36). Samples analyzed by pH PAGE 8.9. Frequencies calculated from subunits identified initially(time 0) in crabs. Number of animals corresponding to percentages are also shown. 100%=36 crabs

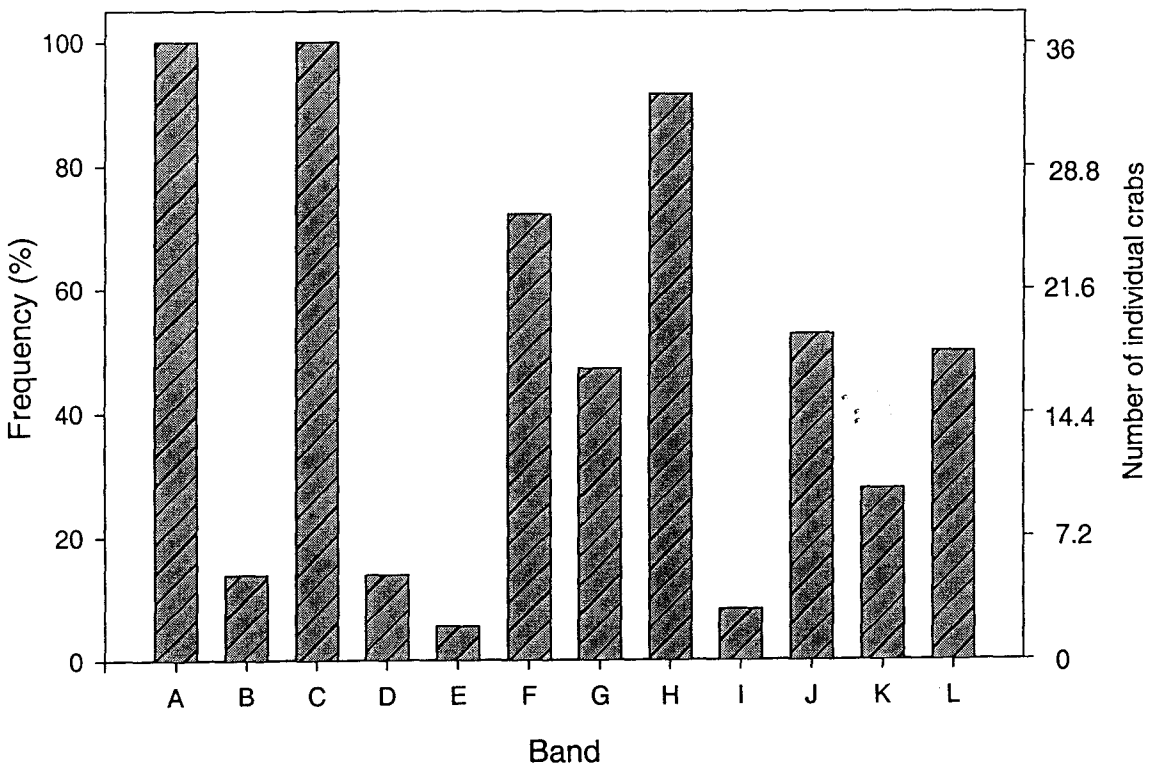

Figure 10. Frequencies of each subunit found in *Cancer productus*. Sample size n=36. Values calculated from initial samples taken from *C.productus*(time=week 0). Samples analyzed via pH 8.9 PAGE.

