THE INFLUENCE OF VERTICAL ZONATION: DIFFERENCES IN HEMOCYANIN STRUCTURE AND FUNCTION BETWEEN THE PORCELAIN CRABS *PETROLISTHES ERIOMERUS* AND *PETROLISTHES CINCTIPES*

by

JENNIFER M. SCHMITT

A THESIS

Presented to the Department of Biology
and the Graduate School of the University of Oregon
in partial fulfillment of the requirements
for the degree of
Master of Science

August 2002

"The Influence of Vertical Zonation: Differences in Hemocyanin Structure and Function Between the Porcelain Crabs *Petrolisthes eriomerus* and *Petrolisthes cinctipes*," a thesis prepared by Jennifer M. Schmitt in partial fulfillment of the requirements for the Master of Science degree in the Department of Biology. This thesis has been approved and accepted by:

_____

Dr. Nora Terwilliger, Chair of the Examining Committee

_____

Date

Committee in charge: Dr. Nora Terwilliger, Chair
Dr. Richard Emlet
Dr. Lynda Shapiro

Accepted by:

_____

Dean of the Graduate School

An Abstract of the Dissertation of

Jennifer M. Schmitt for the degree of Master of Science

in the Department of Biology to be taken August 2002

Title: THE INFLUENCE OF VERTICAL ZONATION: DIFFERENCES IN HEMOCYANIN STRUCTURE AND FUNCTION BETWEEN THE PORCELAIN CRABS *PETROLISTHES ERIOMERUS* AND *PETROLISTHES CINCTIPES*

Approved: _____
Dr. Nora Terwilliger

Petrolisthes cinctipes survives in a high-mid intertidal habitat in which physicochemical changes are more drastic compared to the low intertidal/subtidal zone of *Petrolisthes eriomerus*. This study examined how the hemocyanins of these two species differed functionally in response to physicochemical factors. *Petrolisthes eriomerus* had a lower oxygen affinity than *P. cinctipes*. *Petrolisthes eriomerus* hemocyanin was more sensitive to temperature and lactate (ΔH = -40.81; lactate coefficient = -0.32) compared to *P. cinctipes* (ΔH = -35.99; lactate coefficient = -0.22). *Petrolisthes eriomerus* exhibited a smaller Bohr effect (Φ = -1.07) than *P. cinctipes* (Φ = -1.25). Electrophoretic analysis indicated that the subunit composition of hemocyanin differed between species in both the number and electrophoretic mobility of bands. The structure and function of

each species' hemocyanin are important in maintaining their distinct vertical distributions.

CURRICULUM VITA

NAME OF AUTHOR: Jennifer M. Schmitt

PLACE OF BIRTH: Colden, New York

DATE OF BIRTH: April 5, 1977

GRADUATE AND UNDERGRADUATE SCHOOLS ATTENDED:

University of Oregon
Bucknell University

DEGREES AWARDED:

Master of Science in Biology, 2002, University of Oregon
Bachelor of Science in Biology, 1999, Bucknell University

AREAS OF SPECIAL INTEREST:

Comparative Physiology
Invertebrate Physiology and Ecology

PROFESSIONAL EXPERIENCE:

Teaching Assistant, Department of Biology, Oregon Institute of Marine Biology, University of Oregon, Charleston, Summer 2002

Teaching Assistant, Department of Biology, Oregon Institute of Marine Biology, University of Oregon, Charleston, Summer 2001

Teaching Assistant, Department of Biology, Oregon Institute of Marine Biology, University of Oregon, Charleston, Spring 2001

Teaching Assistant, Department of Biology, University of Oregon, Eugene, Winter 2001

Research Assistant, Department of Biology, Oregon Institute of Marine Biology, University of Oregon, Charleston, Fall 2000

AWARDS AND HONORS:

Neil Richmond Fellowship, September 2000
Robert C. Terwilliger Scholarship, June 2000

ACKNOWLEDGEMENTS

I would like to thank my advisor, Nora Terwilliger, for her help during the course of my thesis. I thank Margie Ryan for sharing her expertise, patience, and lively energy, making my research experience that much more fulfilling. A special thanks to Barb Butler whose friendship and advice have been invaluable. I would like to thank all of my fellow graduate students for their camaraderie, especially Anita McCulloch, Jessica Miller, and Ali Helms. My heartfelt appreciation goes to the researchers at the South Slough National Estuarine Research Reserve, especially Steve Rumrill, Sue Powell and Craig Cornu – whose ideas and enthusiasm are both captivating and contagious. Finally, I would like to thank my family and friends who continually show their support throughout all of my endeavors.

DEDICATION

To my grandparents Wendell and Edna Stackpole who first showed me the ocean

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

INTRODUCTION

Life in the Intertidal

The intertidal environment is a spatially and temporally diverse zone that is in a constant state of flux. As the relatively stable conditions of high tide give way to low tide, rapid physicochemical changes take place, including alterations in temperature, salinity, pH, oxygen levels, and carbon dioxide levels. In temperate rock pools, temperature can vary from –1.5 °C to 30 °C and oxygen partial pressures (Po_2) can fluctuate between 10-500 mm Hg (Truchot and Duhamel-Jouve, 1980; Morris and Taylor, 1983). The extent that these physicochemical factors vary during tidal cycles differs among geographic location. For example, locations that exhibit hot air temperatures will have warmer and more saline pools than cooler climates. Regions that experience heavy annual rainfall will have tide pools with a lower salinity than pools found in more arid climates. On a smaller scale, tide pools exposed to creeks or heavy surface runoff will have lower salinities than those in the same area that receive no runoff. Small isolated pools will undergo evaporation on sunny days, creating warm saline pools, while large connecting pools will not. In addition, the warmer pools will have a more severe change in temperature when the incoming tide washes over them. An intertidal habitat exposed on a north-facing slope will have less extreme temperature

fluctuations than an adjacent one facing south. The importance of substratum angle was demonstrated by Helmuth and Hofmann (2001), who found that maximum monthly temperatures were on average 6.75 ^{o}C warmer on a horizontal site than a north facing vertical site located 20 cm away. On an even smaller scale, there are often differences in conditions within a pool. For example, during daylight, Po_2 values are generally higher near photosynthetic algae, which occur along the bottom and sides of a pool, than along the water's surface (Morris and Taylor, 1983). Temperatures often tend to be warmer along the bottom and sides of a pool than at the surface, as sunlight will warm the rocks within the pool and wind will often cool off the surface of the pool (Morris and Taylor, 1983). Salinity levels can differ within a pool during windless conditions, with a distinct low salinity top layer occurring after heavy rain (Morris and Taylor, 1983).

Vertical zonation at any given geographic location will also create differences in habitat conditions. A tide pool located lower on the intertidal zone will experience a shorter low tide period and therefore a smaller range in changes than one higher up. Physicochemical changes in a tide pool during an incoming tide will be more drastic for high littoral pools than pools lower on the intertidal zone due to the amount of time they have been isolated. For instance, Po_2 levels have been observed to change from 362 mm Hg to 150 mm Hg in less than 10 minutes in a pool being flooded by an incoming tide (Morris and Taylor, 1983).

Diverse environmental conditions combined with diurnal immersion and emersion cause intertidal animals to experience drastic fluctuations in many physicochemical

factors several times a day. Due to this, most intertidal animals are behaviorally and physiologically adapted to respond to these unstable conditions.

Hypoxia

One major consequence of living in the intertidal zone is the potential for emersion during low tide, which can cause an internal hypoxia in many water-breathing species. Hypoxia occurs in obligate aquatic crustaceans upon emersion as the gill structure collapses, coalesces and eventually dries out, causing a reduction in surface area, thereby impairing oxygen transport to the hemolymph system (Truchot, 1992). The results of this are a shift to anaerobic respiration and hypercapnia, which occurs when CO_2 is unable to be excreted into the environment (Morris et al., 1996). Hypercapnia in turn causes a decrease in internal pH, intensifying the metabolic acidosis that comes about from anaerobic respiration.

Behaviorally, many decapods adjust to hypoxic conditions by increasing gill ventilation, which allows the supply of oxygen to the gills to be maintained. Some crabs such as *Cancer productus* can keep their gills moist and oxygenated during short periods of emersion by retaining or recirculating branchial water (DeFur et al., 1983). A slightly different behavioral response can be seen in *Palaemon elegans*. When exposed to oxygen tensions below 20 mm Hg, this prawn will position itself so that it is partially emersed at the air/water interface (Taylor and Spicer, 1988). Then, by a combination of pleopod beating and hyperventilation it is able to maintain aerobic respiration (Taylor and

Spicer, 1988). By using the higher oxygen tensions at the air/water interface, this behavior allows the prawn to obtain higher hemolymph oxygen levels, maintain lower lactate levels, allow for the release of CO_2, and effectively sustain a higher internal pH than it could otherwise (Taylor and Spicer, 1988).

Hemocyanin Oxygen Affinity

One physiological way that intertidal animals can adjust to environmental stresses, such as hypoxia, is through their hemolymph. Most malacostracan crustaceans contain the respiratory pigment hemocyanin dissolved in their hemolymph. Arthropod hemocyanin is composed of subunits 75,000 Da in size, each with a binuclear copper-containing active site that reversibly binds with oxygen. These subunits form hexamers; the number of hexamers forming a functional hemocyanin molecule ranges from one to eight and varies among species (Markl and Decker, 1992). Both the quaternary structure and the subunit composition within the macromolecule are variable, and each variant may have different oxygen binding properties (McMahon, 1986). Differences in hemocyanin structure can be seen among the anomurans *Pagurus bernhardus* and *Pagurus striatus*, both of which have hemocyanins in the hexameric form predominating in the blood (Svedberg, 1933), and the anomuran *Petrolisthes eriomerus*, which has predominantly a dodecameric form (Mangum, 1983b).

Interactions between hemocyanin subunits allow for flexibility in both structure and function. Conformational flexibility and molecular stability directly reflect the oxygen binding capabilities of a hemocyanin molecule. In order to transport oxygen

effectively, hemocyanin must load oxygen at the gills and unload it at various internal tissues. This process requires that hemocyanin respond to different partial pressures of oxygen at each site. The range of Po_2 levels a hemocyanin molecule responds to can be quantified by the oxygen pressure required to fill half of the oxygen binding sites on a molecule (P_{50}). A hemocyanin molecule that has a high oxygen affinity can easily bind oxygen; therefore the pressure it takes for oxygen to bind to hemocyanin is very low. Similarly, a low affinity hemocyanin molecule does not bind easily with oxygen and therefore requires high pressures to bind. The hemocyanin oxygen affinity for most littoral species is fairly low with P_{50} values greater than 10 mm Hg; high affinity hemocyanins in the range of 2-7 mm Hg are only found in several terrestrial crabs and chelicerates (Mangum, 1980; Truchot, 1992).

Cooperative binding is one way that conformational changes can affect hemocyanin oxygen affinity. A single hemocyanin macromolecule has multiple oxygen binding sites, allowing allosteric interactions to occur. As an oxygen molecule binds to one subunit, it changes the binding properties of the hemocyanin macromolecule causing an increase in affinity. This allows subsequent oxygen ligands to more easily bind. As oxygen molecules continue to bind, the affinity of the hemocyanin macromolecule becomes greater still. This happens as the hemocyanin macromolecule changes from its low affinity tense state to a high affinity relaxed state (Monod et al., 1965). The process works in reverse as well; as a fully oxygenated hemocyanin releases an oxygen molecule at the tissue, release of subsequent oxygen molecules becomes easier as the overall affinity of the macromolecule decreases. Native crustacean hemocyanins

generally demonstrate highly cooperative oxygen binding (Mangum, 1983b; Markl and Decker, 1992).

Because hemocyanin oxygen affinity is adjusted alongside changing physicochemical conditions, an organism may be able to maintain a steady supply of oxygen during a variety of environmental stresses. For instance, the large decrease in internal pH caused by hypoxia as described above causes a decline in hemocyanin oxygen affinity. Concurrently, anaerobic metabolism forms lactate as a byproduct, which increases oxygen affinity, allowing for increased oxygen loading at the gills. This lactate response is an adaptation that allows many crustaceans to reduce the effects of the drastic decrease in oxygen affinity brought on by simultaneous metabolic acidosis (Mangum, 1983a; McMahon, 1988). Several crustaceans such as *Carcinus maenas* and *Orconectes rusticus* induce hyperventilation during hypoxia, which increases the pH in their hemolymph (McMahon, 1985 ; Lallier and Truchot, 1989a). This in turn causes an increase in oxygen affinity that compounds the lactate response and allows for even greater oxygen uptake at the gills

The modification of hemocyanin oxygen affinity by allosteric effectors can clearly enable species to become well adapted to their specific environment. The extent of stress an animal can undergo as well as its range in hemocyanin oxygen affinity differ from one species to the next. Species that inhabit hypoxic environments tend to be well adapted, having higher oxygen affinities and oxygen carrying capacities than those in normoxic habitats; this allows oxygen uptake at the gills to be maintained even at low oxygen tensions (Dejours, 1981; Taylor et al., 1985; Taylor, 1988; Taylor et al., 2000;

Chausson et al., 2001; Hagerman and Vismann, 2001). For example, *Palaemon elegans*, a shrimp commonly found in hypoxic tide pools, contains hemocyanin with both a relatively high oxygen affinity and a high oxygen carrying capacity (McMahon, 1988). In conditions where oxygen is rarely limited, such as in the subtidal zone, a high affinity for oxygen is unnecessary. A high oxygen affinity in this circumstance would be maladaptive because the carrier molecules would be unable to easily give up oxygen at the tissues. Taylor (1988), supported this with his comparison of intertidal and open water species, concluding that intertidal species often show higher hemocyanin oxygen affinities, higher pH sensitivities, and higher hemocyanin concentrations in their hemolymph. High hemocyanin oxygen affinity in these intertidal species is adaptive for increasing oxygen uptake at the gills while pH sensitive hemocyanin allows oxygen unloading at the tissues.

As was mentioned previously, various factors such as environmental stress, organic metabolites, and inorganic ions affect the oxygen affinities of respiratory proteins in various ways, as do different combinations of these factors. Decapod crustaceans tend to exhibit the same general trends in their oxygen affinity response to different stresses, with increases in pH, CO_2, calcium, L-lactate, urate, and dopamine causing hemocyanin oxygen affinity to increase, and temperature increases causing a decrease in oxygen affinity (Burnett, 1992).

pH

Acid base balance is extremely difficult to maintain in an intertidal environment (Truchot, 1988). Most respiratory proteins exhibit sensitivities to pH, often decreasing in oxygen affinity as pH becomes more acidic. When pH is lowered, the normally neutrally charged histidines on the hemocyanin molecule gain a positive charge and form bridges with other amino acids. These bridges stabilize the molecule in its deoxygenated form, lowering its affinity for oxygen. Internal pH often fluctuates in relation to environmental factors such as temperature, salinity, and oxygen levels. For instance, a lowering of temperature causes a rise in hemolymph pH, both of which increase oxygen affinity. Increases in either salinity or L-lactate, both of which increase hemocyanin oxygen affinity, cause a lowering of pH, which decreases affinity (Truchot, 1980; Truchot, 1983; Weiland and Mangum, 1975).

The amount of oxygen available in any given tide pool as well as the pH of a tide pool, is directly related to biological processes. Tide pools that are heavily covered with algae will have more oxygen during the day and be more insulated from the sun than pools with little or no algae. Morris and Taylor (1983) demonstrated that diurnal variations in Po_2, partial pressure of carbon dioxide (Pco_2), and pH were directly related to the photosynthetic influence and respiration factors in any given tide pool, with lowest pH and Po_2 levels and highest Pco_2 levels occurring at night as photosynthesis ceased and faunal respiration was maintained. Tide pools measured at night during winter months

have been shown to exhibit extremely high Pco_2 levels, causing the pH to drastically decrease (Morris and Taylor, 1983). Conversely, pools with high Po_2 levels, such as those that are immersed at daytime in the summer, exhibit high pH levels (Morris and Taylor, 1983). Morris and Taylor (1983) demonstrated that changes in CO_2 content in intertidal rock pools in Scotland could result in pH changes as large as pH 6.8 to 9.0. Changes in both oxygen and pH can be drastic and occur very rapidly in shallow isolated pools where both animal and plant populations are present (Truchot and Duhamel-Jouve, 1980).

In addition to external variation in pH, intertidal organisms frequently experience internal fluctuations due to metabolic processes and shifts in ionic concentrations. For instance, an increase in hemolymph salt concentration is accompanied by a decrease in blood pH. Molting processes are also known to affect acid base balance in crustacean hemolymph. Prior to ecdysis, the pH in the hemolymph of many crustaceans, like the crayfish *Astacus leptodactylus*, increases considerably (Dejours and Beekenkamp, 1978). This metabolic alkalosis may be partially due to partial absorption of the calcified exoskeleton before it is shed (Truchot, 1983). Following ecdysis, internal alkalosis is replaced by a slight acidosis (Dejours and Beekenkamp, 1978).

pH can differ along a gradient within the hemolymph system as well, with higher pHs occurring at the gills and lower pHs found at the tissue or oxygen release site. The lowering of pH caused partially by lactic acid in active tissues as well as by CO_2 loading of the hemolymph, and the subsequent lowering of oxygen affinity, allows more oxygen to be released at the tissues than would otherwise occur. The decrease in oxygen affinity

and subsequent increase in oxygen unloading that comes about from the lowering of internal pH is called the Bohr effect. The coefficient for the Bohr effect is generally negative and varies considerably among species. It has been suggested that the possession of hemocyanin that demonstrates a large Bohr effect may be advantageous for species that inhabit environments that are periodically exposed to hypoxia (Truchot, 1992).

Temperature

Temperature is one of the strongest abiotic determinants for the distribution limits of rocky intertidal organisms (Doty, 1946; Hutchins, 1947; Helmuth and Hofmann, 2001). Temperature effects confound the naturally complicated physical aspects of the intertidal environment. This is partially due to the fact that the oxygen capacity of seawater is influenced by temperature. At 10 ^{o}C, oxygen capacity of seawater is 1.79 μmol L $^{-1}$ mm Hg $^{-1}$ while at 30 ^{o}C it is 1.27 μmol L $^{-1}$ mm Hg $^{-1}$ (Dejours, 1981). In many arthropods, a rise in ambient temperature increases the metabolic oxygen requirements but decreases hemocyanin oxygen affinity (Truchot, 1992). Therefore, an increase in temperature reduces the solubility of oxygen in water, decreases hemocyanin oxygen affinity, and increases organismal oxygen demand, creating a situation that is analogous to relatively hypoxic conditions (Truchot, 1980).

The stability and flexibility of hemocyanin is quite sensitive to temperature changes. This is important in a crustacean, where the body temperature is not internally

regulated and approximates that of its surroundings. Therefore, during extremely warm or cold external temperatures, hemocyanin may be so loose (warm temperatures) or inflexible (cold temperatures) that effective oxygen binding can no longer occur (Fields, 2001).

The thermal tolerance of an organism is established by a combination of morphological, physiological and biochemical traits, including thermal sensitivities of enzymatic proteins such as hemocyanin (Alexandrov, 1977). As might be expected, the effect of temperature on oxygen binding can be variable between species. It has long been understood that oxygen affinity of hemocyanin typically decreases as temperature rises (Redfield, 1934), although an exception to this trend can be seen in *Carcinus maenas* where acclimation to high ambient temperatures causes an increase in oxygen affinity (Truchot, 1980). In general, higher oxygen affinities are found in species that are frequently exposed to warmer waters while lower oxygen affinities are seen in species inhabiting colder waters (Redmond, 1968).

Temperature fluctuations are more pronounced and occur more frequently in high intertidal zones than low intertidal or subtidal zones. In temperate regions, low intertidal species experience an annual temperature range of 8-16 °C while mid to high intertidal species can have annual temperature ranges from 0-32 °C (Stillman and Somero, 2000). Low intertidal *Petrolisthes eriomerus*, for example, only experience a 5 °C annual temperature fluctuation associated with upwelling whereas high intertidal *Petrolisthes cinctipes* can experience temperature fluctuations of over 20 °C during a single tide change and over 30 °C annually (Stillman and Somero 1996; Stillman, 1998).

The eurythermal nature of *P. cinctipes* allows it to tolerate both hotter and colder temperatures than *P. eriomerus* (Stillman and Somero 1996; Stillman, 1998). Stillman demonstrated this during his studies of *P. eriomerus* and *P. cinctipes* at Cape Arago, Oregon where he found that temperatures at the top of *P. eriomerus* zone never exceeded 25 °C while temperatures at the top of *P. cinctipes* zone were recorded as high as 32 °C (Stillman and Somero, 1996; Stillman, 1998). He also discovered that *P. eriomerus* did not survive at or below temperatures of 2 °C while *P. cinctipes* survived down to 1.5 °C (Stillman, 1998). A study by Jensen and Armstrong (1991) reported that both species survived in air for 24 hours at 15 °C but at 20 °C *P. eriomerus* only survived an average of 5.5 hours and at 25 °C an average of 2.25 hours, while all *P. cinctipes* survived 24 hours at these higher temperatures with no visible distress. Furthermore, the sensitivity of *P. eriomerus* to the highest temperature was size-specific, with large adults (13.7 mm carapace width) surviving less than 0.5 hours while the smallest crab tested (2.2 mm carapace width) was still alive after 25 hours (Jensen and Armstrong, 1991).

The metabolism of *P. eriomerus* and *P. cinctipes* at high air temperatures has also been studied. *Petrolisthes eriomerus* showed an elevated dependence on anaerobic glycolysis when emersed at 25 °C as seen by a significant rise in total body lactate (Stillman, 1998). *Petrolisthes cinctipes* seemed to rely mainly on aerobic ATP generating pathways during heating and was therefore better able to withstand emersion at high temperatures than *P. eriomerus* (Stillman, 1998). The reason that *P. eriomerus* may not need to be well-adapted to withstand high temperatures, as was clearly demonstrated in each of these studies, is partially due to its choice in habitat. Rock pools

in the low intertidal zone where *P. eriomerus* are found usually harbor massive amounts of algae that act as a thermal buffer from the warm air temperatures (Stillman, 1998).

Lactate

L-lactate is the primary end product of anaerobic metabolism in decapods (Boyland, 1928); it is produced during both prolonged periods of activity and hypoxic conditions (Bridges and Brand, 1980; Graham et al., 1983; Mangum, 1983b). In 1975, Truchot identified L-lactate as a dialyzable factor that increased hemocyanin oxygen affinity in *Carcinus maenas* and *Cancer pagurus*. Since then, the lactate effect has been found in numerous decapods, including anomuran crabs (Mangum, 1983b). In general, L-lactate buildup in the hemolymph causes an increase in oxygen affinity. This occurs because L-lactate preferentially binds to oxygen-bound hemocyanin causing a conformational change that increases oxygen affinity (Johnson et al., 1984). Lactate build-up during strenuous exercise or hypoxia allows an overall stabilization of hemocyanin oxygen affinity to occur as it offsets the low oxygen affinity that concurrently takes place from increased acidosis (McMahon, 1986). This has been demonstrated in *Callinectes sapidus*, where 14 mM of L-lactate can elevate oxygen affinity to a point where similar amounts of oxygen can be obtained during exhaustive activity and at rest. (McMahon, 1986).

The lactate effect is very specific; analogous structures have little or no effect on oxygen affinity. For example, the stereoisomer D-lactate, which is chemically and physically identical to L-lactate and differs only in symmetry, has little effect on

hemocyanin oxygen affinity (Graham et al., 1983). This preferential binding to L-lactate suggests that the lactate binding site on hemocyanin must be asymmetrical (Johnson et al., 1984).

The severity of hypoxia that is necessary to cause lactate accumulation in intertidal animals varies among species. For example, a large accumulation of whole-body lactate was found in specimens of *P. eriomerus* incubated in air at 25 °C over a 5-hour period, but not in *P. cinctipes* from the same treatment group (Stillman and Somero 1996). *Carcinus maenas* is a crab that accumulates L-lactate only in severely hypoxic waters (Lallier et al., 1987). The residence time of lactate in the blood after exposure to hypoxia also varies among crustaceans (Bridges and Brand, 1980). Depending on the species, lactate can remain in the blood from 8 to 32 hours, with shorter residence times corresponding to species that regularly encounter hypoxic conditions in their natural environment (Bridges and Brand, 1980).

Salinity

Intertidal organisms are subjected to diurnal, seasonal and spatial changes in salinity that may be gradual or abrupt (Wheatly, 1988). External salinity rarely changes in isolation from other environmental factors such as oxygen and CO_2 levels, acid-base balance, or temperature (Wheatly, 1988). High temperatures can speed the evaporation process in pools, resulting in increases in salinity; this in turn amplifies another effect of temperature by further reducing the solubility of oxygen in water (Dejours, 1981; McMahon, 1988; Schmidt-Nielsen, 1997). Internally, an organism's hemolymph pH and

hemocyanin P_{50} vary inversely with salinity (Sabourin, 1984). Intertidal species are particularly susceptible to the effects of these combined environmental and internal salinity fluctuations; the magnitude of these effects are largely dependent on the osmoregulatory ability of a particular species (Bridges, 2001).

There are a number of physiological effects caused by hypersaline or hyposaline waters. Ventilation, heartbeat frequency, heart stroke volume, respiratory gas exchange, oxygen transport by hemocyanin, oxygen utilization at the tissues, as well as hemocyanin concentration, all change with salinity, thereby affecting oxygen uptake and delivery (Sabourin, 1984; Wheatly, 1988). For instance, a reduction in external salinity can produce a drastic decrease in hemocyanin oxygen affinity (Mangum, 1983b). Total salinity effects can be seen in *Callinectes sapidus*, where crabs that were acclimated to 10‰ demonstrated a lower oxygen affinity than those acclimated to 30‰ (Sabourin, 1984).

Many inorganic ions are capable of individually affecting crustacean hemocyanin oxygen affinity, thus an increase in ions allows for an increase in oxygen loading at the gills (Wheatly, 1988; Bridges, 2001). The ionic composition that determines total salinity of the hemolymph also directly affects hemocyanin oxygen affinity, with differences in affinity directly correlated to changes in Ca^{++}, Mg^{++} and Cl^{-} concentration (Bridges, 2001). Magnesium and calcium have been shown to distinctly increase oxygen affinity in hemocyanins (Truchot, 1975; Morris, 1990). For example, the oxygen affinity of *Carcinus maenas* hemocyanin notably increases with greater concentrations of Mg^{++}

or Ca^{++} (Truchot, 1975). In another study, it was shown that the conversion of $CaCO_3$ from the carapace to HCO_3^- in the hemolymph could cause an increase in oxygen affinity in decapod crustaceans, partially compensating for the decrease in oxygen affinity that occurs from hemolymph acidosis during anaerobic respiration (Henry et al., 1981).

Not only do many inorganic ions, such as Ca^{++}, Mg^{++}, and Cl^- affect oxygen affinity, they are also necessary for normal functioning in crustaceans. Magnesium is essential for the proper assembly and folding of molecules such as hemocyanin (Truchot, 1992). Magnesium is also important as it influences cell permeability and is crucial in many enzyme reactions (Dehnel and Carefoot, 1965). The regulation of magnesium is also important in the normal functioning of neuromuscular impulse transmission (Dehnel and Carefoot, 1965), as high extracellular levels of Mg^{++} are known to block this process (Katz, 1936). Therefore animals that exhibit high levels of activity generally keep Mg^{++} concentrations low in the hemolymph compared to the surrounding seawater (Sartoris and Portner, 1997). This regulation of magnesium occurs almost immediately upon a change in salinity (Dehnel and Carefoot, 1965). Because of its physiological importance, magnesium effects have been examined in a number of crustaceans (for example: Dehnel and Carefoot, 1965; Truchot, 1975; Terwilliger and Brown, 1993; Sartoris and Portner, 1997).

Petrolisthes

The family Porcellanidae contains 23 genera, the largest of which is the genus *Petrolisthes* (Stillman and Reeb, 2001). There are more than 100 *Petrolisthes* species worldwide, with approximately 45 species distributed across both latitudinal and vertical gradients in the eastern Pacific (Haig, 1960; Stillman and Somero, 2000). Two of these species, *Petrolisthes eriomerus* and *P. cinctipes*, are sympatric, exhibiting a non-overlapping vertical distribution on many rocky shores of the northeastern Pacific, from central California to northern British Columbia (Haig, 1960; Jensen and Armstrong, 1991; Stillman and Somero, 2000). *Petrolisthes eriomerus* occurs under rocks in the relatively stable low intertidal and subtidal zones to 80m while its congener, *P. cinctipes*, occurs under boulders and in mussel beds in the middle and upper intertidal zone (Stillman and Somero 1996). A distinct boundary between the *P. eriomerus* and *P. cinctipes* can be found at approximately the $^{+}$0.8m tidal level, with all sizes and sexes of each species found at the borderline of their distributions (Jensen and Armstrong, 1991). Although this borderline is distinct, the reason for it is not. Both of these *Petrolisthes* species exhibit gregarious settlement, which partially explains how they are able to maintain their distinct distributions; why and how the motile adults remain in these distribution patterns is less clear (Jensen and Armstrong, 1991).

Because of their different vertical distributions, these *P. eriomerus* and *P. cinctipes* experience very different levels of biotic and abiotic stress (Stillman and Somero 1996). Respiratory limitations during emersion may determine the upper limit

for *P. eriomerus*. During emersion, *P. cinctipes* is able to maintain aerobic metabolism while *P. eriomerus* switches to anaerobic metabolism (Stillman and Somero 1996). This difference in emersion response is partially due to distinct body structures in the two species. From measuring lactate levels after 5 hours of air exposure, it has been shown that *P. cinctipes* is able to maintain aerial respiration during emersion by using a membranous structure on the merus of each walking leg (Stillman and Somero 1996). These leg membranes are found in at least 16 *Petrolisthes* species, with relative membrane size varying between species (Stillman and Somero, 1996). The low intertidal and subtidal species that possess leg membranes, such as *Petrolisthes violaceus* and *Petrolisthes tuberculatus*, are unable to remain aerobic during emersion, questioning the true functional significance of the leg membranes (Stillman, 2000). Stillman (2000), suggested that although these membranes apparently did not evolve specifically as a respiratory structure, certain intertidal *Petrolisthes* species are capable of maintaining aerobic respiration during low tide periods with the use of these membranes. In primarily subtidal or low intertidal species, such as *P. eriomerus*, *Petrolisthes manimaculis* and *Petrolisthes crenulatus*, the loss of leg membranes has occurred (Haig, 1960; Stillman 1998) with the meral segments showing complete calcification (Stillman and Somero 1996).

These structural differences between *P. eriomerus* and *P. cinctipes* partially explain the different vertical distributions of these species. Differences in the hemocyanin oxygen binding capabilities and hemocyanin structure between these two species may be another explanation for their distinct distributions.

Research Questions

Apart from the aforementioned experiments, there have been few studies comparing the ecological, physiological, or behavioral adaptations of closely related species in order to explain the establishment and maintenance of vertical zonation. At South Cove, Cape Arago, Oregon there are two species in the genus *Petrolisthes* that exhibit different vertical distributions. Because of this, these two species differ in the severity and extent of intertidal stresses. The present study will compare the physiological adaptations of these two species to environmental stress by examining: 1) how the oxygen binding properties of their hemocyanins change in relation to varying levels of pH, temperature, L-lactate and salinity; and 2) whether or not there are structural differences in the hemocyanins of the two species.

CHAPTER II

MATERIALS AND METHODS

Animals

Approximately 90 *P. eriomerus* and 90 *P. cinctipes* were collected from South Cove, Cape Arago, Oregon (43°21'N; 124°19'W) on several occasions from April 2001 to June 2002. Larger animals were selected from the field to more easily extract hemolymph, to minimize the number of animals needed for each experiment, and to ensure adult specimens. Crabs collected were of both sexes but did not include females that were in berry. *Petrolisthes eriomerus* that were collected tended to be smaller than *P. cinctipes*, which is consistent with the literature where adult *P. eriomerus* ranges from 3.8 mm to15 mm in carapace width and adult *P. cinctipes* ranges from 4.5 mm to 21.1 mm in carapace width (Haig, 1960). The average *P. eriomerus* collected in this study was 9 mm in carapace width while that of *P. cinctipes* was 13 mm. *Petrolisthes eriomerus* were found under boulders in lower intertidal zones, usually immersed in pools that contained kelps. *Petrolisthes cinctipes* were found high in the intertidal zone under boulders that generally were not in standing water.

Crab species were kept separately in covered plastic containers, each with an approximate volume of 600 ml. The containers had windows covered in mesh on two

sides to allow water circulation. Each container held 1-4 crabs, depending on crab size, with the largest crabs having their own containers. A mussel shell for each crab was placed into each container to provide cover. A larger tank (2.5m x 0.5m x 0.2m) in which all small containers were held, possessed two air stones and two seawater inlets that provided fresh running seawater at ambient temperature and salinity (10-12 °C, 32‰). *Petrolisthes* are filter feeders and obtained most of their nutrients from the running seawater. In order to supplement their diets, several homogenized mussels were divided up among the containers biweekly.

Oxygen Binding: Buffers

Before oxygen binding experiments could be embarked upon, a buffer that reflected the osmotic and ionic concentrations of *Petrolisthes* hemolymph needed to be designed for use during dialysis and hemolymph dilution. Magnesium concentrations in the hemolymph of both *P. eriomerus* and *P. cinctipes* were measured spectrophotometrically (Sky-Peck, 1964) using a Beckman Coulter DU series 640 spectrophotometer. Osmolality was also determined, with the use of a Vapro® Vapor Pressure Osmometer (by Wescor®). The equipment to measure potassium, sodium and calcium were unavailable and no information on the concentration of these ions in *Petrolisthes* could be found in the literature. Due to this, known concentrations of these ions in the hemolymph of another decapod crustacean, *Cancer magister*, were used (Brown, 1991). The ionic concentrations of the final *Petrolisthes* buffer are shown in Table 1. Although the measured hemolymph ionic concentrations differ slightly between

these species, this buffer was used in oxygen binding studies for both *Petrolisthes* in order to allow direct comparisons to be made.

Table 1: Ionic concentrations and pH of *Petrolisthes* buffer.

KCl	11.5 mM/L
$CaCl_2$	13.5 mM/L
Na_2SO_4	23.5 mM/L
NaCl	379 mM/L
$MgCl_2$	35.5 mM/L
HCl	50 mM/L
pH	7.9
Total osmolality	975 m•osmol/kg

For experiments testing the effect of pH on oxygen affinity, buffers were titrated to the experimental pH using Trizma Base (Tris), while temperature was held constant (Table 2A). During temperature manipulations, buffers were titrated to pH 7.9 at each experimental temperature (Table 2B). The temperature and pH for lactate experiments were held constant; buffers differed only in the amount of lactate stock solution (lithium salt) and NaCl stock solution that were added to the hemolymph (Table 3). The addition of NaCl stock solution was used to maintain a constant osmotic strength in the buffer. Salinity buffers were manipulated either by varying the concentration of Mg^{++} (Table 4A) or the percentage of total salinity (Table 4B). Temperature and pH remained the same throughout all salinity manipulations.

Table 2: Outline of experimental groups for: (A) pH manipulations; (B) temperature manipulations.

A.

pH	Temperature (°C)
7.45	12
7.7	12
7.95	12
8.2	12
8.4	12
8.6	12

B.

pH	Temperature* (°C)
7.9	10
7.9	15
7.9	20
7.9	25
7.9	30

* The temperature under rocks at Cape Arago ranges from 10 °C to 30 °C (Stillman, 1998). Even though *P. eriomerus* has been shown to die ≥ 25 °C, hemolymph from each species was tested at both 25 °C and 30 °C to determine if their hemolymph was thermally stable *in vitro*.

Table 3: Experimental protocol for each lactate manipulation.

[Lactate] (mM)	NaCl** (µl)	Lactate** (µl)	Hemolymph (ml)	Total vol (ml)	pH	Temperature °C
2	28.6	5.2	1.266	1.3	7.9	15
5	20.8	13	1.266	1.3	7.9	15
8	13	20.8	1.266	1.3	7.9	15
10	7.8	26	1.266	1.3	7.9	15
13	0	33.8	1.266	1.3	7.9	15

** NaCl and Lactate are in 0.5M stock solutions diluted with *Petrolisthes* Buffer (pH 7.9 @ 15°C).

Table 4: *Petrolisthes* buffer ionic concentrations for each salinity manipulation: (A) solutions differed in [Mg++] by changing $MgCl_2$ while all other components of the buffer remained the same; (B) solutions differed in all components to give a total reduction/increase in salinity.

A.

$MgCl_2$ mM/L	pH	Temperature (°C)
2	7.9	15
35.5	7.9	15
71	7.9	15

B.

	50%	100%	150%
KCl mM/L	5.75	11.5	17.25
$CaCl_2$ mM/L	6.75	13.5	20.25
Na_2SO_4 mM/L	11.75	23.5	35.25
NaCl mM/L	189.5	379	568.5
$MgCl_2$ mM/L	17.75	35.5	53.25
HCl mM/L	25	50	75
pH	7.9	7.9	7.9
Temperature (°C)	15	15	15
Total osmolality (m•osmol/kg)	487.5	975	1462.5

Oxygen Binding: Spectrophotometry

Hemolymph from each species was collected from the basal membrane of the 3rd or 4th walking leg using a microcapillary pipette (size 10 µl). Oxygen binding experiments required hemolymph samples from each species to be pooled in order to acquire an adequate volume for experimentation. For each sample, approximately 40 *P. cinctipes* and 50 *P. eriomerus* were bled and the hemolymph placed into 1.5 ml Eppendorf tubes. Hemolymph samples were held at room temperature for 10 minutes to allow clot formation and then spun at 12,000 g for 4 minutes in a refrigerated centrifuge (4 °C; Eppendorf centrifuge 5415 D), to remove cellular material. Absorbances of the supernatants were read spectrophotometrically, after which samples were diluted in *Petrolisthes* buffer until an absorbance between 0.6 and 0.8 was obtained at wavelength

335nm (approximately 1:7 dilution). Samples were then refrigerated until use or used immediately for oxygen binding experiments.

Oxygen binding experimentation was similar to the tonometric method described in Benesch et al. (1965). Approximately 1.5 ml of diluted sample were dialyzed (Spectra/Por® membrane, molecular weight cutoff point 12-14,000 Da) against a total of 500 ml of *Petrolisthes* buffer. For pH, temperature, and salinity manipulations, the buffer was changed after approximately 5 hours. For lactate manipulations, buffers were changed twice in the first 5 hours in order to more effectively remove any residual organic cofactors such as lactate. After a total of approximately 17 hours, samples from all experimental groups were removed from dialysis and measured into tonometers. For L-lactate manipulations, L-lactate and NaCl were then added to the dialysed hemolymph. Samples were equilibrated to either 12 °C for the pH manipulations, 15 °C for L-lactate and salinity experiments, or the experimental temperature for temperature manipulations, by being placed into a thermostated water bath for 10 minutes. The pH measurement for samples was taken after dialysis, prior to deoxygenation, similar to Sanders et al. (1988), using an Orion Ross pH electrode.