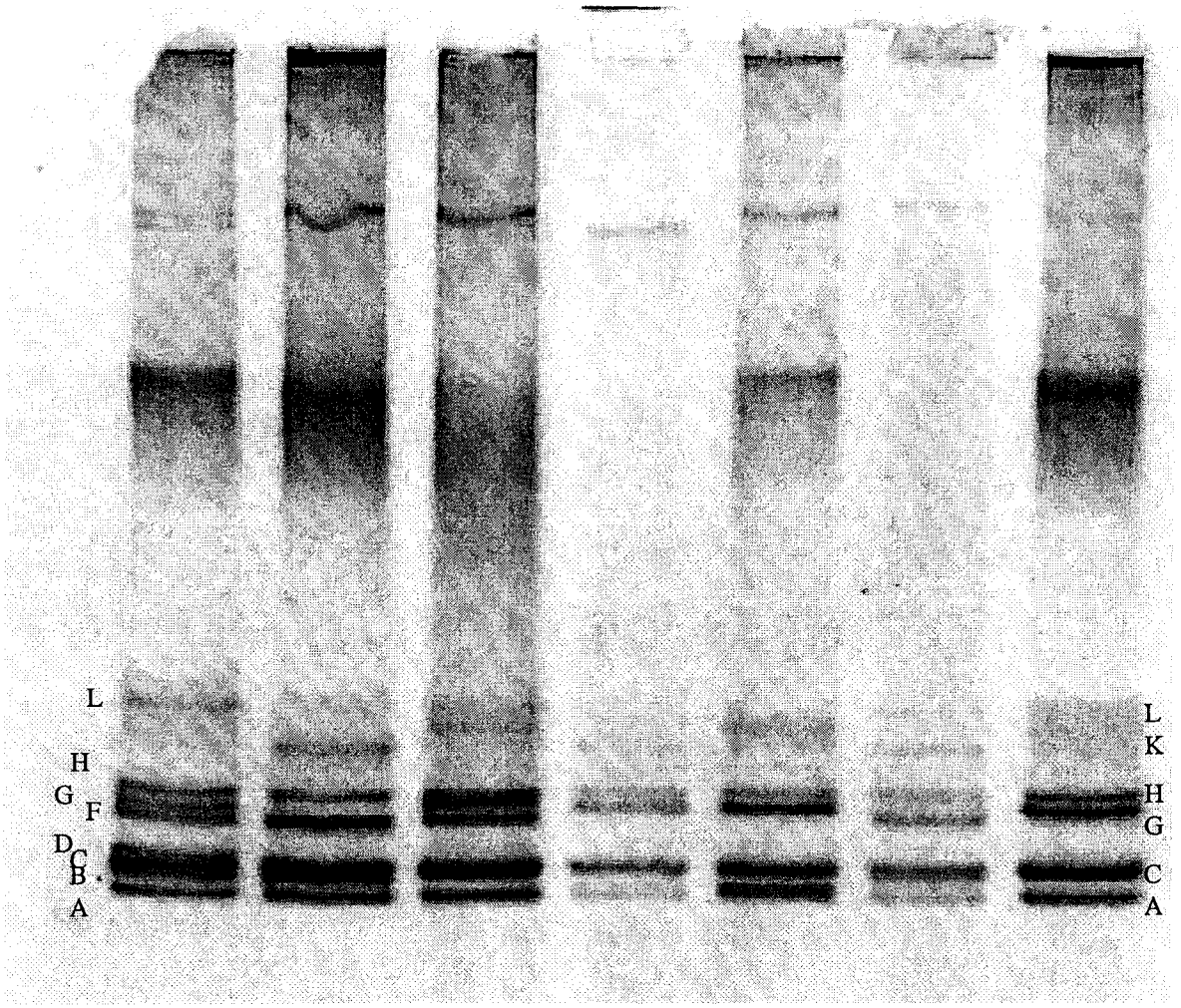

Figure 11. Subunit banding patterns of *C. productus* whole hemolymph on pH 8.9 PAGE 7.5% acrylamide. Hemolymph was dialyzed against Tris-HCl, 0.239mM EDTA, pH 8.9 buffer..Each of 7lanes represents hemolymph from a different crab.

Cancer productus phenotypes

												# of subunits
*	A		C			F		H		J		L	6
	A		C			F		H	I		K		6
	A		C			F	G	H		J		L	7
	A	B	C	D		F	G	H				L	8
	A		C			F		H			K		5
	A		C				G	H		J			5
	A		C			F		H				L	5
	A		C				G	H			K	L	6
	A		C			F		H			K	L	6
	A		C			F		H		J	K		6
	A		C			F		H		J			5
	A		C				G	H		J		L	6
	A	B	C			F		H	I		K		7
	A		C	D		F		H				L	6
	A		C			F	G	H		J			6
	A		C		E		G	H					5
	A		C			F	G				K	L	6
	A	B	C				G	H			K		6
	A		C			F		H	I	J			6
	A		C		E		G	H		J		L	7
	A		C			F	G	H		J	K		7
	A	B	C			F		H					5
	A		C	D		F		H					5
	A		C	D			G						4

Figure12. Phenotypes observed in *Cancer productus*. Phenotypes identified from results of pH 8.9 PAGE analysis. * Denotes most common phenotype among crabs sampled (characteristic of 6 crabs, n=36).

was characteristic of six of the 36 *C. productus* sampled. Several other phenotypes consisted of 6 subunits.

Hemolymph, from the five crabs analyzed in each treatment, showed changes in the concentration and composition of subunits throughout the experiment. The changes in subunit concentrations and compositions are illustrated for 1 crab from each treatment in Figures 13a, b and c. Subunit concentrations among the three treatments did not differ significantly; $p=0.82$. Although there were variations in subunit concentrations over time within all three treatments, subunit composition and concentrations did not differ significantly; $p>0.05$.

The results of the effect of elevated temperature and decreased salinity compared to ambient seawater conditions on subunit composition of *C. productus* hemocyanin were not normally distributed for 14°C 32ppt, 14°C, 16ppt or 19°C, 32ppt. Neither treatment expressed sphericity. Temperature and salinity experiment results did express homogeneity of variance; $p>0.05$.

Oxygen Binding

The 16S Hc fraction of *C. productus* Hc was purified twice through LPLC to obtain a sample without detectable amounts of 25S Hc. PAGE 8.9 analysis of 16S versus 25S Hc oligomer yielded different subunit compositions for the two oligomers. Similar results were obtained for two different crabs, #41 and #22 (Figure 14a and b). The 25S Hc of both crabs had a banding pattern similar to the whole Hc of the crab. The purified 16S Hc of each crab was missing one subunit. The missing subunit, in both crabs, was