The original absorbance at 335nm, the absorption maximum that is seen in most crustacean hemocyanins (Jokumsen and Weber, 1982), was read spectrophotometrically for each sample; samples were then deoxygenated after approximately 12 pulls from a vacuum pump (with 5 minutes equilibration between pulls). After deoxygenation, samples were again immersed in the waterbath for 10 minutes, followed by a deoxygenated absorbance reading. Recirculating water from the waterbath to the cuvette

holder allowed the tonometer to remain at the experimental temperature for the duration of the absorption reading (approximately 15 seconds). To reoxygenate, known amounts of air were introduced into each tonometer using a 10 ml syringe fitted with rubber tubing. Samples were allowed to equilibrate at their experimental temperature for 10 minutes after each increment before absorbances were read. Samples were opened to air after approximately 6 incremental readings, and a final absorption reading was taken. Hemolymph was discarded 6 days following each hemolymph extraction.

Oxygen Binding: Data Analysis

To find P_{50} values, Po_2 was first calculated. Barometric and air temperature readings were taken using an Eberbach barometer and thermometer. Relative humidity readings were assumed to be 100% as the internal portions of the syringe were coated with dH_20 causing the adjacent air to be completely saturated. Partial pressure of oxygen in the tonometers was calculated using the formula:

$$Po_2 = \text{ml air injected } (0.21)\ (B\text{-}Hp)\ T_i / V\ T_o$$

Where B is barometric pressure, H is relative humidity, p is vapor pressure of water, T_i is temperature of the sample, V is volume of air in the tonometer and T_o is air temperature.

The percent of hemocyanin saturated with oxygen (y) was calculated using the formula:

$$y/100 = (A_d - A_i)/ (A_d - A_o)$$

Where A_d is the absorbance of the sample at the deoxygenated state, A_i is the incremental reoxygenated absorbance, and A_o is the original fully oxygenated absorbance.

Using this data, graphs of y vs. Po_2 were made to examine the basic differences between species for each individual experiment. A Hill plot was also constructed where the $\log_{10}$ of (y/1-y) was plotted against the $\log_{10}$ of Po_2. Using this Hill plot, cooperativity (n_{50}) was determined by measuring the slope of the portion of line transecting the log P_{50} reference line.

For pH manipulations, the Bohr effect was determined by plotting the average log P_{50} at each pH versus pH. The slope of the resultant line was then calculated (Δ log P_{50}/ Δ pH) in order to determine the Bohr effect. Cooperativity values were plotted against pH to determine the effect of pH on cooperativity.

For temperature experiments, van't Hoff plots were created by plotting log P_{50} vs. 1/T (degrees Kelvin). To calculate the heat of oxygenation (ΔH), regression analysis over the entire temperature range was used to determine the slope of each van't Hoff plot. The heat of oxygenation was then calculated in order to determine the thermal sensitivity of each species' hemocyanin by using the formula:

$$\Delta H = 2.303R \text{ (slope)}$$

Where R is the universal gas constant = 8.314 J/mol-^{o}K. This method allows for the determination of the effects of temperature on oxygen affinity at constant pH. Cooperativity values versus temperature were plotted to determine the effect of temperature on hemocyanin cooperativity.

Plots of [L-lactate] vs. P_{50} were created to demonstrate the general effects of lactate on the hemocyanin of each *Petrolisthes* species. L-lactate data was further analyzed by determining the slope of the line when plotting the average log P_{50} against each log L-lactate concentration (Δ log P_{50}/ Δ log [L-lactate]). The resultant number is called the L-lactate effect coefficient. A plot of cooperativity values versus [L-lactate] was created to determine the effect of L-lactate on cooperativity.

The oxygen binding effects of total salinity and magnesium on hemocyanin of each *Petrolisthes* species were determined by plotting log P_{50} against either total salinity or [Mg^{++}]. A plot of cooperativity values versus total salinity or [Mg^{++}] was generated to determine the effect of each on hemocyanin cooperativity.

To test for significance, statistical analyses were done on cooperativity and oxygen affinity data from all experimental groups. Two assumptions of parametric testing, normality and homogeneity of variance, were met; data was analyzed for these assumptions using Levene's test, Hartly, Cochran, and Bartlett test, and normal distribution curves. Linear regression was used initially in order to determine whether or not slopes of data were significantly different from zero. A t-test was used on any groups where slopes were not significantly different from zero, in order to determine if there was a significant difference in line elevation between the two species. Analysis of covariance (ANCOVA) was used on any groups that had a slope significantly different from zero in order to determine if there was a significant difference in the slope and/or elevation between *P. eriomerus* and *P. cinctipes*. ANCOVA was used despite the repeated use of pooled samples in this study. Due to hemolymph pooling, the data points collected

across experimental factors and within an experimental range were not independent, violating one assumption of ANCOVA.

Hemocyanin Structure: PAGE

Hemocyanin structure was examined in addition to function, by the use of polyacrylamide gel electrophoresis (PAGE). Hemolymph from individual crabs was spun for 4 minutes at 12,000 g in order to remove cellular debris. The supernatant was then used to characterize and compare protein bands between the two species by the use of pH 7.4 PAGE, pH 8.9 PAGE, and SDS-PAGE gels (Terwilliger and Terwilliger, 1982; Davis, 1964; Laemmli, 1970). Samples used for SDS-PAGE (7.5% acrylamide) were first diluted to 20% with dH_2O. Samples were then mixed 1:1 with SDS Incubation Buffer (SDS Sample Buffer (0.0625 M Tris-OH, 2% SDS, 10% glycerin, 0.001 M EDTA, 0.01% Bromphenol Blue, HCl, dH_2O), 0.1M dithiothreitol, 0.03M phenylmethyl sulfonyl fluoride, dH_2O), followed by a 1:1 dilution with diluted SDS Sample Buffer (mixed 1:1 with dH_2O). Samples were then boiled for 90 seconds to inactivate proteases. The dissociated, denatured samples were loaded onto gels in volumes of 1 – 2 µl. SDS electrode buffer consisted of 0.025 M Tris-OH, 0.192 M glycine, 0.1% SDS, 1 mM EDTA. SDS gels were run at 200 volts followed by a staining in Coomassie blue and a destaining in AcOH. SDS samples were stored in a –20 ^{o}C freezer.

Samples for non-dissociating, non-denaturing pH 7.4 PAGE (5% acrylamide) experiments were prepared by mixing 3 µl supernatant, 12 µl pH 7.4 upper gel buffer (0.05 M maleic acid; 0.05 M NaOH; 0.05 M Tris; titrated to pH 7.4) and 3 µl bromphenol

blue:glycerol (3:1). Samples were then loaded onto gels in volumes of 1 µl and run at 35 mA per gel. Electrode buffers included the upper gel buffer (see above) and lower gel buffer (0.05 M HCl; 0.05 M Tris; titrated to pH 6.8). Protein bands were resolved by staining with Coomassie blue and destaining with 10% AcOH.

Several bands from the pH 7.4 PAGE were then further characterized using SDS PAGE. The upper band from pH 7.4 PAGE, which corresponds to *Cancer magister* 25S two hexamer hemocyanin, and the lower band, which corresponds to *C. magister* 16S one hexamer hemocyanin (Terwilliger and Terwilliger, 1982), were excised, rinsed and incubated overnight at –20 °C in SDS Incubation Buffer diluted 1:1 with dH_2O. The two bands were then run on an SDS gel alongside a sample of that individual crab's respective whole hemolymph.

Samples for non-denaturing, dissociating pH 8.9 PAGE (7.5% acrylamide) were prepared identically to pH 7.4 PAGE samples except for the dilution of hemolymph samples in pH 8.9 upper gel buffer (0.052 M Tris; 0.052 M Glycine; 0.5 mM EDTA, pH 8.9). Loading volume and procedure was identical to pH 7.4 PAGE. The lower gel electrode buffer for pH 8.9 PAGE consisted of 0.05 M Tris, 0.05 M HCl and 0.5 mM EDTA, pH 8.1.

Hemocyanin Structure: Data Analysis

All gels were scanned for analysis using a Umax® PowelookIII scanner and software with constant parameters (transmissive, gray 256 scale, 600 dpi resolution, no filter, no descreen). The major bands in each lane of all gels were recognized using Gel

Pro Analyzer 4.0 (by Media Cybernetics). Standardization across gels included background subtraction, fixed lane width, and fixed band height. The relative protein concentration of the major bands in individual gels of pH 7.4 PAGE and pH 8.9 PAGE was calculated. This was done by measuring the integrated optical density (IOD) of each band and dividing it by the total IOD of the corresponding lane to give a percentage of protein in that band in relation to the amount of protein loaded into the lane. The molecular weights of all SDS bands were calculated with Gel Pro Analyzer, by referencing the high molecular weight standards (Biorad) that were run alongside hemolymph samples on each gel.

CHAPTER III

RESULTS

Results of the magnesium assay established that *P. eriomerus* hemolymph had an average Mg^{++} concentration of 36 mM (n = 5) and *P. cinctipes* an average concentration of 35 mM (n = 5). Osmolality measurements determined *P. eriomerus* hemolymph osmolality at 989 +/- 10 m•osmol/kg and *P. cinctipes* at 996 +/- 14 m•osmol/kg. This is similar to a literature value of 985 +/- 17 m•osmol/kg for *P. cinctipes* (Hunter and Kirschner, 1986).

Oxygen Binding: pH

Dialyzed hemolymph of both *P. eriomerus* and *P. cinctipes* significantly increased oxygen affinity as pH increased (Tables 5 and 6; Figure 1). Bohr effect values were calculated between pH 7.7 and pH 8.6 from Figure 1, giving *P. eriomerus* a Bohr coefficient of -1.07 and *P. cinctipes* a Bohr coefficient of -1.25.

Oxygen binding experiments demonstrated obvious differences between the dialyzed hemolymph of the two *Petrolisthes* species over a range of pH. With the exception of several pH 7.45 experiments, in individual paired experiments at each pH *P. eriomerus* hemolymph had a lower affinity for oxygen than that of *P. cinctipes* (Table 6). After combining data, *P. eriomerus* hemolymph demonstrated higher average log P_{50}

values than *P. cinctipes* (Figure 1), although this effect was not statistically significant (Table 7).

On average, *P. eriomerus* hemolymph tended towards a slightly higher cooperativity than that of *P. cinctipes* (Figure 2). The cooperativity of neither species was influenced by pH (Table 8; Figure 2). Average cooperativity values for *P. eriomerus* ranged from 4.2 to 3.1 (at pH 7.7 and pH 8.2 respectively) while the average cooperativity values for *P. cinctipes* ranged from approximately 4.0 to 2.4 (at pH 7.7 and pH 8.6 respectively). Results from a statistical t-test gave a p value of 0.16, demonstrating that there was no significant difference in cooperativity between *P. eriomerus* and *P. cinctipes* hemocyanin across the range of pHs tested (Table 9).

Interestingly, the oxy-deoxy spectra for both *P. eriomerus* and *P. cinctipes* were anomalous at the lower pHs of pH 7.7 and especially pH 7.45. The spectra were characterized by an abnormally low oxygenated absorbance with a deoxygenated absorbance reading not far below the original. The absorbance readings at 335nm would fluctuate higher than the original oxygenated absorbance value and lower than the deoxygenated absorbance value during incremental reoxygenation stages. This effect was more distinct in *P. eriomerus* at both pHs. The absorption spectra for both species at each of these lower pHs compared with a more typical absorption spectra at pH 7.95 are shown in Figures 3A and 3B. The reaction of *Petrolisthes* hemocyanin to these low pH conditions suggests that it was unable to fully oxygenate at any time during a low pH treatment. These findings may reflect a possible Root effect (see Discussion).

Table 5: Simple linear regression on the P_{50} values of dialyzed hemolymph from (A) *P. eriomerus* and (B) *P. cinctipes*, the results of which demonstrated that the slope in each experimental group $\neq 0$, allowing for subsequent ANCOVA analysis.

A.

	F value	df	P
pH*	170.5	1:15	<0.001
Temperature	71.422	1:18	<0.001
[L-lactate]**	9.71	1:10	0.01
$[Mg^{++}]$	29.95	1:10	<0.001
Total salinity	35.91	1:8	<0.001

B.

	F value	df	P
pH*	117.5	1:19	<0.001
Temperature	53.99	1:18	<0.001
[L-lactate]**	5.88	1:10	0.036
$[Mg^{++}]$	20.08	1:10	0.001
Total salinity	55.38	1:10	<0.001

* Only pH values in the range pH 7.95 to pH 8.6 for *P. eriomerus* and pH 7.7 to pH 8.6 for *P. cinctipes* were used for linear regression analysis.

** Only data points for [L-lactate] between 2 mM and 8 mM were used for linear regression analysis.

Table 6: P_{50} values for the dialyzed hemolymph of *P. eriomerus* and *P. cinctipes* at each experimental pH, at 12 °C. Shaded boxes show the only values where *P. cinctipes* had a lower affinity than *P. eriomerus*.

pH	P_{50} *P. cinctipes*	P_{50} *P. eriomerus*
8.6	3.35	7.7
8.6	3.8	7.2
8.6	4.8	8
8.6	5.2	7.9
8.4	7.2	10.5
8.4	4.7	9.5
8.4	4.6	11
8.4	16	21
8.2	17	21
8.2	16	26.8
8.2	28	32.9
8.2	21	27

pH	P_{50} *P. cinctipes*	P_{50} *P. eriomerus*
7.95	39	48.5
7.95	13.5	43
7.95	52	63.5
7.95	35.5	41.8
7.7	45.8	56.3
7.7	55.5	79
7.7	49.5	52
7.7	61.5	64
7.45	79	83
7.45	55	56.8
7.45	52.5	45
7.45	62	53

Table 7: Statistical results demonstrating the differences in log P_{50} values between dialyzed hemolymph of *P. eriomerus* and *P. cinctipes* using analysis of covariance (ANCOVA). The 1st step of ANCOVA demonstrated that the slopes were not significantly different from one another. The following table represents values from the 2nd step of ANCOVA, which tests whether or not the elevation is significantly different between species. Note: Results found using SYSTAT® 9 (SYSTAT, Inc., 1999).

	F value	df	P
pH*	1.54	1:26	0.23
Temperature	126.95	1:37	< 0.001
[L-lactate]**	2.40	1:21	0.14
$[Mg^{++}]$	6.59	1:21	< 0.02
Total salinity	2.65	1:19	0.12

* Only pH values in the range pH 7.95 to pH 8.6 were used for ANCOVA analysis.
** Only data values for [L-lactate] between 2 mM and 8 mM were used for ANCOVA analysis.

Table 8: Simple linear regression results demonstrating the effect of each condition on n_{50} values from the dialyzed hemolymph of (A) *P. eriomerus* and (B) *P. cinctipes*. Since the cooperativity did not significantly differ from zero across the range of conditions tested, t-tests were subsequently used. Note: Results found using SYSTAT® 9 (SYSTAT, Inc, 1999).

A.

	F value	df	P
pH	1.03	1:20	0.32
Temperature	0.28	1:18	0.61
[L-lactate]	0.29	1:19	0.60
$[Mg^{++}]$	0.26	1:9	0.63
Total salinity	1.35	1:8	0.28

B.

	F value	df	P
pH	2.14	1:19	0.16
Temperature	0.29	1:18	0.60
[L-lactate]	0.46	1:19	0.51
$[Mg^{++}]$	0.04	1:10	0.85
Total salinity	4.66	1:10	0.06

Table 9: Results from t-tests on the cooperativity values of *P. eriomerus* and *P. cinctipes* for each tested condition. Analysis was done using a 95% confidence interval. Note: Results found using SYSTAT® 9 (SYSTAT, Inc, 1999).

	df	t	P
pH	42	-1.44	0.16
Temperature	42	-1.07	0.29
[L-lactate]	40	-2.03	0.05
$[Mg^{++}]$	21	0.17	0.86
Total Salinity	20	-0.57	0.57

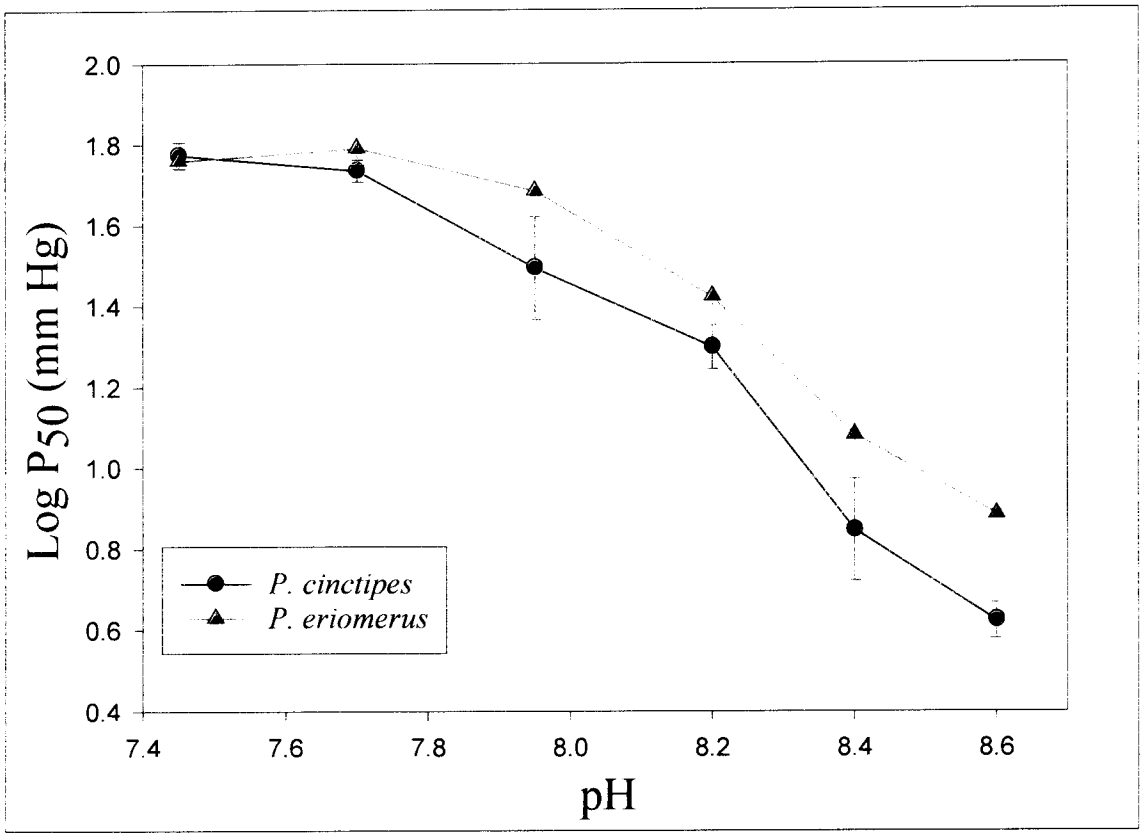


Figure 1: Average log P_{50} values for the dialyzed hemolymph of *P. eriomerus* and *P. cinctipes* at constant temperature of 15 °C, demonstrating the effects of pH on oxygen affinity. Symbols represent mean values +/- standard error, n = 4.