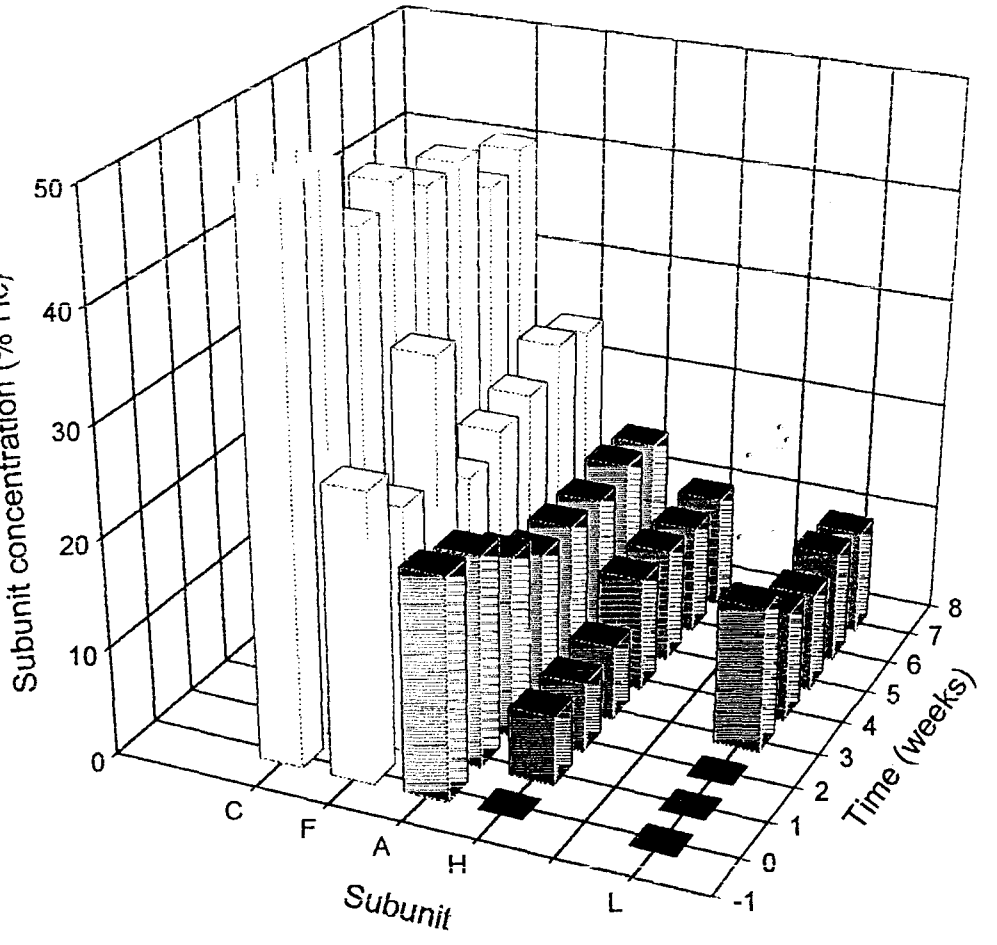

Figure 13a. Subunit composition and change in subunit concentration (%Hc) for *C.productus* #13. 14^{o}C,32ppt treatment sample. Subunits are depicted in order of overall concentration for graphical purposes.

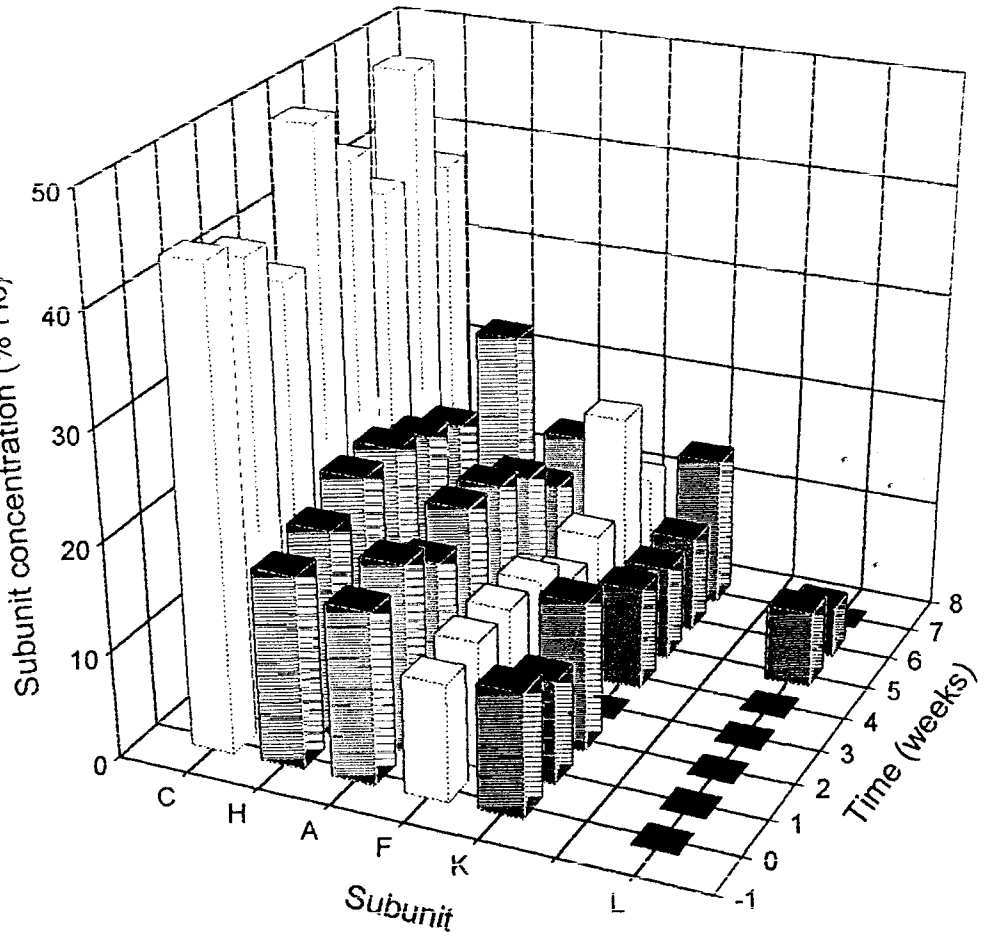

Figure 13b. Subunit compostition and change in subunit concentration (% of Hc) for *C.productus # 22.* 14^{o}C,16ppt treatment sample. Subunits are depicted in order of overall concentration for graphical purposes.