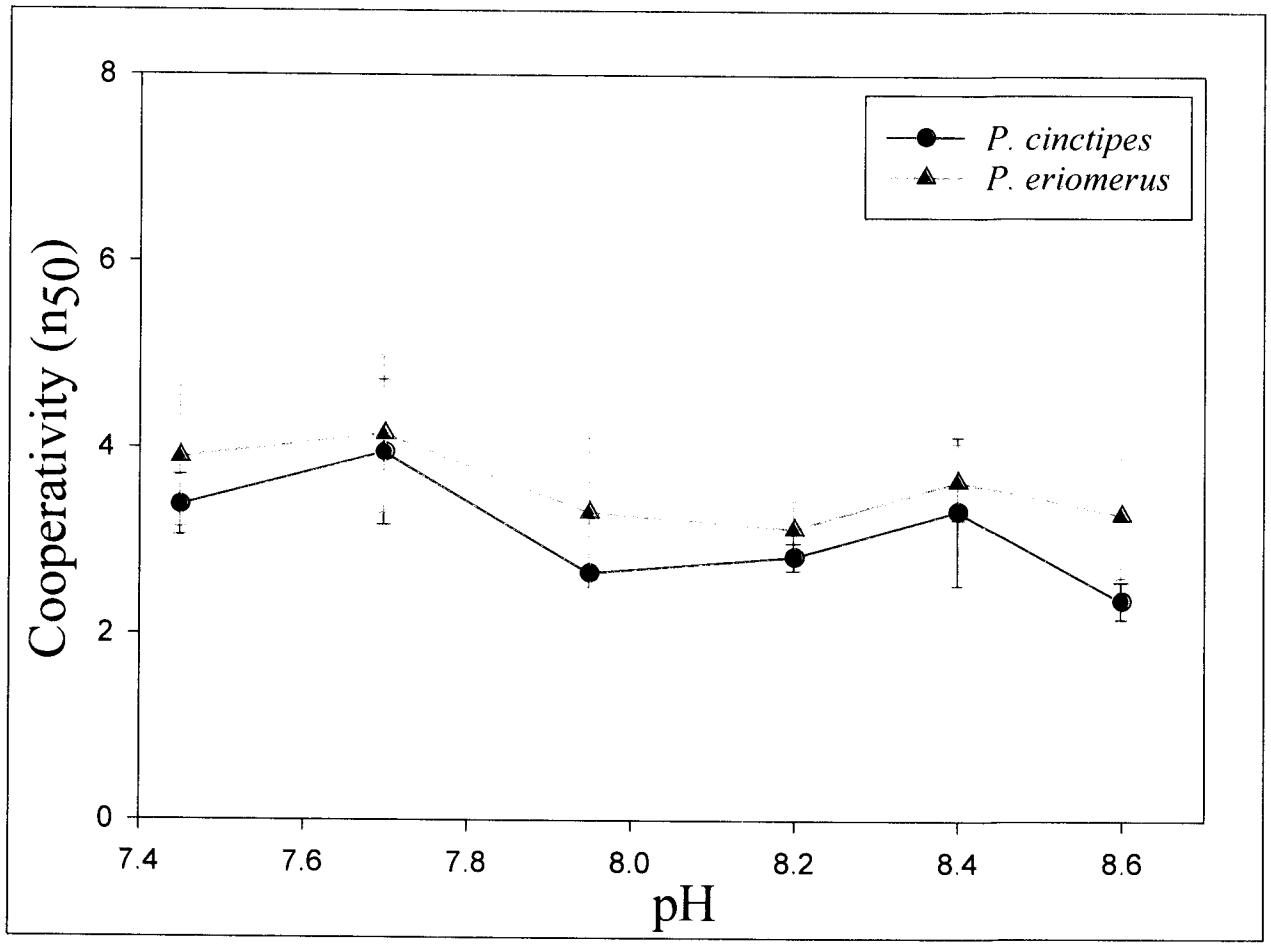


Figure 2: Average cooperativity for *P. eriomerus* and *P. cinctipes* dialyzed hemolymph at each experimental pH. Symbols represent mean values +/- standard error, n = 4.

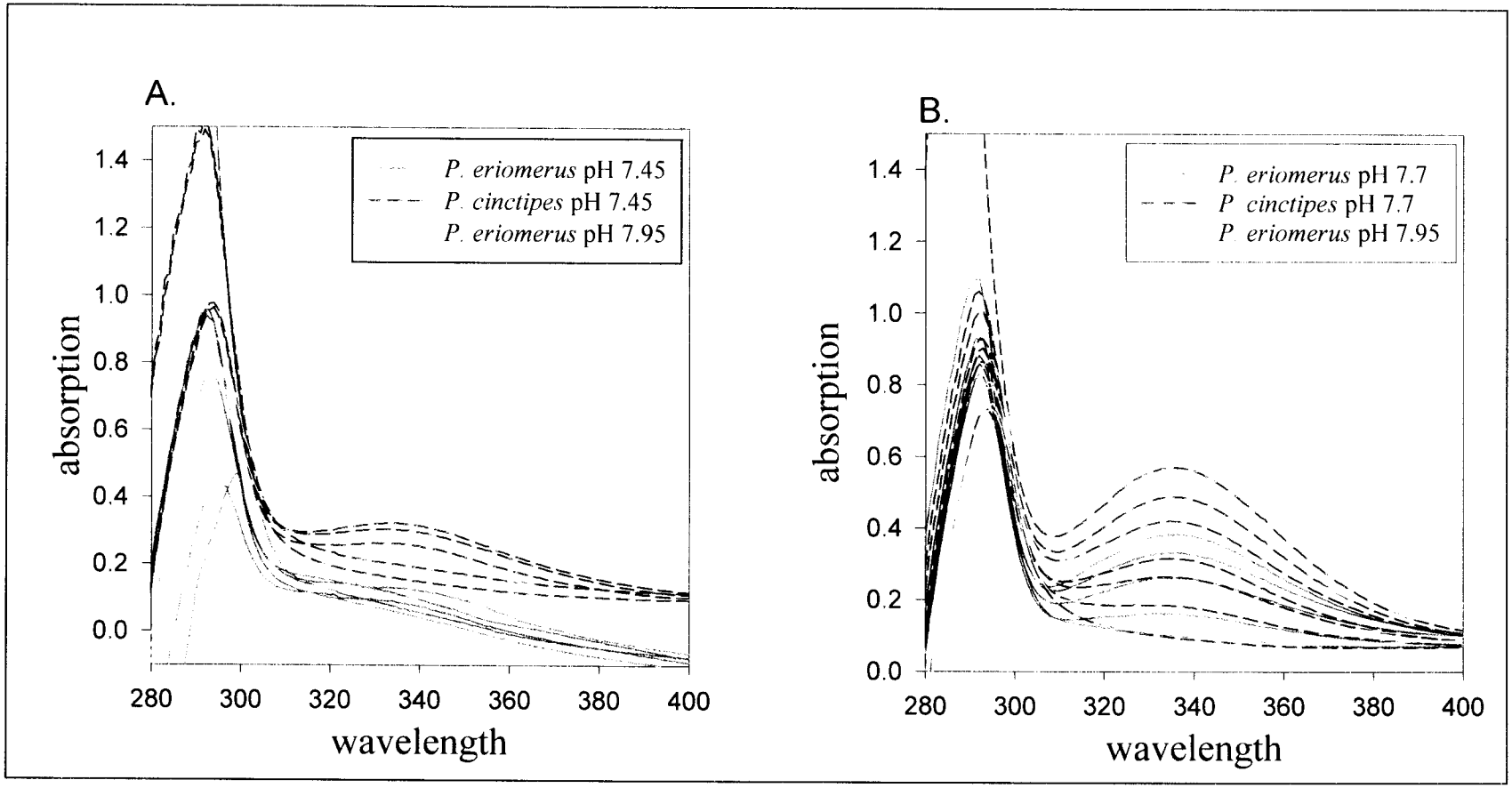


Figure 3: Absorption spectra of the deoxygenated and various degrees of oxygenated hemocyanin from *P. eriomerus* and *P. cinctipes* dialyzed hemolymph at low pH, showing (A) pH 7.45, and (B) pH 7.7. In both (A) and (B) a *P. eriomerus* absorption spectra at pH 7.95 are shown for comparison.

Oxygen Binding: Temperature

As temperature increased, P_{50} values for both species increased, representing a significant decrease in oxygen affinity (Table 5; Figure 4). The effect of temperature on hemocyanin oxygen affinity was very similar for *P. eriomerus* and *P. cinctipes* as was quantified by their heat of oxygenation values. Between 10 °C and 30 °C at pH 7.9, ΔH was calculated as -40.81 kJ/mol for *P. eriomerus* and -35.99 kJ/mol for *P. cinctipes*, providing evidence that *P. eriomerus* hemocyanin is slightly more sensitive to temperature than *P. cinctipes*. Across the entire temperature range, *P. eriomerus* hemocyanin demonstrated a significantly lower affinity for oxygen than *P. cinctipes* (Table 7).

There were no apparent differences in cooperativity between the two species (Figure 5). In addition, the effects of temperature on hemocyanin cooperativity were statistically insignificant for both *P. eriomerus* and *P. cinctipes* (Table 8). The average cooperativity values ranged from 3.8 at 20 °C to 5.5 at 25 °C for *P. eriomerus* and 3.5 at 25 °C to 4.6 at 30 °C for *P. cinctipes*. A t-test on the cooperativity data of both species concluded that there was no significant difference in cooperativity across the full range of temperatures tested ($p = 0.29$) (Table 9).

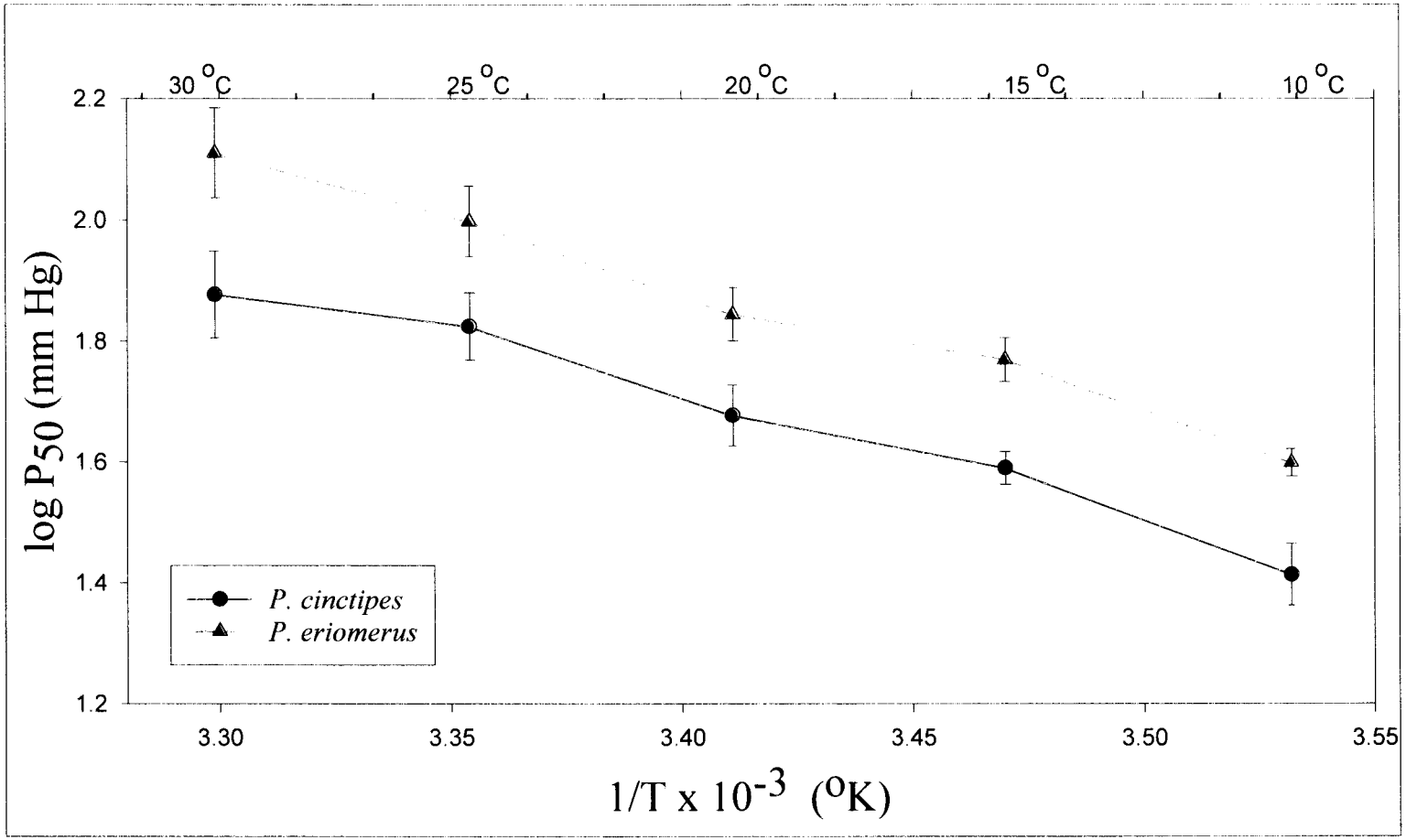


Figure 4: Van't Hoff plots demonstrating hemocyanin oxygen affinity in relation to temperature variation at a constant pH 7.9 for both *P. eriomerus* and *P. cinctipes*. Symbols represent mean values +/- standard error, n = 4.

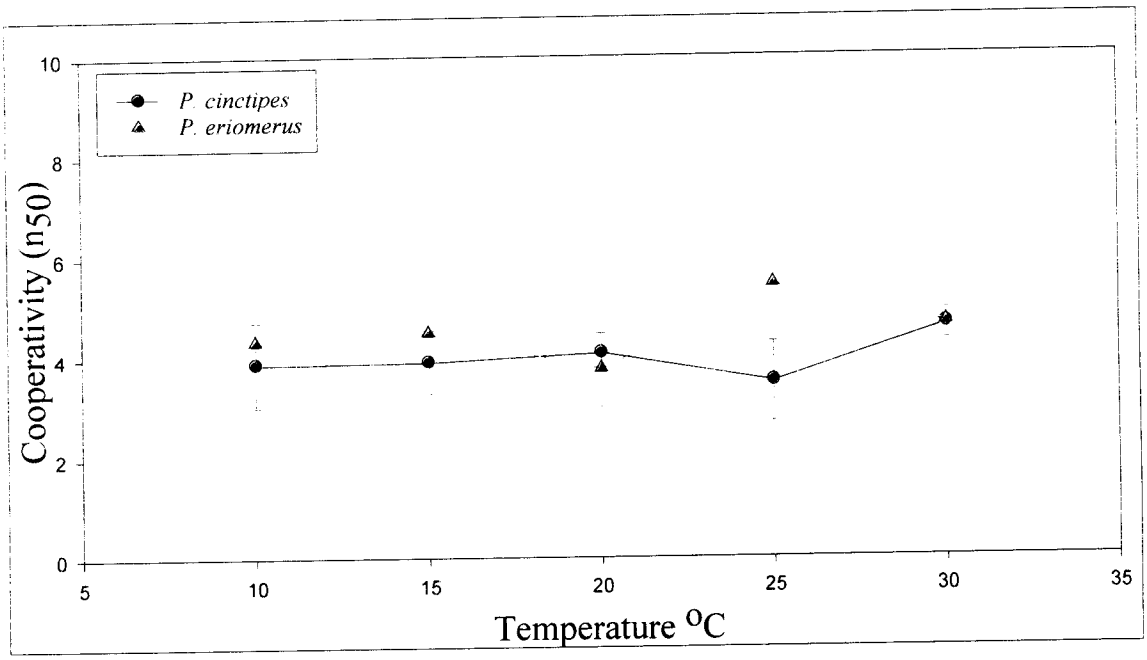


Figure 5: Average cooperativity values for the dialyzed hemolymph of *P. eriomerus* and *P. cinctipes* at each experimental temperature. Symbols represent mean values +/- standard error, n = 4.

Oxygen Binding: Lactate

Increases in L-lactate levels caused a significant increase in hemocyanin oxygen affinity for both *Petrolisthes* species (Figure 6; Table 5). The magnitude of this effect reached a plateau at concentrations higher than 8 mM. For each individual paired experiment at 2 mM and 5 mM lactate, *P. eriomerus* had a lower affinity than *P. cinctipes*, although the combined values were not statistically significant (Figure 6; Table 7). At 8 mM lactate concentrations and higher, there was no obvious trend towards one species having a higher affinity than the other during individual experiments (Figure 6).

The lactate coefficients ($\Delta \log P_{50}/ \Delta \log$ [L-lactate]) between 2 mM L-lactate and 8 mM L-lactate, calculated using the slope of the lines in Figure 7, were -0.32 for *P. eriomerus* and -0.22 for *P. cinctipes.*

Across the experimental range of [L-lactate], *P. eriomerus* had a significantly higher cooperativity than *P. cinctipes* (Table 8). L-lactate did not appear to affect the cooperativity of either species at any of the experimental concentrations, changing the n_{50} values only subtly (Figure 8). Mean cooperativity values ranged between 2.6 at 5 mM and 3.3 at 2mM for *P. eriomerus* and 2.1 at 5 mM and 2.6 at 10mM for *P. cinctipes*. A t-test on the cooperativity data of both species concluded that there was a significant difference in line elevation between the two species ($p = 0.05$), signifying an overall higher cooperativity for *P. eriomerus* hemocyanin (Table 9).

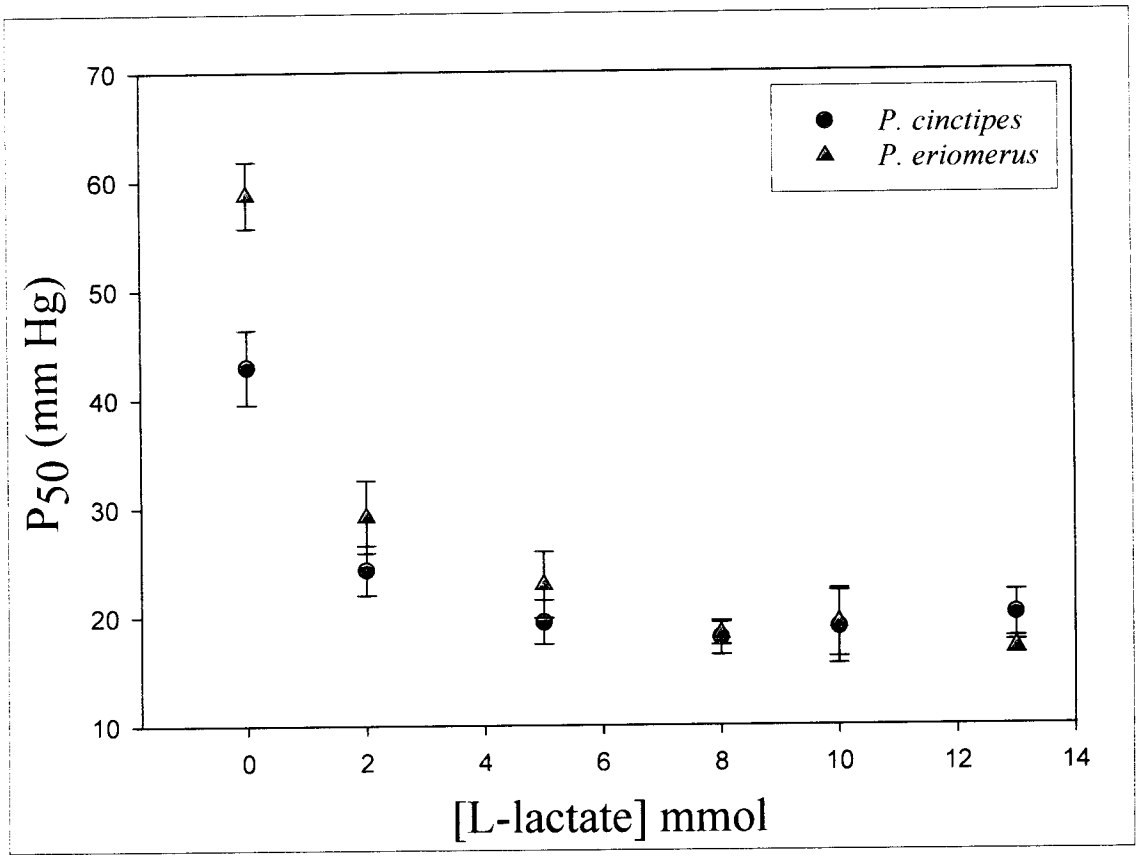


Figure 6: Effect of varying [L-lactate] on hemocyanin oxygen affinity for both *P. eriomerus* and *P. cinctipes* at pH 7.9, 15 °C. Symbols represent mean values +/- standard error. [L-lactate] = 0, n = 8; [L-lactate] = 2 mM, 5 mM and 8 mM, n = 4; [L-lactate] = 10 mM and 13 mM, n = 5.

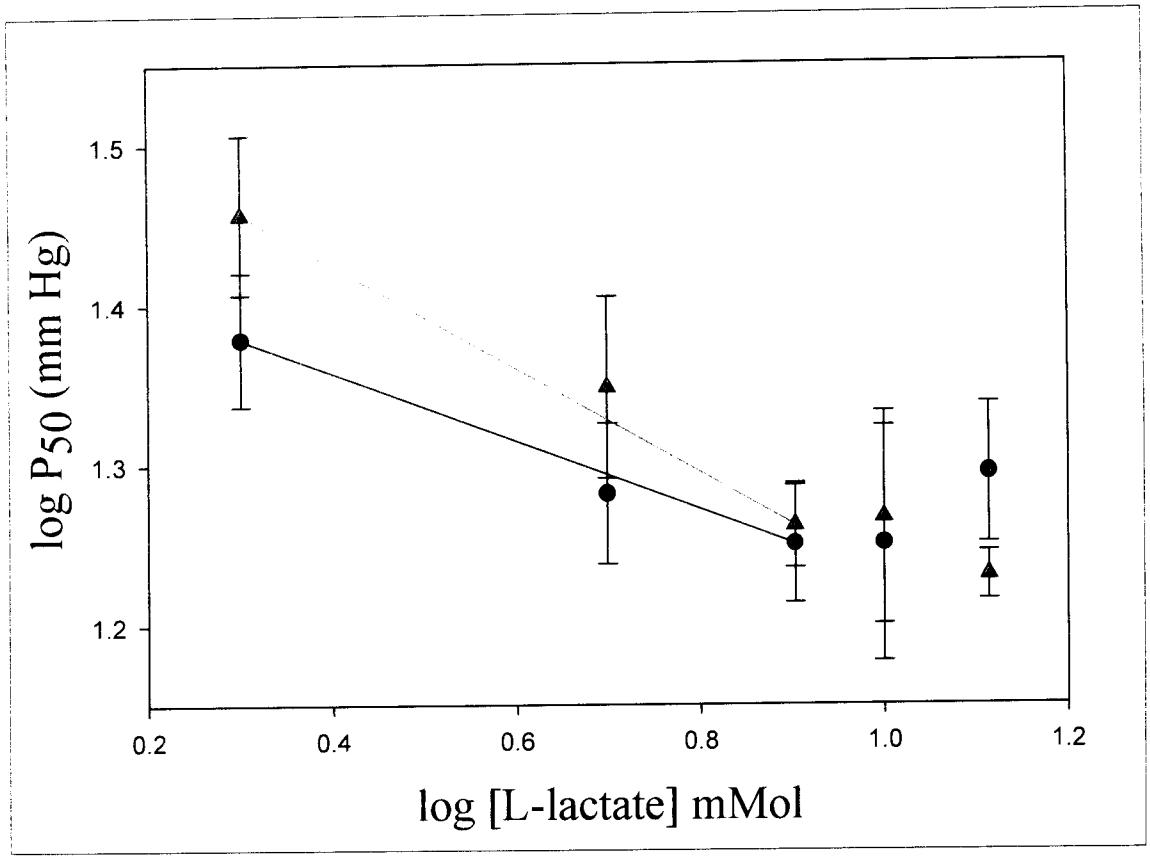


Figure 7: Plot of log [L-lactate] vs. log P_{50} at pH 7.9, 15°C. The slope of the blue line represents the lactate coefficient for *P. eriomerus*. The slope of the red line represents the lactate coefficient for *P. cinctipes*. Symbols (blue triangle for *P. eriomerus*, red circle for *P. cinctipes*) represent mean values +/- standard error. [L-lactate] = 2 mM, 5 mM and 8 mM, n = 4; [L-lactate] = 10 mM and 13 mM, n = 5.

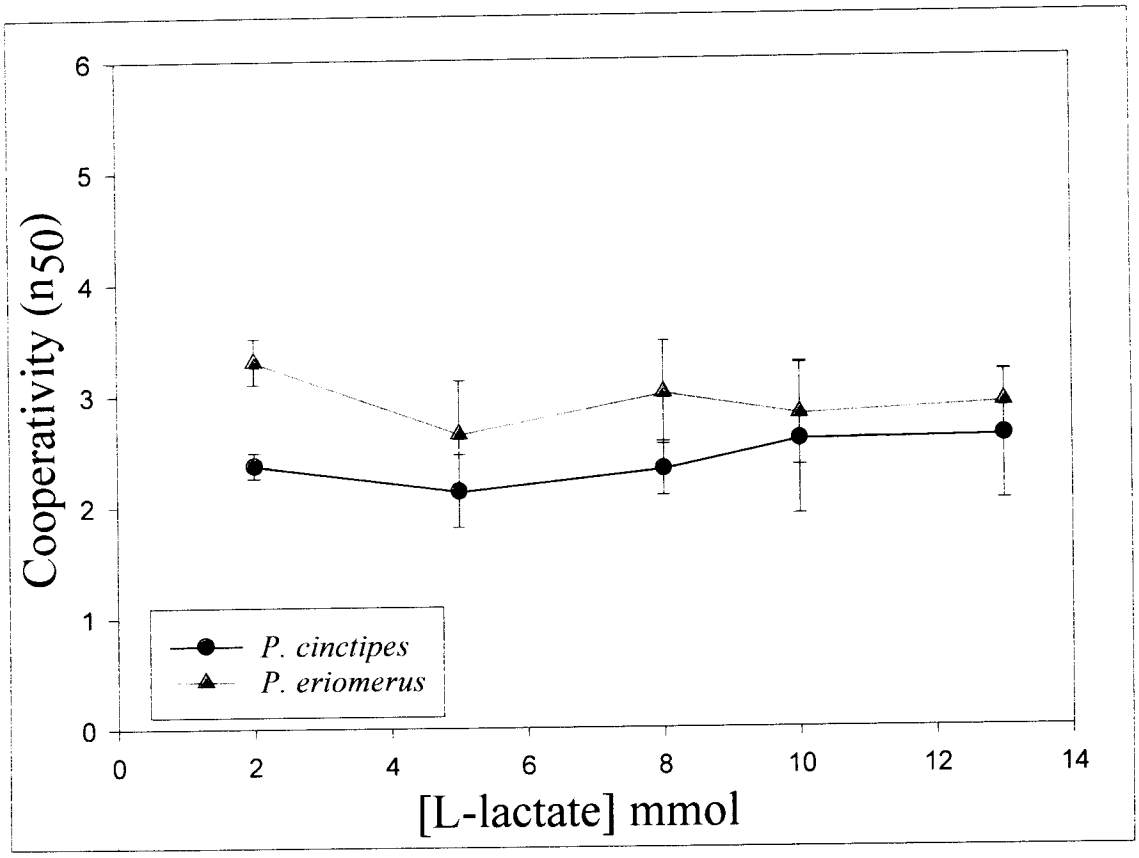


Figure 8: Average n_{50} values for *P. eriomerus* and *P. cinctipes* at each experimental [L-lactate], demonstrating the effects of varying lactate on the cooperativity of oxygen binding. Symbols represent mean values +/- standard error. [L-lactate] = 2 mM, 5 mM and 8 mM, n = 4; [L-lactate] = 10 mM and 13 mM, n = 5.

Oxygen Binding: Salinity

In both *Petrolisthes* species, total salinity and magnesium each had the effect of increasing oxygen affinity as ion concentration increased (Figures 9 and 10 respectively; Table 5). In both experiments, *P. eriomerus* had a lower overall affinity than *P. cinctipes*; this difference was statistically significant for the magnesium experiment (Table 7). In every individual salinity experiment except the 50% total salinity group, *P. eriomerus* had a lower affinity than *P. cinctipes*. During the 50% total salinity

experiments, both *P. eriomerus* and *P. cinctipes* hemolymph acted similarly to the pH 7.45 experiments mentioned above where their oxy absorbances were much reduced. Absorption spectra for these low total salinity experiments looked similar to those shown in Figure 3A.

The effects of both total salinity and magnesium on cooperativity did not differ between species (Figures 11A and 11B). In both species, increasing total salinity appeared to have a negative effect on cooperativity (Figure 11A), although this was not statistically significant (Table 8). Magnesium had no effect on the cooperativity of either *P. eriomerus* or *P. cinctipes* (Figure 11B; Tables 8 and 9).

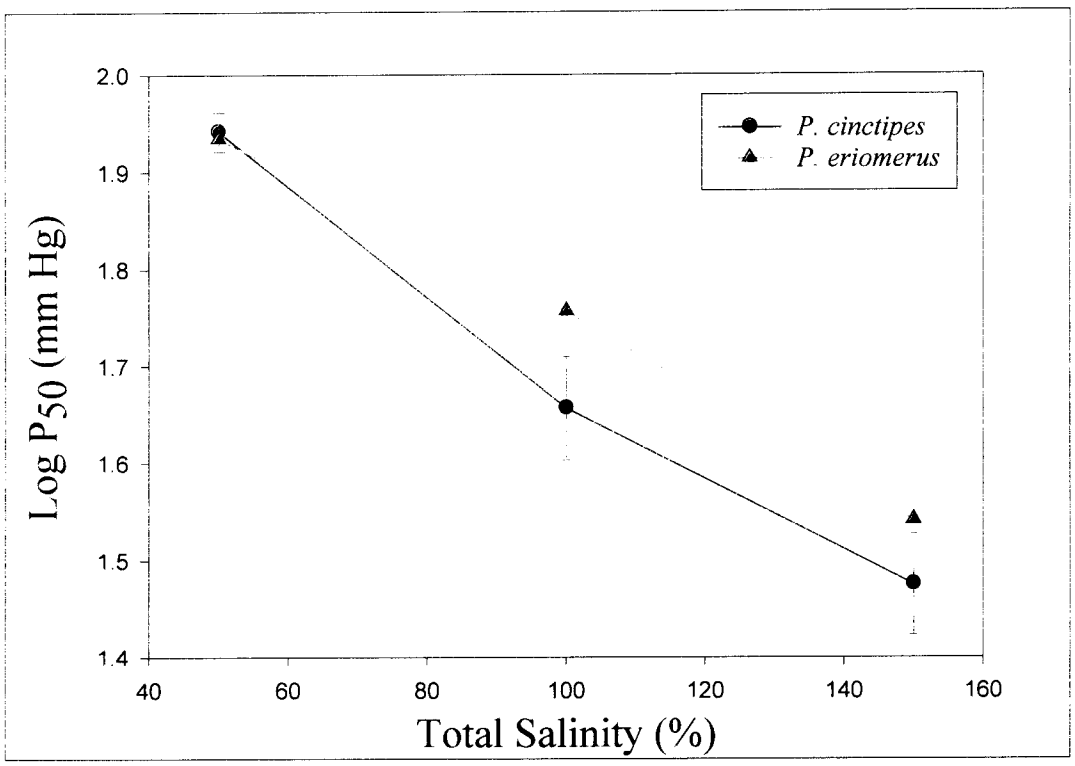


Figure 9: Effect of total salinity on the oxygen affinity of the dialyzed hemolymph from *P. eriomerus* and *P. cinctipes* at pH 7.9, 15 °C, demonstrated by average log P_{50} values. Symbols represent mean values +/- standard error, n = 4

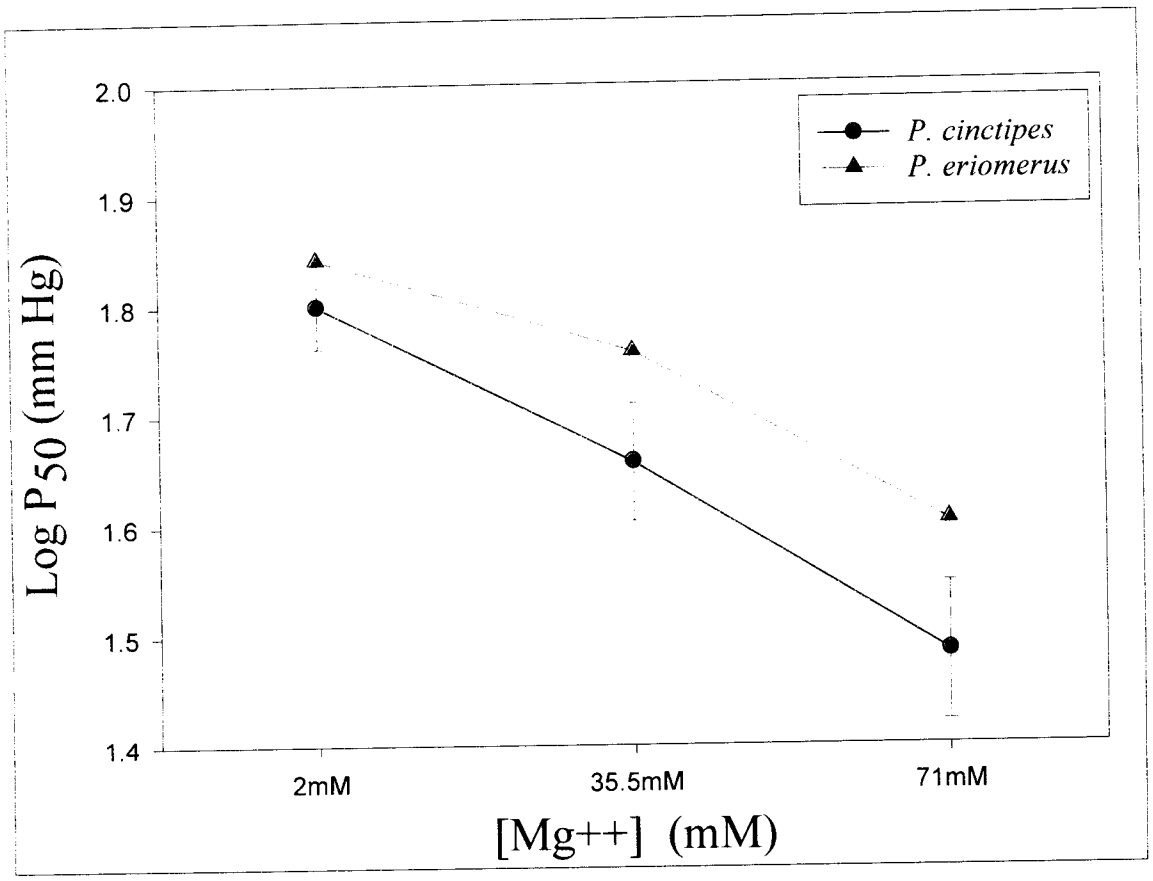


Figure 10: The effect of different magnesium concentrations on the oxygen affinity of the dialyzed hemolymph from *P. eriomerus* and *P. cinctipes* at pH 7.9, 15 °C. Symbols represent mean values +/- standard error, n = 4.

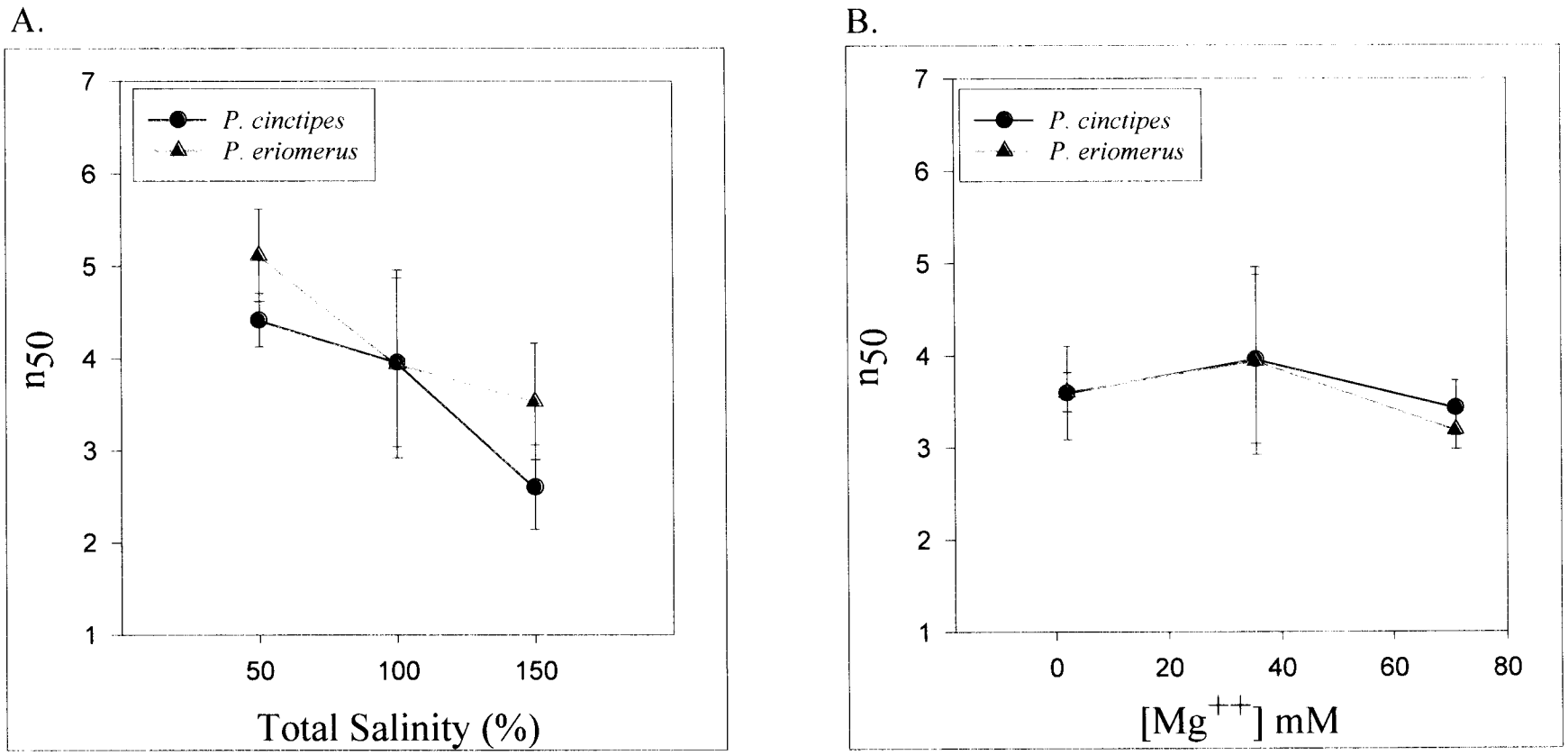


Figure 11: Average cooperativity for *P. eriomerus* and *P. cinctipes* in response to (A) the three different total salinity manipulations, (B) each experimental [Mg^{++}]. Symbols represent mean values +/- standard error, n = 4.

Hemocyanin Structure

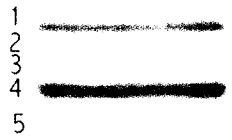
Results from gel electrophoresis experiments show several differences between *P. eriomerus* and *P. cinctipes* in protein banding patterns. Banding patterns were labeled sequentially, starting with 1 as the highest molecular weight. Bands were numbered separately for each species. Whole hemolymph characterization using SDS-PAGE generally resulted in 4 distinct bands (bands 1,3,4,5) present in *P. eriomerus* hemolymph in the molecular weight range corresponding to crustacean hemocyanins, although occasionally 5 bands were resolved from *P. eriomerus* hemolymph (Figure 12A). The banding pattern for *P. cinctipes* whole hemolymph varied between 3 distinct bands (bands 1,3,4) and 4 bands (Figure 12B). The banding patterns between the two species can be easily distinguished from one another by the high molecular weight subunit (band 1) of *P. eriomerus* hemocyanin and the low molecular weight subunit (band 4) of *P. cinctipes* hemocyanin.