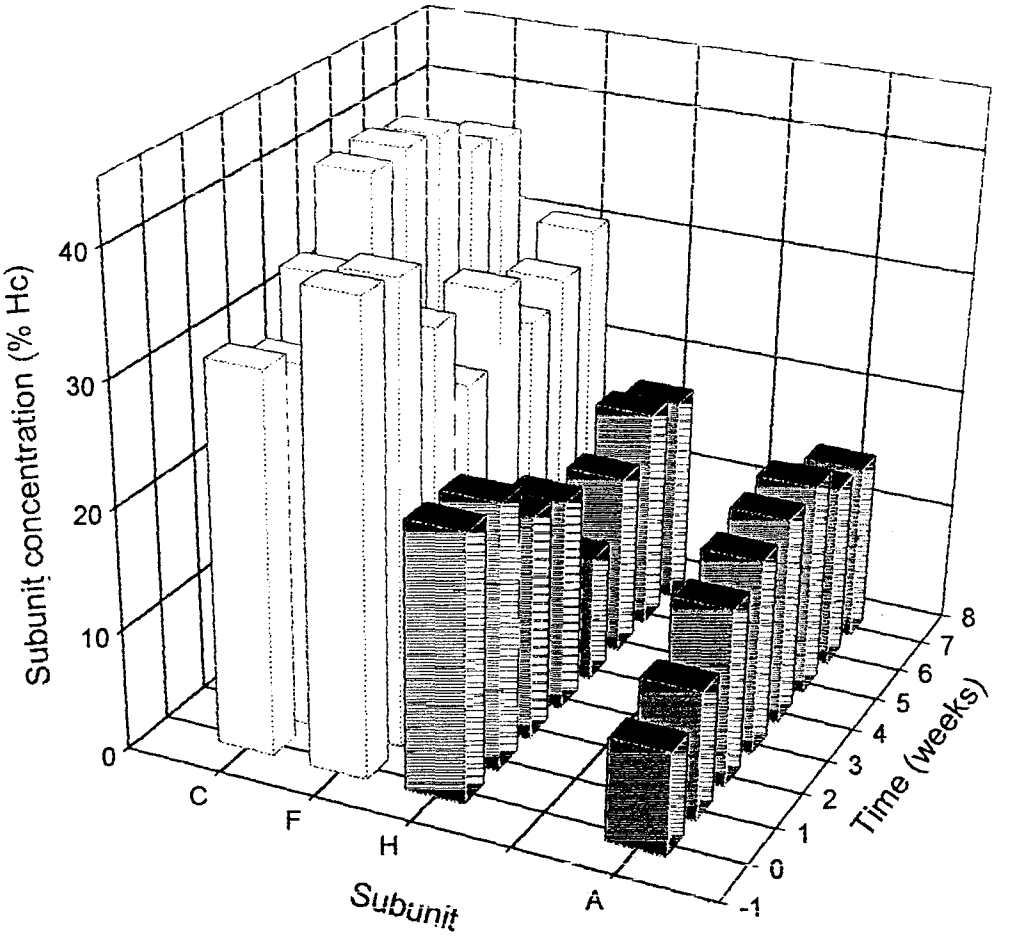

Figure 13c. Subunit composition and changes in subunit concentrations (%of Hc) for *C.productus* #33. 19°C,32ppt treatment sample. Subunits are depicted in order of overall concentration for graphical purposes.

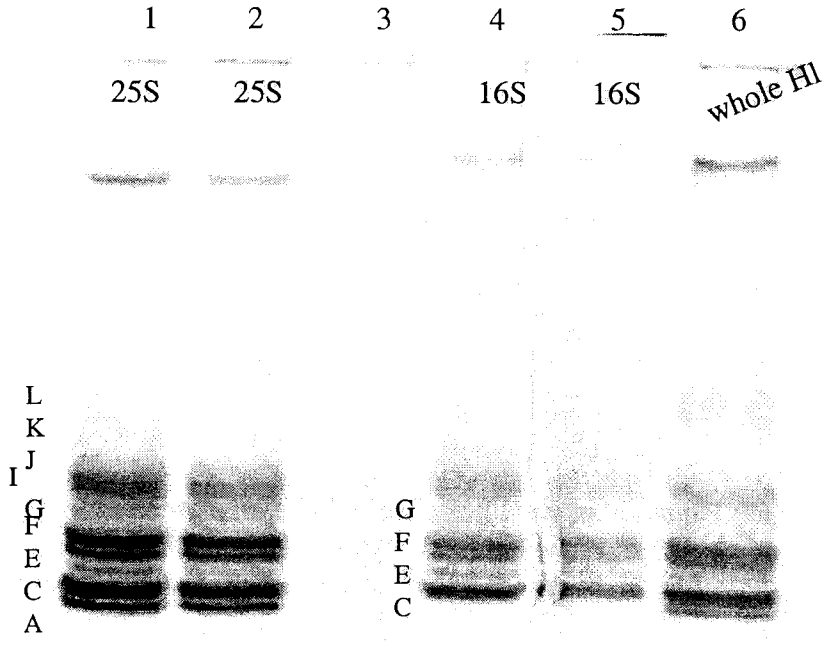

Figure 14a.