Banding patterns from pH 7.4 PAGE demonstrated two distinct bands for each species, a faster migrating band corresponding to *Cancer magister* 16S hemocyanin and a slower migrating band that corresponded to *C. magister* 25S hemocyanin (Figure 13). In both species, the slower band (25S) had a higher concentration of protein than the faster band (16S) (Table 10). The 16S band from *P. eriomerus* hemolymph consistently migrated further on each gel than that of *P. cinctipes*, signifying a lower molecular weight and/or a stronger negative charge than the 16S band for *P. cinctipes* (Figure 13).

The 16S and 25S bands were cut from each pH 7.4 gel and run on an SDS-PAGE to determine the exact subunit composition of the hemocyanin macromolecule of each species. In both species, the SDS pattern of the 25S excised band was indistinguishable from that of whole hemolymph for the respective species (Figure 14). The 16S subunit for both species generally showed 2 distinct bands, but occasionally 3 bands were present, and so differed from whole hemolymph by having fewer bands (Figure 14). These patterns were verified from calculating the molecular weight of each band. The 25S excised bands had identical molecular weights to the whole hemolymph bands for both species (Table 11A and 11B). A strong high molecular weight band (approximately 227 kDa) was consistently present in the 25S lane for both species and was occasionally present in whole hemolymph samples as well.

The pH 8.9 PAGE electrophoresis also demonstrated obvious differences between the two species. *Petrolisthes eriomerus* generally had one distinct band present (band C) in the pH 8.9 PAGE while *P. cinctipes* had 2 distinct bands (bands A and B) (Figure 15). Band C from *P. eriomerus* ran lower on the gel than either *P. cinctipes* bands, but was fairly close to the *P. cinctipes* band B. Occasionally a second band was present just below band B or C, forming a doublet. In some samples of both species, pH 8.9 PAGE procedure did not seem to fully dissociate the hemolymph proteins. This was characterized by large smears of protein in each lane as well as a lack of a strong banding pattern (not shown). This effect was quantified by examining the IOD of each band; total IOD of all bands in each lane were a very small percentage of the total protein in that lane

(Table 12). When pH 8.9 PAGE was replicated in late April and June, the smearing phenomenon was intensified, causing the banding patterns to diminish greatly.



A.

B.

Figure 12: SDS-PAGE from whole hemolymph samples showing: (A) all 5 bands in the hemocyanin range for *P. eriomerus* (left lane) and the most frequent banding pattern for *P. eriomerus* (right lane); (B) the two banding patterns of *P. cinctipes* demonstrating 4 bands (left lane) and 3 bands (right lane). Bands are labeled 1-5 in accordance with their molecular weight, with band 1 having the highest molecular weight. Bands are numbered separately for each species (e.g. *P. eriomerus* band 1 is not equivalent to *P. cinctipes* band 1). Only bands in the mobility range corresponding to crustacean hemocyanin subunits were analyzed.

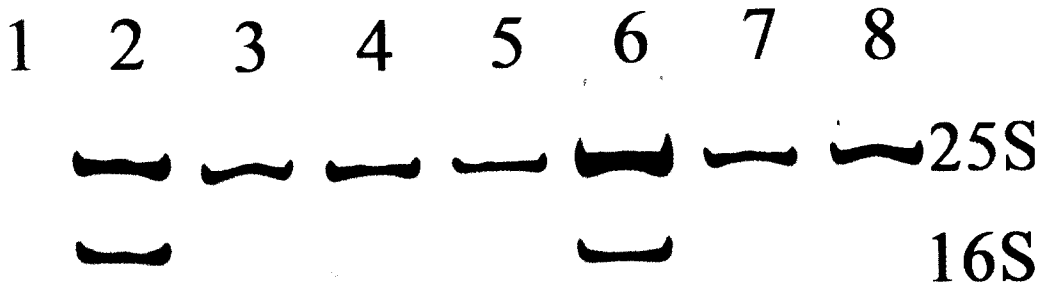


Figure 13: Typical results from pH 7.4 PAGE showing two distinct bands that correspond with *Cancer magister* 25S and 16S hemocyanin. Lanes are, from left to right: 1) Blank (negative control); 2) *Cancer magister* (positive control); 3-5) three individuals of *P. eriomerus*; 6-8) three individuals of *P. cinctipes*.

Table 10: Gel Pro results of % IOD (IOD in band / Total IOD) in each major band from pH 7.4 PAGE.

Species	Major Band	% IOD	+/- SD	n
P. eriomerus	25S	62.8	8	9
P. eriomerus	16S	6.9	3.4	9
P. cinctipes	25S	59.2	7.1	9
P. cinctipes	16S	13.6	10.2	9

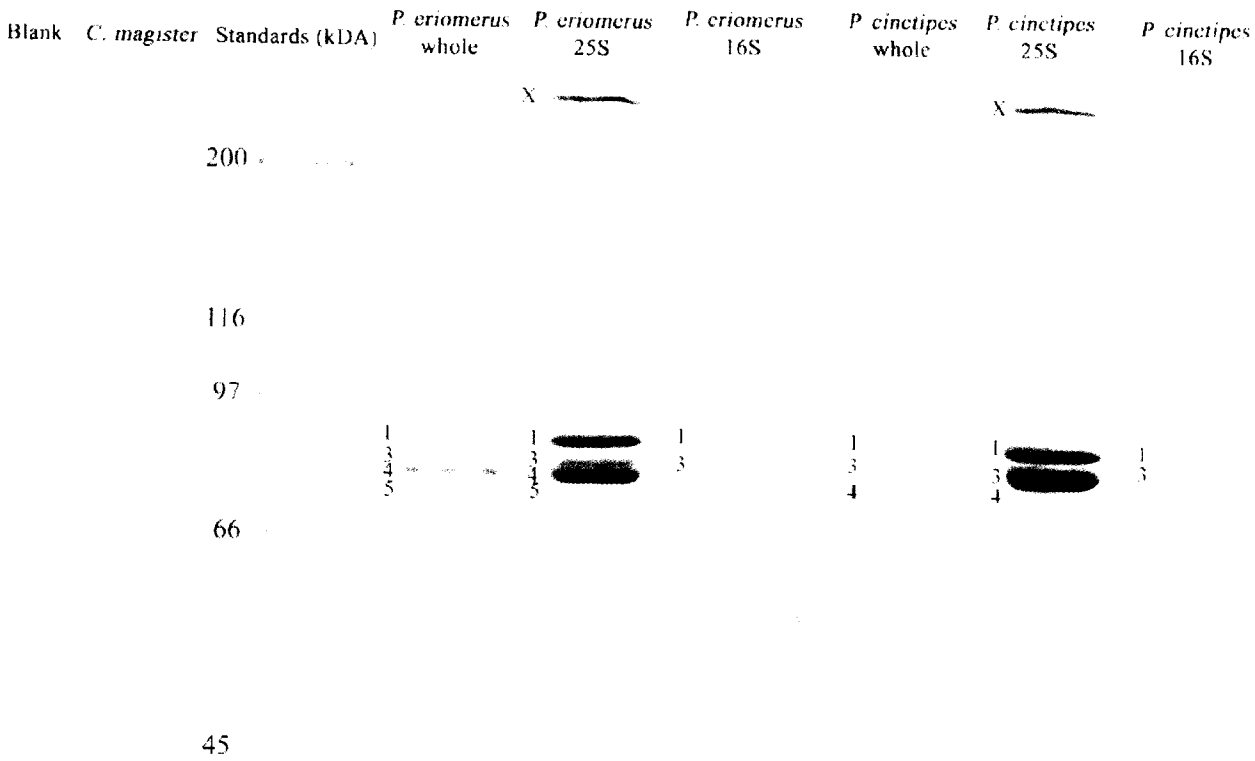


Figure 14: Example of SDS-PAGE showing whole hemolymph along with 25S and 16S excisions from pH 7.4 PAGE. Bands are labeled 1-5 in accordance with their molecular weights. The label "X" denotes a high molecular weight band found in the 25S lanes of both species. Lanes 1 (Blank) and 2 (*C. magister* 25S hemocyanin) are negative and positive controls respectively. Lane 3 (Standards) shows the high molecular weight standards that were used to calculate the molecular weights of the subunits from *P. eriomerus* and *P. cinctipes* hemocyanin.

Table 11: Molecular weights (kDa) of major bands from whole hemolymph compared with molecular weights of 25S and 16S bands, determined using SDS-PAGE, for A) *P. eriomerus* and B) *P. cinctipes*. Molecular weights are shown as means +/- standard deviation with sample size (n).

A.

Species	Band #	Whole hemolymph	25S excised	16S excised
P. eriomerus	Band 1	91.1 +/- 2.0 (8)	91.3 +/- 2.4 (9)	91.8 +/- 1.8 (12)
P. eriomerus	Band 2	89.4 +/- 2.6 (4)	*	89.1 +/- 2.2 (6)
P. eriomerus	Band 3	84.2 +/- 1.9 (2)	86.2 +/- 2.3 (5)	85.9 +/- 2.6 (6)
P. eriomerus	Band 4	83.4 +/- 2.3 (8)	84.5 +/- 2.1 (7)	
P. eriomerus	Band 5	78.6 +/- 2.1 (8)	79.8 +/- 2.4 (9)	

* Although band # 2 was present in several individuals of *P. eriomerus*, molecular weight data was not used for those individuals due to overloaded lanes.

B.

Species	Band #	Whole hemolymph	25S excised	16S excised
P. cinctipes	Band 1	87.6 +/- 1.6 (9)	88.1 +/- 1.5 (5)	89.0 +/- 1.6 (13)
P. cinctipes	Band 2	84.3 +/- 2.4 (3)	85.5 +/- 0.7 (2)	85.0 +/- 1.7 (13)
P. cinctipes	Band 3	82.0 +/- 1.9 (9)	82.3 +/- 0.7 (5)	81.4 +/- 2.5 (3)
P. cinctipes	Band 4	76.8 +/- 1.4 (9)	77.7 +/-0.3 (5)	

Table 12: Gel Pro results of % IOD (IOD in band / total IOD) in each major band from pH 8.9PAGE. *Cancer magister* is included for comparison.

Species	Band	% IOD	+/- SD	n
P. eriomerus	C	6.7	2.7	7
P. cinctipes	A	12.9	2.1	9
P. cinctipes	B	22.4	6.3	9
C. magister	major bands combined	59.5	4.4	3

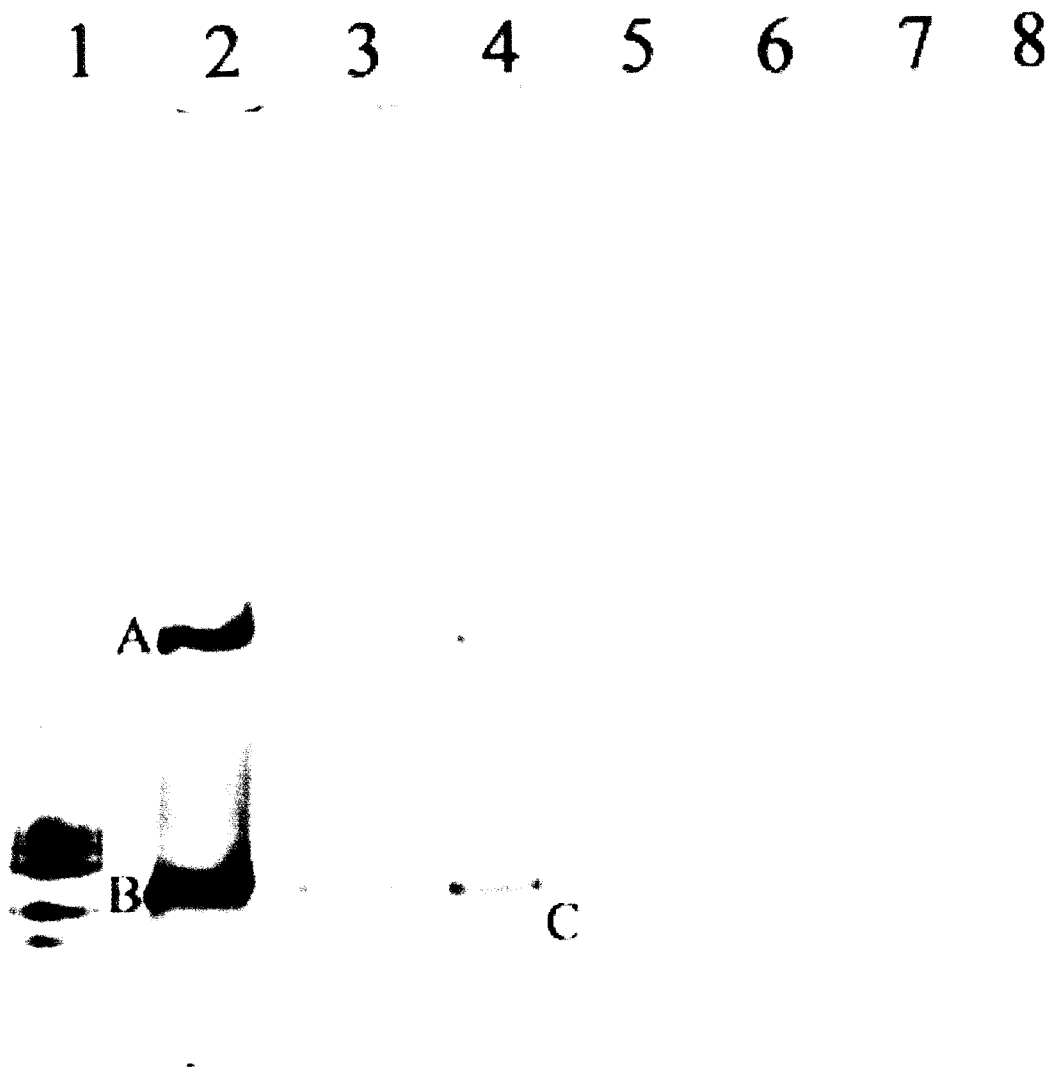


Figure 15: Typical pH 8.9 PAGE showing Lane: 1) *Cancer magister* (positive control); 2-4) three individuals of *P. cinctipes*; 5-7) three individuals of *P. eriomerus*; 8) blank (negative control). A and B denote the mid and lower bands of *P. cinctipes* respectively; C denotes the major band in *P. eriomerus*.

CHAPTER IV

DISCUSSION

Oxygen Binding: pH

The hemocyanin of *P. eriomerus* and *P. cinctipes*, like that of many decapod crustaceans, responds to increases in pH by an increase in oxygen affinity. In some species, the change in P_{50} values across a pH range can be quite large. The difference in P_{50} values for both *Petrolisthes* species was large; values ranged from 79 mm Hg to 7.7 mm Hg in *P. eriomerus* and 62 mm Hg to 3.4 mm Hg in *P. cinctipes* along the pH range 7.7 to 8.6. In comparison, Miller and Van Holde (1981) found that in the anomuran *Neotrypaea [Callianassa] californiensis*, the difference in P_{50} was smaller with a change in P_{50} values from approximately 15 mm Hg to 1 mm Hg along a pH range of 7.57 to 8.73.

The direct relationship between oxygen affinity and pH is an adaptive response to hypoxic situations when environmental pH increases, allowing an organism to increase oxygen unloading at the tissues during activity or hypoxic situations when lactic acid buildup occurs. Because *P. eriomerus* hemocyanin tends towards an overall lower affinity across the pH range and demonstrates a substantial decline in oxygen carrying capacity at low pHs (a possible Root effect), it is suggested that this species is less adapted to changes in pH than *P. cinctipes*.

The Bohr coefficients for both *Petrolisthes* species are moderate to large, reflecting a greater ease in oxygen unloading at the tissue level during low pH than if they demonstrated a small Bohr effect. Bohr coefficients between pH 7.7 and pH 8.6 at 15 °C were -1.07 for *P. eriomerus* and -1.25 for *P. cinctipes*. Mangum (1983b) found a slightly higher Bohr coefficient of -1.2 for *P. eriomerus*, although this was calculated from a pH range of 7.6 to 7.9 at 13.5 °C. Two other anomurans, *Galathea strigosa* and *Eupagurus bernhardus*, had Bohr coefficients similar to *Petrolisthes* at -0.96 and -1.17 respectively, through an approximate pH range of 7.5 to 8.2 at 10 °C (Bridges, 1986). The Bohr coefficient for the brachyuran *Menippe mercenaria* is also similar with a value of -0.9 between pH 7.4 to pH 7.8 at 15 °C (Mauro and Mangum, 1982b). Bohr coefficients in general are widely variable among crustacean species (Table 13). Those species that exhibit large Bohr effects seem to live in habitats that are frequently exposed to low oxygen conditions, such as the high intertidal zone. This could be largely due to the fact that animals living in hypoxic conditions often have hemocyanin with a high oxygen affinity, and therefore a large Bohr effect would allow for an efficient release of oxygen to the tissues. It is therefore reasonable that *P. cinctipes* exhibits a larger Bohr effect than *P. eriomerus*.

Hemocyanin cooperativity values in the current study fluctuated between approximately 2.5 and 5.0 in both *Petrolisthes* species. This is similar to values measured in the anomuran *Eupagurus bernhardus* where approximate n_{50} values between 2.5 and 5.5 were observed between pH 7.4 and pH 8.2 (Bridges, 1986). Large

Table 13: Bohr coefficients of various crustacean species demonstrating both the high degree of variability among species and the sensitivity of the Bohr effect to temperature and pH conditions within species.

Species	Temperature (°C)	pH range	Bohr Coefficient	Reference
Astacura				
Nephrops norvegicus	10	7.5 - 8.0*	-1.19	Bridges, 1986
Brachyura				
Atelecyclus rotundatus	10	7.4 - 8.1*	-0.92	Taylor et al., 1985
Corystes cassivelaunus	10	7.2 - 8.0*	-1.48	Bridges, 1986
Cyanagraea praedator	15	7.0 - 7.8*	-1.8	Chausson et al., 2001
Goneplax rhomboides	10	7.4 - 8.1*	-0.59	Taylor et al., 1985
Hemigrapsus nudus	15	7.3 - 8.1*	-0.84	Morris et al., 1996
Hemigrapsus nudus	15	6.6 - 8.2*	-0.71	Nyren, 1989
Liocarcinus depurator	10	7.7 - 8.2*	-1.43	Taylor et al., 1985
Menippe mercenaria	15	7.4 - 7.8	–0.9	Mauro and Mangum, 1982b
Anomura				
Eupagurus bernhardus	10	7.4 - 8.2*	-1.17	Bridges, 1986
Eupagurus bernhardus	15	7.8 - 8.0	–1.55	Jokumsen and Weber, 1982
Galathea strigosa	10	7.4 - 8.2*	–0.96	Bridges, 1986
Petrolisthes eriomerus	13.5	7.6 - 7.9	-1.2	Mangum, 1983b
Petrolisthes eriomerus	15	7.7 - 8.6	-1.07	present study
Petrolisthes cinctipes	15	7.7 - 8.6	-1.25	present study
Amphipoda				
Gammarus locusta	10	7.5 - 8.3*	-1.47	Spicer and Taylor, 1994
Echinogammarus pirloti	10	7.5 - 8.0*	-1.16	Spicer and Taylor, 1994
Echinogammarus marinus	10	7.6 - 8.1*	-1.16	Spicer and Taylor, 1994
Hyale nilsonni	10	7.4 - 7.8*	-1.2	Spicer and Taylor, 1994
Orchestia gammarellus	10	7.4 - 8.1*	-0.77	Spicer and Taylor, 1994

* This denotes approximate values for pH range

differences in cooperativity have also been documented in the shore crab *Hemigrapsus nudus*, with values fluctuating between approximately 2.5 and 4.5 across a pH range of 7.3 to 8.15 (Morris et al., 1996). The lack of a pH effect on cooperativity, as was demonstrated in this study, has been found in many species. The crabs *Bythograea thermydron*, *Atelecyclus rotundatus*, *Goneplax rhomboides*, *Liocarcinus depurator*, and *Hemigrapsus nudus* are among those whose cooperativity is independent of pH (Sanders et al., 1988; Taylor et al., 1985; Morris et al., 1996).