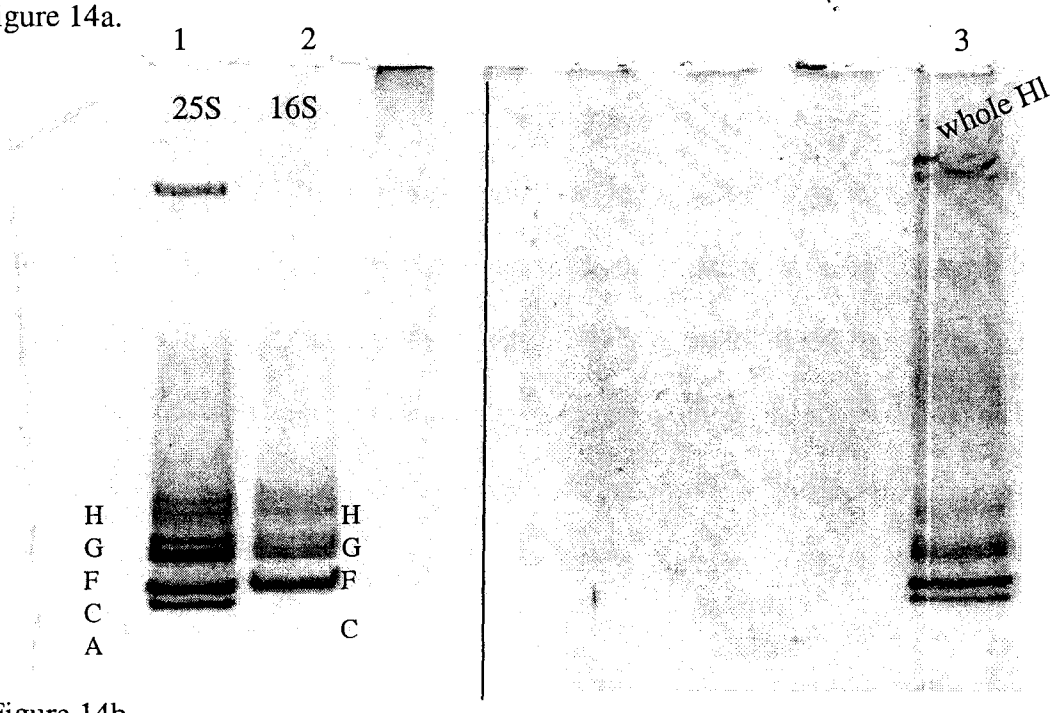

Figure 14b.

Figures 14a and 14b. Examples of the subunit compositions for *C. productus* 25S and 16S oligomers separated from whole hemolymph through LPLC and used for oxygen binding studies.

13a. Samples from crab #41: lanes 1 and 2, 25S fraction at two loading volumes; lanes 4 and 5, 16S fraction at two loading volumes; lane 6 whole hemolymph

13b. Samples from crab #22: lane 1, 25S fraction; lane 2, 16S fraction; lane 3 whole hemolymph.

subunit A. *Cancer productus* #41 whole hemolymph and 25S Hc contained 9 subunits (Figure 14a). The 16S oligomer fraction contained only 8 of the 9 subunits identified in the whole hemolymph. Subunit A was missing.

Slight variations in oxygen affinities of 25S Hc compared to 16S Hc were measured within the pH range of 7.3 to 8.8 (Figure 15). For both 25S Hc and 16S Hc, oxygen affinities increased as pH increased (log P_{50} decreased). The oxygen affinity of 16S Hc was consistently higher than 25S Hc within the pH range of 7.0 to 8.75. Oxygen affinities of 25S Hc and 16S Hc at pH 8.0 were similar to each other with log P_{50} values of 2.90. At higher pH levels, 16S Hc oxygen affinity was higher than 25S Hc. The Bohr effect of 25S Hc was –1.10 and of 16S Hc was –1.33. There were no significant differences in the Bohr effects of 25S Hc and 16S Hc, $p= 0.10$.

Increases in lactate caused an increase in oxygen affinity for both 25S Hc and 16S Hc (Figure 15 and Figure 16). In the presence of lactate at pH 8.0 the oxygen affinity of 16S Hc was increased more than the oxygen affinity of 25S Hc. At a given lactate concentration, the effect of lactate on the oxygen binding of 25S compared to 16S was significantly different, $p<0.01$. Although the magnitude of the lactate effect (Δ log P_{50}/ Δ log lactate) on 25S Hc was –0.52 whereas the lactate effect on 16S Hc was –0.29 these magnitudes were not significantly different; $p=0.284$. Oxygen affinity for 25S Hc steadily increased as lactate concentrations increased. Oxygen affinity of 16S Hc also increased as lactate increased, but to a lesser extent than 25S Hc oxygen affinity so that at lactate concentrations near 10mM the oxygen affinities of 25S Hc and 16S Hc were similar.

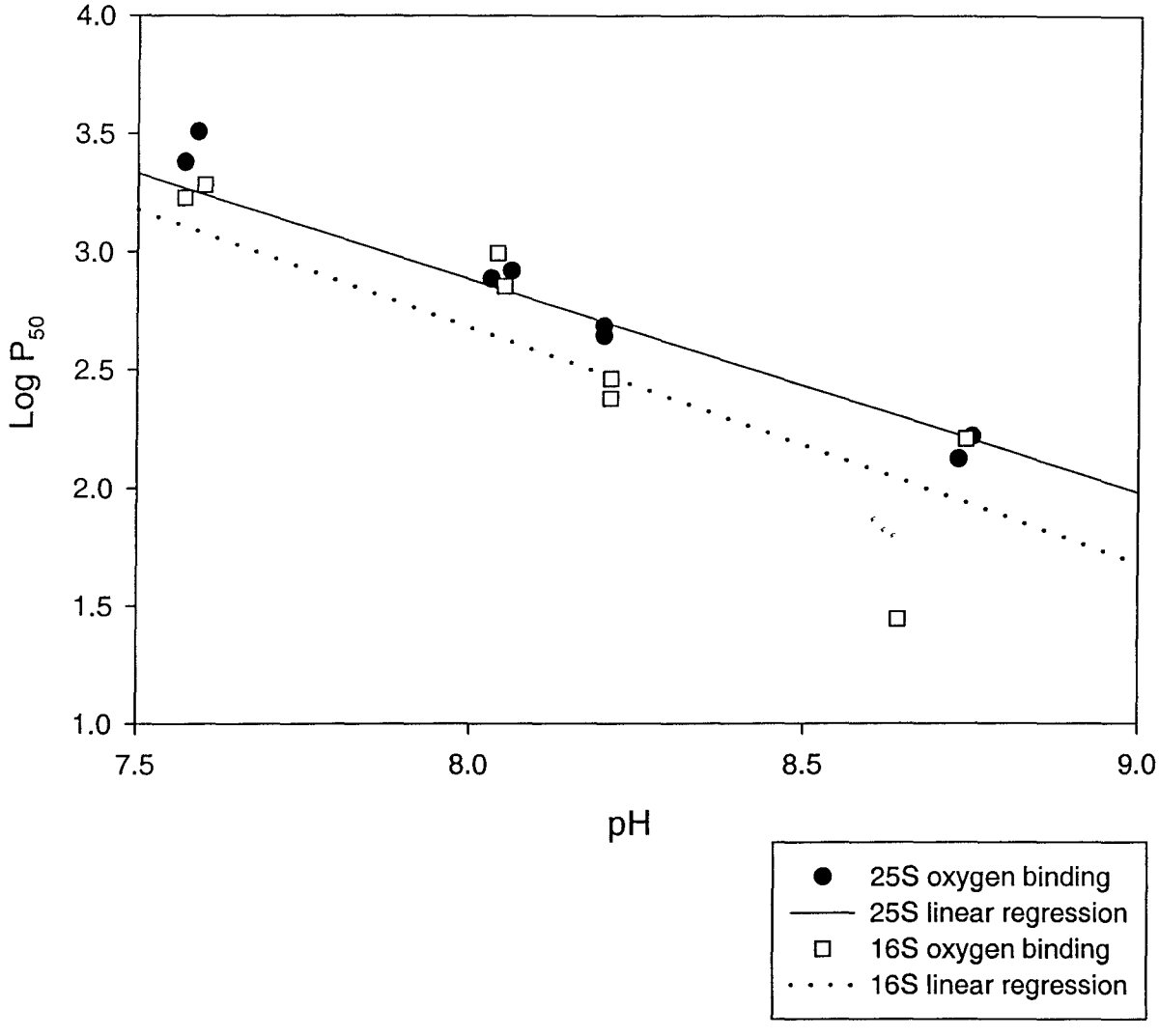

Figure 15. The Bohr curve of *C.productus* hemocyanin 25S and 16S in 1M Tris buffer of 0.1M HCl, 0.1M NaCl, 0.01M $CaCl_2$ and 0.01M $MgCl_2$ titrated with 1M tris to the designated pH at $10^{o}C$. Log P_{50} in mm Hg. Replicate samples for 16S and 25S were measured. Linear regression lines are depicted for each oligomer. The Bohr effect on 25S was -1.1 and the Bohreffect on 16S was -1.33.