The spectra for *Petrolisthes* at pH 7.7 and pH 7.45 suggest that *Petrolisthes* hemocyanin is undergoing a Root effect at low pHs. A Root effect can be defined as a dramatic decrease in protein oxygen binding capacity induced by low pH (Root, 1931; Dejours, 1981; Miller and Mangum, 1988; Bridges, 1994). A Root effect comes about from a progressive stabilization of the low affinity tense state of the oxygen carrying protein with a concurrent destabilization of the high affinity relaxed state, occurring even at full oxygen saturation (Kuiper et al., 1980, Brittain, 1987). This is characterized by an inability of the protein to achieve full oxygen saturation in air along with a sharp decline in cooperativity at low pH. Hemoglobin from the Atlantic bluefin tuna, *Thunnus thynnus*, demonstrates this process; the oxygen carrying protein remains in the low affinity tense state at low pH even when fully saturated (Brittain, 1987).

A common notion is that the oxygen carrier can exhibit either a true Root effect or an apparent Root effect; in the latter case, the protein actually undergoes an exaggerated Bohr effect and only needs higher oxygen tensions to bring it back to its original oxygen carrying capacity (Root, 1931; Miller and Mangum, 1988; Bridges, 1994). A true Root

effect occurs in some fish that cannot fully saturate their hemoglobin even at pressures as high as 100 atmospheres (Scholander and Van Dam, 1954). An apparent Root effect caused by a very large Bohr effect has been demonstrated in the hemocyanin of *Octopus dofleini*, which will not reach full saturation below pH 7.0 even when exposed to pure oxygen (Miller and Mangum, 1988). There are varying degrees of Root effect among species, and these are often not well defined. The arthropod *Panulirus interruptus* and the molluscs *Buccinum undatum* and *Octopus dofleini* are among the few invertebrate species studied that have demonstrated some form of a Root effect (Kuiper et al., 1980; Brix and Torensma, 1981; Miller and Mangum, 1988).

The physiological role for the Root effect in the well-studied fish hemoglobin can be linked to either gas secretion to the swim bladder or oxygenation of the poorly vascularized retina of many fish species (Ingerman and Terwilliger, 1982; Brittain, 1987). Although Root effects in vertebrate hemoglobins have been studied in great detail, there is relatively less knowledge of it for invertebrate hemocyanins. The physiological significance of a true Root effect or an exaggerated Bohr effect in molluscan and arthropod hemocyanins is unknown. For most intertidal species, the oxygen pressure that would be needed (greater than 1 atmosphere) to distinguish between a true and an apparent Root effect would not occur *in vivo* as it would for the many fish that have exhibited Root effects (Bridges, 1994; Pelster and Weber, 1991).

Whether the inability of *P. eriomerus* hemocyanin to become fully saturated in air at low pH is a true Root effect or solely an exaggerated Bohr effect remains to be seen. Although *P. eriomerus* hemocyanin exhibits a loss of oxygen carrying capacity at low

pH, it does not appear to demonstrate the concurrent decrease in cooperativity. However, the current study examined pHs only as low as pH 7.45 in order to limit the study to the physiological range of oxygen binding responses. Most Root effect studies on teleost fish, where most of the work on Root effect has been concentrated, examine affinity and cooperativity at pH 6.5 or lower. Furthermore, spectrophotometric readings of samples exposed to pure oxygen would be necessary in order to distinguish between Bohr and Root effects (Bridges, 1994). Along with this, the extinction ratio of absorbances at 335 nm and 280 nm would need to be measured at a higher pH in order to extrapolate the low pH extinction ratio back to maximum saturation so that the true percent oxygenation in pure oxygen could be obtained for lower pHs (Miller and Mangum, 1988). Perhaps by examining hemocyanin oxygen affinity in a lower pH range, measuring the affinity in pure oxygen, and extrapolating for true values of percent oxygenation, the question of whether or not *Petrolisthes* hemocyanin demonstrates a Root effect could be answered.

Oxygen Binding: Temperature

In both *Petrolisthes* species, a temperature increase caused a decrease in hemocyanin oxygen affinity as would be expected for arthropod hemocyanins (Redfield, 1934). The extent of this change (ΔH = -40.1 kJ mol^{-1} for *P. eriomerus* and -35.99 kJ mol^{-1} for *P. cinctipes*) was within the widely variable range seen for most crustacean hemocyanins (Table 14). Heat of oxygenation values, which quantify temperature sensitivity, have been found to vary between -158 kJ mol^{-1} and 0 kJ mol^{-1} (Jokumsen et al., 1981; Jokumsen and Weber, 1982), although crustacean hemocyanins normally

exhibit a ΔH between approximately -35 kJ mol^{-1} to -55 kJ mol^{-1} (Mangum, 1980; Morris, 1991). In general, there is an inverse relationship between the value of ΔH and the range of temperatures at which hemocyanin has to function *in vivo*; lower values of ΔH are thought to either be adaptive to or reflect poikilothermic animals living in thermally unstable environments (Greenaway et al., 1992; Toulmond, 1992). Therefore it could be suggested that the lower heat of oxygenation value for *P. cinctipes* reflects the fact that it lives in an environment where there is a high degree of temperature fluctuation. An alternate theory is that differences in oxygen affinity with respect to temperature (i.e. slopes) are relatively constant among species regardless of habitat, while a more important reason for such a varying range of ΔH values has to do with the relationship between pH and temperature (Burnett et al., 1988). The inverse relationship between Bohr effect and temperature dependence allows oxygen binding changes due to temperature fluctuations to be minimized in animals that exhibit large Bohr effects (Burnett et al., 1988). *Petrolisthes* appears to better support the second theory as the two species experience very different thermal fluctuations yet their hemocyanins have very similar ΔH values. A study specifically designed to determine the relationship between hemolymph pH and temperature and their interactions with oxygen affinity would need to be done to help clarify the understanding of the physiological effects of heat of oxygenation in *Petrolisthes*.

Table 14: Heat of oxygenation (ΔH) values for different crustaceans, demonstrating the high variability in values both intraspecifically and interspecifically.

Species	Temperature range (°C)	pH	ΔH (kJ mol $^{-1}$)	Reference
Astacura				
Nephrops norvegicus	10 - 15	7.9	-5.1	Bridges, 1986
Brachyura				
Atelecyclus rotundatus	5 - 15	7.8	-7.2	Taylor et al., 1985
Callinectes sapidus	5 - 15/15 - 25	7.7	-21/-8	Mauro and Mangum, 1982a
Cancer borealis	5 - 15	7.6	-31	Mauro and Mangum, 1982b
Corystes cassivelaunus	10 - 15	7.9	-13.6	Bridges, 1986
Goneplax rhomboides	5 - 15	7.8	–64	Taylor et al., 1985
Goniopsis cruentata	20 - 36	7.9	-47	Young, 1972
Hemigrapsus nudus	5 - 25	7.8/7.4	–30/-45.1	Morris et al., 1996
Hemigrapsus nudus	10 - 25	7.8	-24.3	Nyren, 1989
Hyas araneus	-1.5 - 25	7.9	-20	Morris and Bridges, 1989
Hyas coarctatus	-1.5 - 25	7.9	-22	Morris and Bridges, 1989
Liocarcinus depurator	5 - 15	7.8	–14.5	Taylor et al., 1985
Menippe mercenaria	15 - 25	7.6	-13	Mauro and Mangum, 1982b
Anomura				
Eupagurus bernhardus	10 - 15	7.9	-17.8	Bridges, 1986
Eupagurus bernhardus	5 - 25	7.8/8.0	0	Jokumsen and Weber, 1982
Galathea strigosa	10 - 15	7.9	–18.1	Bridges, 1986
Petrolisthes eriomerus	10 - 30	7.9	⁻40.1	present study
Petrolisthes cinctipes	10 - 30	7.9	⁻35.99	present study
Isopoda				
Glyptonotus antarcticus	0 - 5	8.2	-158.3	Jokumsen et al., 1981

Interestingly, *P. eriomerus* hemocyanin demonstrated normal oxygen binding curves at high temperatures despite the fact that individual crabs can not survive in air at temperatures above 20 °C, and temperatures have not been measured above 25 °C in this species distribution range (Jensen and Armstrong, 1991; Stillman, 1998). Normal oxygen binding curves at high temperatures imply that the hemocyanin protein from this species does not denature and can continue to bind with oxygen at temperatures up to 30 °C. At the same time, the affinity for oxygen at 30 °C was so low (~ 135 mm Hg) in *P. eriomerus* that the hemocyanin could be physiologically non-functional at extreme temperatures.

Temperature had no influence on hemocyanin cooperativity for either *P. eriomerus* or *P. cinctipes* although overall cooperativity values across the temperature range were high for both species. Lack of a temperature effect on cooperativity is not rare; it can be seen in hemocyanins from many different decapods such as *Cyanagraea praedator* (Chausson et al., 2001), *Hemigrapsus nudus* (Morris et al., 1996), *Galathea strigosa*, *Eupagurus bernhardus*, *Corystes cassivelaunus*, *Nephrops norvegicus* (Bridges, 1986) and *Bythograea thermydron* (Sanders et al., 1988), as well as in the isopod *Glyptonotus antarcticus* (Jokumsen et al., 1981). The high, variable cooperativity values found in *Petrolisthes* hemocyanin during temperature manipulations are not unusual either. The crab *Callinectes sapidus* is among those that show variable, high cooperativity values; at pH 7.4 and 25 °C, the n_{50} value is close to 6.0 in this species (Mauro and Mangum, 1982a).

Oxygen Binding: Lactate

Petrolisthes eriomerus hemocyanin had a lower oxygen affinity than *P. cinctipes* during individual lactate experiments at low lactate concentrations within the range of 0 – 8 mM lactate. At higher lactate concentrations, there were no obvious differences in oxygen affinities between the two species. One explanation for this is that the hemocyanin of both species is fully saturated at lactate concentrations higher than 8 mM. Therefore, the difference in lactate sensitivity between these two species can be best expressed at lactate concentrations lower than 8 mM.

The lactate coefficients of -0.32 for *P. eriomerus* and -0.22 for *P. cinctipes* are both moderate, falling within the wide range of known values (Table 15). The lactate coefficient for most decapod crustaceans falls between -0.04 and -0.56 (Bridges and Morris, 1986; Bridges et al., 1984). In general, the adaptation to a more terrestrial way of life is correlated with a decrease in the sensitivity of hemocyanins to lactate and therefore a smaller lactate coefficient (Morris and Bridges, 1994; Morris et al., 1996). Although the difference is not pronounced, *P. eriomerus* is more sensitive to lactate than *P. cinctipes*, suggesting a possible advantage for *P. cinctipes* to live in a habitat where aerial exposure is more frequent. A possible explanation for the similar lactate coefficients would be that the normal range of lactate concentrations does not differ greatly between the two species. Although *P. cinctipes* lives in higher intertidal zones that are potentially hypoxic, their leg membranes allow lactate levels to remain at a minimum for at least 5 hours during emersion (Stillman and Somero, 1996). Due to this, the normal *in vivo*

lactate levels may not differ greatly between these two species, providing an explanation as to why the lactate coefficients do not exhibit a pronounced difference.

Table 15: Lactate coefficients for hemocyanins of various decapod crustaceans.

Species	Temperature (°C)	pH	Lactate effect	Reference
Brachyura				
Atelecyclus rotundatus	10	7.8	-0.33	Taylor et al., 1985
Cancer magister	13.5	7.8	-0.25	Graham et al., 1983
Cancer pagurus	15	7.9	-0.21	Truchot, 1980
Carcinus maenas	15	7.8	-0.096	Truchot, 1980
Goneplax rhomboides	10	7.8	-0.18	Taylor et al., 1985
Hemigrapsus nudus	15	7.7	–0.1	Nyren, 1989
Hyas araneus	2/25	7.8	–0.50/-0.30	Morris and Bridges, 1989
Liocarcinus depurator	10	7.8	-0.29	Taylor et al., 1985
Maia squinado	15	7.8	-0.13	Bridges et al., 1984
Caridea				
Palaemon elegans	10	7.8	-0.56	Bridges et al., 1984
Anomura				
Petrolisthes eriomerus	15	7.9	-0.32	Present study
Petrolisthes cinctipes	15	7.9	-0.22	Present study

Both *P. eriomerus* and *P. cinctipes* demonstrated an increase in hemocyanin oxygen affinity as lactate levels increased, as is found in many crustaceans (ex. Truchot, 1980; Booth et al., 1982; Graham et al., 1983; Johnson et al., 1984; Bouchet and Truchot, 1985; Lallier and Truchot, 1989b; Spicer and Taylor, 1994). In addition, they both showed the normal response of an asymptotic decrease in lactate sensitivity.

Because L-lactate is an allosteric effector, it has been suggested that it is likely to affect the cooperativity of hemocyanin (Johnson et al., 1984). At the same time, the

hemocyanin of many crustaceans studied thus far have exhibited no cooperativity response to lactate. Species whose hemocyanins follow this trend include *Squilla empusa*, *Homarus americanus*, *Procambarus clarki*, *Sicyonia ingentis*, *Emerita talpoida*, *Panulirus interuptus* (Mangum, 1983a), *Palaemon elegans* (Bridges et al., 1984), *Goneplax rhomboides* (Taylor et al., 1985), *Hemigrapsus nudus* (Nyren, 1989), *Petrolisthes eriomerus* and *Petrolisthes cinctipes*.

A future study combining the effects of low lactate levels and varying pH could be done to determine the physiological significance of a low pH effect on *Petrolisthes*. It would be interesting to see if the low affinity response that was seen in this study during very low pHs can be offset by low lactate levels. Perhaps the rare emersion or exposure to hypoxic waters in subtidal *P. eriomerus*, the ability of *P. cinctipes* to undergo aerobic respiration at high temperatures during extended emersion (Stillman, 1998; Stillman and Somero, 1996), and the inactive lifestyles of both *P. eriomerus* and *P. cinctipes*, allow only a small amount of lactic acid to accumulate in the blood of these species. During normal physiological conditions, this acidosis may be small enough that lactate levels can offset any resultant changes in oxygen affinity.

Oxygen Binding: Salinity

Responses to changes in salinity vary among crustacean species, both behaviorally and physiologically. A behavioral way to cope with varying salinity levels has been shown in the porcelain crab, *Porcellana platycheles*. The osmoreceptors located on the walking legs of this crab allow it to move along a salinity gradient in order to

avoid salinities below 10.2 ‰ (Davenport, 1972; Davenport and Wankowski, 1973). In many ways *Porcellana platycheles* is similar to *Petrolisthes cinctipes* while its congener, *Porcellana longicornis* shares similar features to *Petrolisthes eriomerus*. For instance, both *P. cinctipes* and *P. platycheles* occur under rocks in higher intertidal zones and are vulnerable to desiccation during low tide (Davenport and Wankowski, 1973). On the other hand, *P. eriomerus* and *P. longicornis* are both found in low intertidal and subtidal zones and are rarely found away from standing water (Davenport, 1972). Higher intertidal *P. platycheles* exhibits a wider tolerance to salinities than the stenohaline *P. longicornis* (Davenport, 1972); it would be interesting to see if a similar difference occurred between *P. eriomerus* and *P. cinctipes*.

In addition to behavioral responses, crustaceans can deal with salinity fluctuations by osmoconforming and/or osmoregulating. For instance, *Cancer antennarius*, commonly found under rocks in the lower intertidal, is a known osmoconformer (Jones, 1941), but it can strongly regulate specific ions such as Ca^{++} and Mg^{++} (Freel, 1978). Alternatively, *Hemigrapsus nudus* is an osmoconformer at normal and high salinities but an osmoregulator at low salinities (Jones, 1941). In contrast, the anomurans *Neotrypaea [Callianassa] californiensis* and *Emerita analoga* are strict osmoconformers (Freel, 1978). It is generally believed that porcelain crabs as a whole tend to conform to external salinities (Smaldon, 1973; Jones and Greenwood, 1982). For example, Hunter and Kirschner (1986) demonstrated that *P. cinctipes* conformed to external salinities between 60% and 100% sea water.

Another typical reaction to reduced salinity for most decapod crustaceans is a measurably lower hemocyanin oxygen affinity. The effect of Mg^{++} on oxygen affinity is similar; a decrease in the concentration of this divalent cation causes a decrease in hemocyanin oxygen affinity. Both total salinity and magnesium effects have been demonstrated in the hemocyanins of the brachyurans, *Callinectes sapidus* (Mason et al., 1983), *Carcinus maenas* (Truchot, 1975), *Cancer magister* (Terwilliger and Brown, 1993), the isopod, *Saduria entomon* (Hagerman and Vismann, 2001), and now the anomurans *P. eriomerus* and *P. cinctipes*, as well as in other crustaceans. Most crabs respond to dilute salinities by undergoing hyperventilation, which requires high levels of ATP and therefore a large oxygen demand at the tissue level. Due to this, it is advantageous for a crab to have a low oxygen affinity under these low salinity conditions. Magnesium and calcium cations are believed to be largely responsible for the total salinity effects on oxygen binding (Truchot, 1992), so it is not surprising that the effects of Mg^{++} and total salinity on oxygen binding were similar in this study.

Magnesium cations are often closely regulated in crustaceans because high Mg^{++} levels can block neuromuscular transmission (Katz, 1936), yet magnesium is required for proper assembly of hemocyanin molecules (Truchot, 1992). *Petrolisthes eriomerus* and *P. cinctipes* did not differ from one another in hemocyanin oxygen affinity across the range of magnesium concentrations tested. Because Mg^{++} is often highly regulated, it is not surprising that there was no significant difference between *P. eriomerus* and *P. cinctipes* in either their hemolymph Mg^{++} concentrations or their oxygen affinities across the range of Mg^{++} tested.

A cooperativity independence within the physiological range of total salinity is another effect that has been seen in the hemocyanin of many decapods (Mangum, 1983b; Mason et al., 1983; Miller and Van Holde, 1981), including *P. eriomerus* and *P. cinctipes*. Hemocyanin cooperativity in crustaceans has been shown to be largely independent of magnesium as well. For example, differences in magnesium concentrations have no effect on the hemocyanin cooperativities of adult *Cancer magister* (Terwilliger and Brown, 1993), *Callinectes sapidus* (Mason et al., 1983), *Hemigrapsis nudus* (Morris et al., 1996) or *P. eriomerus*. *Petrolisthes cinctipes* demonstrated a decrease in cooperativity as total salinity increased, although this was not significant ($p = 0.06$).

Hemocyanin Structure

There were several clear differences when comparing the hemocyanin structure of *P. eriomerus* and *P. cinctipes*. The banding patterns and the size of subunits that emerged on SDS-PAGE were distinct for each species. *Petrolisthes eriomerus* hemocyanin is made up of subunits with molecular weights ranging from approximately 79,000 Da to 90,000 Da while the molecular weight of *P. cinctipes* subunits range from about 76,000 Da to 87,000 Da. The pH 7.4 PAGE demonstrated that the 16S band for *P. eriomerus* migrated faster than that of *P. cinctipes*.