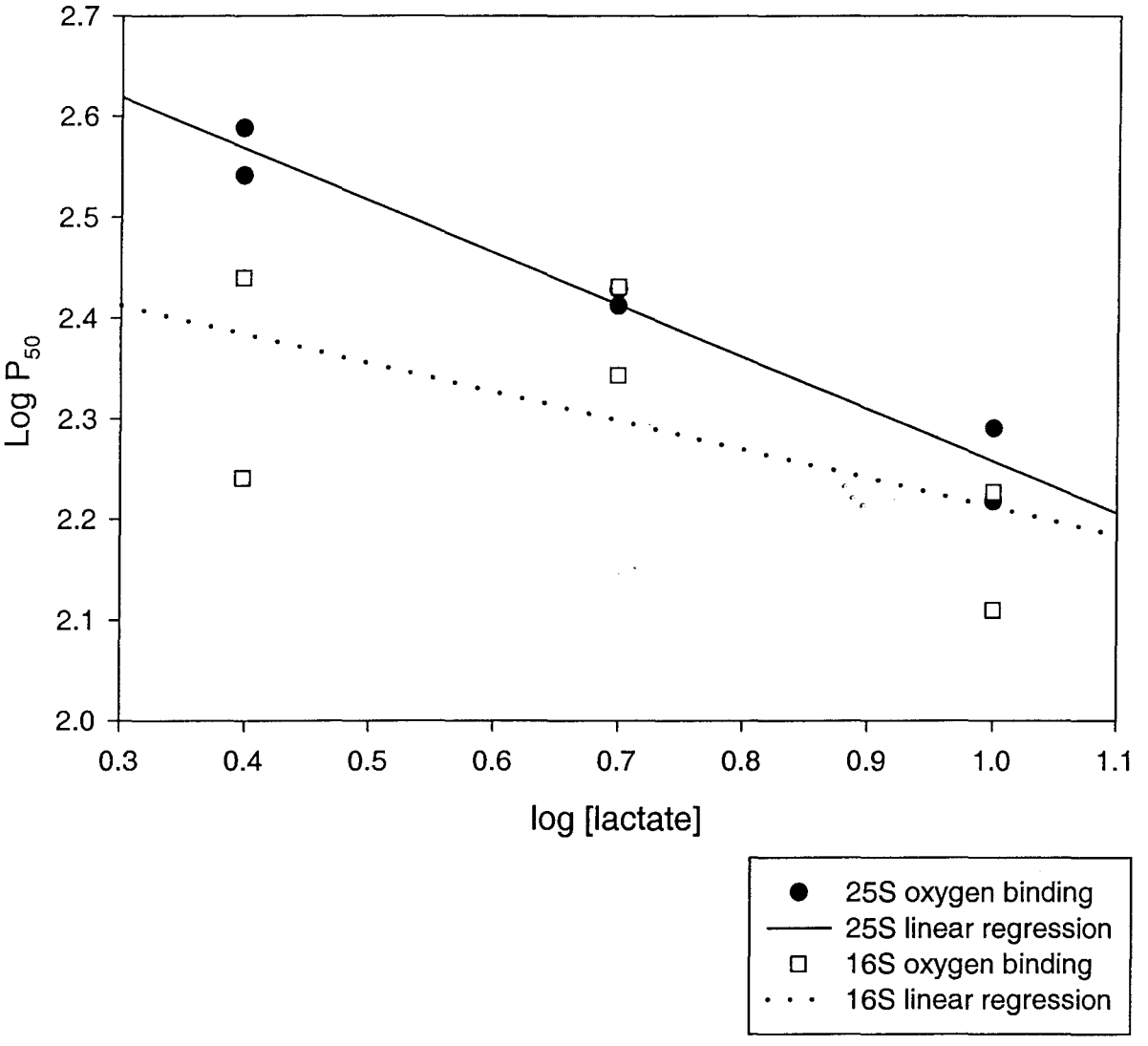

Figure 16. Effect of [lactate] on the oxygen binding of *C. productus* Hc 16S and 25S oligomer fractions at 10°C and pH 8.0. Lactate concentrations used were 0mM, 2.5mM, 5mM and 10mM. Replicate samples of 25S and 16S were measured. P_{50} measured in mmHg. Linear regression lines are depicted for each oligomer. The lactate effect on 25S was -0.52 and the lactate effect on 16S was measured at -0.29.

DISCUSSION

Male *Cancer productus* exhibit many Hc phenotypes. The subunit concentrations of the various phenotypes are flexible. The present study suggests temperature and salinity do not play a role in phenotypic plasticity of Hc's within *C. productus*. Oxygen binding differences between 25S Hc and 16S Hc may explain a functional role for the presence of the two forms as well as phenotypic differences among the crabs studied. Oxygen binding of 25S differed from 16S fractions of Hc within a pH range from 7.757 to 8.715. Oxygen binding results suggest that while pH is important in modulating the oxygen binding of *C. productus*, lactate concentration plays an even greater role in the oxygen binding difference between 25S Hc and 16S Hc.

Temperature and Salinity Experiments.

Only male *C. productus* were used throughout this study. Males were selected to reduce the added complications of reproductive proteins that may be present in female hemolymph. A single sex of crabs was also selected to reduce any effects sex may have on Hc in *C. productus*. There are reports of sex differences in Hc concentrations. Horn and Kerr (1963) found female *C. sapidus* possessed greater Hc concentrations than males. In comparison, Spicer and Baden (2000) found none of the species examined in their study (*Nephrops norvegicus, Liocarcinus depurator and Hyas aranaeus*) exhibited detectable differences in Hc concentrations between males and females. Differences in Hc concentration between male and female crabs may be related to differences in timing of ecdysis. Premolt *C. magister* have increased levels of Hc and females usually molt

prior to males (N. Terwilliger, pers. observation). Increased Hc concentration in one sex may cause subunits low in concentration to be more detectable in that sex, which may affect the interpretation of the effects of various treatments.

Whole hemolymph from male *C. productus* consists of 25S Hc and 16S Hc, a pattern typical of crustaceans. Whole hemolymph levels did not vary significantly throughout the experiment. 25S Hc and 16S Hc electrophoresed at pH 7.5 appeared as two bands (Figure 2). These results are similar to those found by Wache (1985). The ratio of 25S Hc to 16S Hc within individual crabs varied throughout the experiment, but not significantly.

Subunit structure of Hc analyzed by pH 8.9 PAGE is more complex than previously thought. Research by Wache (1985) identified 7 bands which varied in intensities. This study identified 12 subunits that formed various combinations with 4 to 9 subunits. Twenty four phenotypes were characterized within the 36 crabs sampled. The intensities of subunits also varied throughout the experiment. Although subunit concentrations and compositions varied, there was no significant difference in the concentration of any one subunit throughout the experiment as a function of salinity or temperature

The hypothesis that salinity and temperature may elicit the expression of phenotypic plasticity in *C. productus* Hc was not supported by these results. Even though subunit concentrations changed, phenotypic variations in oligomers and subunit concentrations within crabs were not significantly different among treatments. The lack of correlation between phenotypic plasticity of *C. productus* Hc and exposure to

hyposalinity differs from previous research on another braychuran crustacean, *C. sapidus* (Mangum and Rainer, 1988; and Mangum et al.; 1991). Research by Mangum and Rainer (1988) found Hc subunit composition shifted when crabs were transferred from high to low salinity and vice versa (see also Mangum et al. 1991). A review by Cameron and Mangum also suggested synthesis of aggregates of the Hc molecules may be a possible response to acclimation to new temperature (1983). Results of the effects of temperature on phenotypic plasticity were consistent with the results of Rutledge's experiment (1981). Previous research by Rutledge (1981) was unable to demonstrate a difference in electrophoretic patterns of cold-and warm-adapted crayfish Hc.

Oxygen partial pressures throughout the experiment for all treatments were maintained at high levels in order to alleviate the possibility of differences in oxygen levels affecting the crabs' oligomer and subunit concentrations. While oxygen concentrations among treatments varied significantly, all concentrations were higher than normoxic levels. The oxygen levels for each treatment match the order of concentrations expected based on the temperatures and salinities of the treatments. 14°C, 16ppt and 14°C, 32ppt oxygen levels were greater than 265μMol/L throughout the experiment; the normoxic oxygen levels for seawater at 15°C is 258μMol/L. Oxygen concentrations in 19°C, 32ppt treatment were consistently higher than 240μMol/L; the normoxic level for seawater at 20°C is 237μMol/L (Knut Schmidt-Nielsen, 1997). More importantly PO_2 for all treatments was at normal levels. The PO_2 is 150 mmHg in seawater at the surface (Krogh, 1941). The lowest PO_2 in any treatment was during weeks 1-4 in 14°C, 16ppt treatment. During weeks 1-4 PO_2 levels dropped down to 148mmHg. This level was not

significantly lower than the other treatments. These results suggest although oxygen concentrations varied significantly they were still at or slightly above normal levels, and PO_2 did not vary significantly from ambient conditions.

Oxygen equilibrium of *Cancer productus*

Phenotypic plasticity of Hc subunits can be related to environmental factors other than temperature and salinity. As already mentioned, oxygen levels have been linked to phenotypic plasticity in *C. sapidus* and *Uca pugilator* (Mangum 1997, Mangum 1993). Allosteric effectors such as Ca^{2+}, H^+ and Mg^{2+}, L-lactate and urate may also be linked to phenotypic plasticity. Previous research by Johnson et al. (1987) has demonstrated that *Panulirus* Hc subunits respond differently to lactate.

Efforts in looking at the effects of oxygen and lactate concentrations on *C. productus* Hc oligomers focused on determining whether there were intrinsic oxygen binding differences between 25S Hc and 16S and whether lactate affected the two oligomers differently. Factors tested were pH or lactate concentration.

The present data provide further information on the variability of the two native oligomers in *C. productus*. Chromatography of hemolymph from two different crabs clearly demonstrates there are different subunit compositions between 25S Hc and 16S Hc. The subunits composing 25S Hc were the same as those found in whole hemolymph analyzed through pH 8.9 PAGE (Fig. 13a and 13b). The subunits comprising 16S Hc were identical to whole hemolymph and 25S Hc except for one subunit, A. This pattern

of an additional subunit present in 25S Hc is consistent with that seen in other brachyuran crabs' Hcs (Terwilliger and Terwilliger, 1982).

Oxygen binding results suggest there is a functional difference between 25S Hc and 16S Hc oligomers. There is a slight difference between 16S Hc and 25S Hc oxygen binding within the pH range of 7.3 to 8.8. 16S Hc expresses a slightly higher affinity (lower P_{50}) for oxygen than 25S Hc, but not significantly greater (Fig. 15). Nonetheless, the greater Bohr effect of the 16S Hc fraction will allow it to unload O_2 at the tissues over a smaller pH gradient than the 25S Hc. Results suggest lactate plays an important role in the oxygen binding differences between 25S Hc and 16S Hc oligomers. At pH 8.0 with no added lactate 25S Hc and 16S Hc have similar oxygen affinities. The addition of lactate increases the oxygen affinity of both oligomers, however, the effect of lactate concentration on 16S Hc oxygen affinity is significantly greater than its effect on 25S Hc oxygen binding. The log P_{50} values of 16S Hc were consistently lower than 25S Hc log P_{50} values (Figure 16).

These findings demonstrate different oxygen binding properties in the two native oligomers in response to lactate. 16S Hc has a higher oxygen affinity than 25S Hc within the physiological range of lactate concentrations (0-10mM). The variable subunit A, which has not been tested to influence oxygen affinity directly, could have an influence on oxygen affinity of 25S Hc. It is also important to remember that in these studies the Log P_{50} values are given for the functional molecule (25S and 16S), each of which contains mixtures of subunits. The individual subunits may exhibit different properties.

Two mechanisms for lactate binding have been suggested by Johnson et al. (1984 and 1987). One mechanism suggests the lactate binding site occurs between subunits within the protein. The second possibility is that lactate does not bind between subunits but rather only to certain subunits. Neither mechanism has been tested in *C. productus,* but results of these experiments suggest lactate may bind to specific subunits. The presence of subunit A is associated with a decreased lactate effect in 25S Hc compared to 16S Hc. Subunit A in 25S Hc may displace a lactate binding subunit present in 16S Hc. 16S Hc would have more subunits bound to lactate which would result in a greater lactate effect under identical conditions with 25S Hc.

Several hypotheses can account for the phenotypic plasticity of Hc observed in *C. productus*. First, the lack of subunit A in 16S Hc compared to its presence in 25S Hc and the significant differences in oxygen binding affinities between the two oligomers suggest phenotypic plasticity may be a response to lactate produced by the crabs under hypoxic conditions and during exercise. The best way to further study the lactate effect would be to separate individual subunits and recombine them into functional hexamers consisting of one type of subunit. This can be done in some species by eluting Hc subunits on a DEAE Sephacel column under varying NaCl and pH levels. The procedure of dissociation and reassociation of subunits in *Panulirus* hemolymph has been described in detail by Johnson et al. (1987). The study of hexamers composed of a single type of subunit would allow for the study of oxygen binding properties of individual subunits rather than combinations of subunits found naturally within the crab. Another approach to specifically test the effect of PO_2 on Hc would be to subject crabs to

consistently hypoxic conditions and monitor oligomer and subunit structure over a period of time.

Secondly, differences in phenotypic plasticity may have been caused by prior exposure to changes in temperature and salinity outside the ranges examined in this study. This is not a likely explanation, however, based on the tolerance ranges of *C. productus* and the levels tested. *C. productus* tolerate a range of salinity from 21.7ppt to 33.0ppt and are collected within the temperature range of 11°C to 17°C (Rudy and Rudy, 1983). The salinities used in this experiment (16ppt and 32ppt) included a salinity considerably lower than a level normally experienced by *C. productus*. Temperature levels up to 19°C were also extreme enough, without causing mortality, to elicit a response similar to what may be seen in the natural environment where intertidal crabs are exposed to elevated temperatures.

Thirdly, the phenotypic plasticity observed in *C. productus* may be a result of varying sensitivity among Hc subunits to inorganic and organic modulators, such as Ca^{+}, Mg^{2+} and urate, not investigated in this study. Previous studies by Terwilliger et al. (1986, 1993) demonstrated that shifts in *C. magister* Hc subunit composition resulted in functional changes both in intrinsic oxygen affinity and in Mg^{2+} sensitivity. Further oxygen binding research needs to be conducted to fully understand the relationship between allosteric modulators and the phenotypic plasticity observed in *C. productus*.

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