The pH 8.9 PAGE also showed distinct differences in banding patterns between the two species, although comparison between species was inhibited during replicate experiments. In samples obtained in late April, a lack of a distinct banding pattern

occurred for hemolymph of both *Petrolisthes* species. Distinct bands were either absent or obscured by a strong smear that spanned nearly the entire length of the lane. The early June hemolymph samples for pH 8.9 PAGE resulted in several samples demonstrating the original banding pattern and others showing the strong protein smear, for both *Petrolisthes* species. The variation in these gels are most likely due to seasonal differences such as molting and/or reproduction events. The original pH 8.9 PAGE data was gathered in early March, early in the breeding season for *P. eriomerus* and *P. cinctipes*. For *P. eriomerus*, brood laying occurs from February to mid-April and/or mid-May to August, and hatching occurs from May through October; this appears to be similar for *P. cinctipes* (Strathmann, 1987).

In an attempt to clarify this phenomenon, hemolymph samples from crabs showing the strong smear and crabs with the original pH 8.9 patterning were run on pH 7.4 PAGE. In both species, the hemolymph samples that demonstrated a smear on pH 8.9 PAGE also had high protein concentrations of several slowly migrating bands present on pH 7.4 PAGE. Several of these slowly migrating bands from each species were subsequently excised and characterized on SDS-PAGE, resulting in a unique high molecular weight band around 109 kDa in size. It is possible that the strong smear on the pH 8.9 PAGE, the slowly migrating bands on the pH 7.4 PAGE and the high molecular weight band on the SDS-PAGE are due to vitellogenins, which are often found in the hemolymph during the reproductive cycle of females. Supporting this, the hemolymph of crabs demonstrating the above phenomenon was a dark red/orange, while hemolymph from other crabs was clear. The distinct orange in the hemolymph is almost certainly due

to carotenoid-containing lipoproteins, of which vitellogenins are representative (Kerr, 1969). Other carotenoid-containing proteins used in the formation of exoskeleton can be found in the hemolymph of both male and female crabs during certain phases of the molt cycle (Gilchrist and Lee, 1972). Further work specifically designed to examine the seasonal variations in hemolymph proteins as well as the interactions between hemocyanin and other hemolymph proteins would need to be done in order to understand the variable protein patterns that were seen in this study.

Although there were clear differences between the structures of these two *Petrolisthes* species in every technique employed, the specific effects that these differences have on the oxygen binding properties of hemocyanin are less clear. Future work of x-ray crystallography or sequencing the hemocyanin macromolecule might help clarify exactly how the differences in polypeptide chains are related to the intrinsic differences in oxygen binding properties.

Conclusions

Petrolisthes eriomerus and *P. cinctipes* are adapted to very different environmental conditions in spite of their close proximity to one another. Neither species extends far beyond its normal vertical range for various reasons. The oxygen transport system often fails near the limits of a species range (Mangum, 1980), suggesting that the properties of a hemocyanin macromolecule are important in determining where an intertidal crab can successfully inhabit. Differences in the structure and function of hemocyanins of these two closely related species appear to be an important reason as to

why zonation patterns have been sustained as well as a possible reason for their original formation. *Petrolisthes eriomerus* and *P. cinctipes* exhibited many similar oxygen binding characteristics, but those of *P. cinctipes* were more consistent with a species adapted to the extremely variable environment of the high intertidal zone. As Bridges (2001) noted, the substantial amount of oxygen binding data that is now present demonstrates how important even small changes in oxygen affinity can be to fine-tune a hemocyanin molecule so that it is most efficient in its natural environment. In the current study, *P. cinctipes* tended to have an overall higher oxygen affinity than *P. eriomerus* throughout each of the experimental regimes, indicative of a relatively inactive species that regularly experiences low oxygen conditions (McMahon, 1988; Truchot, 1992). Because *P. cinctipes* lives in a potentially hypoxic environment, it is advantageous for it to have a high affinity hemocyanin in order to more easily extract oxygen from the environment. It is also an advantage for *P. cinctipes* to have a large Bohr effect, as it is necessary for hemocyanin to release oxygen at the tissue level despite a high overall oxygen affinity. Low intertidal/ subtidal *P. eriomerus* had a lower overall oxygen affinity than *P. cinctipes*; as oxygen is not limiting in the natural habitat of *P. eriomerus*, a low overall affinity can pick up oxygen at the gill level and allow oxygen to be easily released at the tissue level. *Petrolisthes eriomerus* had a smaller lactate coefficient, indicative of it undergoing anaerobic respiration less frequently than the high intertidal *P. cinctipes*. *Petrolisthes eriomerus* hemocyanin was more temperature sensitive than that of *P. cinctipes*, and demonstrated a severely low affinity at high temperatures; these both suggest that *P. eriomerus* does not often experience widely fluctuating temperatures in its

habitat. All of the results from this study suggest that the hemocyanin molecules of *P. eriomerus* and *P. cinctipes* are well suited for the specific environmental stresses that each species is exposed to in its respective habitat.

Although well studied, the exact physiological significance of cooperativity is still unclear in arthropod hemocyanins (Truchot, 1992). Due to this, no direct conclusions can be made from the lack of a significant difference in cooperativities between these two *Petrolisthes* species. The hemocyanins of both species were highly cooperative but none of the tested environmental stresses significantly affected their cooperativities; whether or not this reflects their suitability to their intertidal environment remains to be seen.

The high interindividual variability in both protein concentration and oxygen capacity that is often seen in arthropods is due to the fact that hemocyanin is not only an oxygen carrier but is also an organic reserve, as can be seen by the steady decrease in hemocyanin concentrations in nutritionally deprived organisms (Truchot, 1992). Nutritional factors and therefore hemocyanin concentrations and oxygen capacities fluctuate seasonally. It would therefore be quite interesting to see how the hemocyanin oxygen affinity and cooperativity in individuals of *P. eriomerus* and *P. cinctipes* change seasonally. One way to examine this variability would be to use a methodology that would allow for small hemolymph samples and therefore individual rather than pooled samples to be examined.

Since physicochemical factors such as those discussed in this study are inherently intertwined with one another, major ecosystem changes will influence these factors in potentially unpredictable ways. The impact these changing factors will have on the

physiological capabilities of intertidal species such as *Petrolisthes* could cause dramatic changes in their distribution ranges. Species of the intertidal habitat are not fixed in either their distributions or their ecosystem roles; reflecting the habitat in which they live, their position in the ecosystem is in a state of flux.

BIBLIOGRAPHY

Alexandrov, V. I. A. 1977. Cells, molecules and temperature: conformational flexibility of macromolecules and ecological adaptation. Springer-Verlag, New York. 330 pp.

Benesch, R., G. MacDuff, and R. E. Benesch. 1965. Determination of oxygen equilibria with a versatile new tonometer. —Analytical Biochemistry, 11: 81-87.

Booth, C. E., B. R. McMahon, and A. W. Pinder. 1982. Oxygen uptake and the potentiating effects of increased hemolymph lactate on the oxygen transport during exercise in the blue crab, *Callinectes sapidus*. —Journal of Comparative Physiology B, 148: 111-121.

Bouchet, J. Y., and J. P. Truchot. 1985. Effects of hypoxia and L-lactate on the haemocyanin-oxygen affinity of the lobster, *Homarus vulgaris*. —Comparative Biochemistry and Physiology, 80A: 69-73.

Boyland, E. 1928. Chemical changes in muscle. Part II. Invertebrate muscle. — Biochemical Journal, 22: 362-376.

Bridges, C. R. 1986. A comparative study of the respiratory properties and physiological function of haemocyanin in two burrowing and two non-burrowing crustaceans. — Comparative Biochemistry and Physiology, 83A: 261-270.

——. 1994. Bohr and Root effects in cephalopod haemocyanins – paradox or pressure in *Sepia officinalis*? ——Marine And Freshwater Behaviour And Physiology, 25: 121-130.

——. 2001. Modulation of haemocyanin oxygen affinity: properties and physiological implications in a changing world. —Journal of Experimental Biology, 203: 1021-1032.

——, and A. R. Brand. 1980. The effect of hypoxia on oxygen consumption and blood Lactate levels of some marine crustacea. —Comparative Biochemistry and Physiology, 65A: 399-409.

——, and S. Morris. 1986. Modulation of haemocyanin oxygen affinity by L-lactate – A role for other cofactors. Pp. 373-429 *in* B. Linzen, ed. Invertebrate Oxygen Carriers. Springer-Verlag, Berlin.

——, ——, and M. K. Grieshaber. 1984. Modulation of haemocyanin oxygen affinity in the intertidal prawn *Palaemon elegans* (Rathke). —Respiration Physiology, 57: 189-200.

Brittain, T. 1987. The Root effect. —Comparative Biochemistry and Physiology, 86B: 473-481.

Brix, O.; Torensma, R. 1981. The molecular basis for the reversed Root effect in the blood of the marine prosobranch *Buccinum undatum*. —Molecular Physiology, 1:209-212.

Brown, A. C. 1991. Effects of salinity and temperature on the respiratory physiology of the Dungeness crab, *Cancer magister*, during development. —Ph.D. dissertation, University of Oregon, Eugene, Oregon. 183 pp.

Burnett, L. 1992. Integrated function of the respiratory pigment hemocyanin in crabs. —American Zoologist, 32: 438-446.

——, D. Scholnick, and C. Mangum. 1988. Temperature sensitivity of molluscan and arthropod hemocyanins. —Biological Bulletin, 174: 153-162.

Chausson, F., C. R. Bridges, P. M. Sarradin, B. N. Green, R. Riso, J. C. Caprais, and F. H. Lallier. 2001. Structural and functional properties of hemocyanin from *Cyanagraea praedator*, a deep-sea hydrothermal vent crab. —Proteins, 45: 351-359.

Davenport, J. 1972. Salinity tolerance and preference in the porcelain crabs, *Porcellana platycheles* and *Porcellana longicornis*. —Marine Behaviour and Physiology, 1: 123-138.

——, and J. Wankowski. 1973. Pre-immersion salinity-choice behaviour in *Porcellana platycheles*. —Marine Biology, 22: 313-316.

Davis, B. 1964. Disc gel electrophoresis. II. Method and application to human serum proteins. —Annals. New York Academy of Sciences, 12: 404-427.

deFur, P. L., B. R. McMahon, and C. E. Booth. 1983. Analysis of hemolymph oxygen levels and acid-base status of *Cancer productus* Randall during emersion *in situ*. —Biological Bulletin, 165: 582-590.

Dehnel, P. A. and T. H. Carefoot. 1965. Ion regulation in two species of intertidal crabs. —Comparative Biochemistry and Physiology, 15: 377-397.

Dejours, P. 1981. Principles of comparative respiratory physiology. Elsevier North-Holland Biomedical Press, New York, NY. 253 pp.

——, and H. Beekenkamp. 1978. Acid-base balance during molting in the crayfish. — Comptes rendus hebdomadaires des séances de l'Académie des sciences, 286: 1895-1898.

Doty, M. S. 1946. Critical tide factors that are correlated with the vertical distribution of marine algae and other organisms along the Pacific coast. —Ecology, 27: 315-328.

Fields, P. A. 2001. Review: protein function at thermal extremes: balancing stability and flexibility. —Comparative Biochemistry and Physiology Part A, 129: 417-431.

Freel, R. W. 1978. Patterns of water and solute regulation in the muscle fibers of osmoconforming marine decapod crustaceans. —Journal of Experimental Biology, 72: 107-126.

Gilchrist, B. M., and W. L. Lee. 1972. Carotenoid pigments and their possible role in reproduction in the sand crab, *Emerita analoga* (Stimpson, 1857). —Comparative Biochemistry and Physiology, 42B: 263-294.

Graham, R. A., C. P. Mangum, R. C. Terwilliger, and N. B. Terwilliger. 1983. The effect of organic acids on oxygen binding of hemocyanin from the crab *Cancer magister*. —Comparative Biochemistry and Physiology, 74A: 45-50.

Greenaway, P., S. Morris, N. Sanders, and A. Adamczewska. 1992. Blood gas transport and oxygen consumption in a supralittoral crab, *Leptograpsus variegatus* (Crustacea: Brachyura). —Australian Journal of Marine and Freshwater Research, 43: 1573-1584.

Hagerman, L., and B. Vismann. 2001. Modulators of haemocyanin oxygen affinity in the hypoxia and sulphide tolerant Baltic isopod *Saduria entomon* (L.). —Journal of Comparative Physiology B, 171: 695-699.

Haig, J. 1960. The Porcellanidae (Crustacea Anomura) of the Eastern Pacific. University of Southern California Press, Los Angeles, California. 440 pp.

Helmuth, B., and G. E. Hofmann. 2001. Microhabitats, thermal heterogeneity, and patterns of physiological stress in the rocky intertidal zone. —Biological Bulletin, 201:374-384.

Henry, R. P., G. A. Kormanik, N. J. Smatresk, and J. N. Cameron. 1981. The role of $CaCO_3$ dissolution as a source of HCO_3^- for the buffering of hypercapnic acidosis in aquatic and terrestrial decapod crustaceans. —Journal of Experimental Biology, 94:269-274.

Hunter, K. C. and L. B. Kirschner. 1986. Sodium absorption coupled to ammonia excretion in osmoconforming marine invertebrates. —American Journal of Physiology, 250: R957-R962.

Hutchins, L. W. 1947. The bases for temperature zonation in geographical distribution. —Ecological Monographs, 17: 325-335.

Ingermann, R. L. and R. C. Terwilliger. 1982. Presence and possible function of Root effect hemoglobins in fishes lacking functional swim bladders. —Journal of Experimental Zoology, 220: 171-177.

Jensen, G. C. and D. A. Armstrong. 1991. Intertidal zonation among congeners: factors regulating distribution of porcelain crabs *Petrolisthes* spp. (Anomura: Porcellanidae). —Marine Ecology Progress Series, 73: 47-60.

Johnson, B. A., C. Bonaventura, and J. Bonaventura. 1984. Allosteric modulation of *Callinectes sapidus* hemocyanin by binding of L-lactate. —Biochemistry, 23: 872-878.

Jokumsen, A., R. M. G. Wells, H. D. Ellerton, and R. E. Weber. 1981. Hemocyanin of the giant Antarctic isopod, *Glyptonotus antarcticus*: structure and effects of temperature and pH on its oxygen affinity. —Comparative Biochemistry and Physiology, 70A: 91-95.

——, and R. E. Weber. 1982. Hemocyanin-oxygen affinity in hermit crab blood is temperature independent. —The Journal of Experimental Zoology, 221: 389-394.

Jones, L. L. 1941. Osmotic regulation in several crabs of the Pacific coast of North America. —Journal of Cellular and Comparative Physiology, 18: 79-92.

——, and J. G. Greenwood. 1982. Water loss of a porcelain crab, *Petrolisthes elongates* (Milne Edwardes, 1837) (Decapoda, Anomura) during atmospheric exposure. —Comparative Biochemistry and Physiology, 72A:631-636.

Katz, B. 1936. Neuromuscular transmission in crabs. —Journal of Physiology, London, 187: 199-220.

Kerr, M. S. 1969. The hemolymph proteins of the blue crab, *Callinectes sapidus*. II. A lipoprotein serologically identical to oocyte lipovitellin. —Developmental Biology, 20: 1-17.

Kuiper, H. A., M. Coletta, L. Zolla L, E. Chiancone, and M. Brunori. 1980. Root effect of *Panulirus interruptus* hemocyanin. —Biochimica et Biophysica Acta, 626: 412-416.

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

Lallier, F. and J. P. Truchot. 1989a. Hemolymph oxygen transport during environmental hypoxia in the shore crab, *Carcinus maenas*. —Respiration Physiology, 77: 323-336.

——, and ——. 1989b. Modulation of haemocyanin oxygen-affinity by L-lactate and urate in the prawn *Penaeus japonicus*. —Journal of Experimental Biology, 147: 133-146.

——, F. Boitel, and J. P. Truchot. 1987. The effect of ambient oxygen and temperature on haemolymph L-lactate and urate concentrations in the shore crab *Carcinus maenas*. —Comparative Biochemistry and Physiology, 86A: 255-260.

Mangum, C. P. 1980. Respiratory function of the hemocyanins. —American Zoologist, 20: 19-38.

——. 1983a. On the distribution of lactate sensitivity among the hemocyanins. — Marine Biology Letters, 4: 139-149.

——. 1983b. Oxygen transport in the blood. Pp. 373-429 *in* L. H. Mantel, ed. The Biology of Crustacea. Vol. V: Internal Anatomy and Physiological Regulation. Academic Press, New York.

Markl, J., and H. Decker. 1992. Molecular structure of the arthropod hemocyanins. Pp. 325-376 *in* C. P. Mangum, ed. Blood and Tissue Oxygen Carriers, Advances in Comparative and Environmental Physiology. Springer-Verlag, Berlin.

Mason, R. P., C. P. Mangum, and G. Godette. 1983. The influence of inorganic ions and acclimation salinity on hemocyanin-oxygen binding in the blue crab *Callinectes sapidus*. —Biological Bulletin, 164: 104-123.

Mauro, N. A., and C. P. Mangum. 1982a. The role of the blood in the temperature dependence of oxidative metabolism in decapod crustaceans. I. Intraspecific responses to seasonal differences in temperature. —The Journal of Experimental Zoology, 219: 179-188.

——, and ——. 1982b. The role of the blood in the temperature dependence of oxidative metabolism in decapod crustaceans. II. Interspecific adaptations to latitudinal changes. —The Journal of Experimental Zoology, 219: 189-195.

McMahon, B. R. 1985. Functions and functioning of crustacean hemocyanin. Pp. 35-58 *in* J. Lamy, J. P. Truchot, and R. Gilles, eds. Respiratory Pigments in Animals: Relation, Structure, Function. Springer-Verlag, Berlin.

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

——. 1988. Physiological responses to oxygen depletion in intertidal animals. — American Zoologist, 28: 39-53.

Miller, K. I. and C. P. Mangum. 1988. An investigation of the nature of Bohr, Root, and Haldane effects in *Octopus dofleini* hemocyanin. —Journal of Comparative Physiology, B, 158: 547-552.

——, and K. E. Van Holde. 1981. The effect of environmental variables on the structure and function of hemocyanin from *Callianassa californiensis*. —Journal of Comparative Physiology, 143: 253-260.

Monod, J., J. Wyman, and J. P. Changeux. 1965. On the nature of allosteric transition: a plausible model. —Journal of Molecular Biology, 12: 88-118.

Morris, S. 1990. Organic ions as modulators of respiratory pigment function during stress. —Physiological Zoology, 63: 253-287.

——. 1991. Respiratory gas exchange and transport in crustaceans: ecological determinants. —Memoirs of the Queensland Museum, 31: 241-261.

——, and C. R. Bridges. 1989. Interactive effects of temperature and L-lactate on the binding of oxygen by the hemocyanin of two Arctic boreal crabs, *Hyas araneus* and *Hyas coarctatus*. —Physiological Zoology, 62: 62-82.

——, and ——. 1994. Properties of respiratory pigments in bimodal breathing animals: air and water breathing by fish and crustaceans. —American Zoologist, 34: 216-228.

——, P. Greenaway, and B. R. McMahon. 1996. Air breathing by the purple shore crab, *Hemigrapsus nudus* (Dana). III. Haemocyanin function in respiratory gas transport. — Physiological Zoology, 69: 839-863.

—— and A. C. Taylor. 1983. Diurnal and seasonal variation in physicochemical conditions within intertidal rock pools. —Estuarine, Coastal and Shelf Science, 17: 339-355.

Nyren, P. 1989. Effects of temperature, salinity, and L-lactate on hemocyanin oxygen binding in the purple shore crab *Hemigrapsus nudus*. —MS thesis, University of Oregon, Eugene, Oregon. 55 pp.

Pelster, B. and R. E. Weber. 1991. The physiology of the Root effect. Pp. 51-77 *in* R. Gilles, ed. Advances in Comparative and Environmental Physiology. Springer-Verlag, New York.

Redfield, A. C. 1934. The haemocyanins. —Biological Reviews, 9: 175-212.

Redmond, J. R. 1968. The respiratory function of hemocyanins. Pp. 5-23 *in* F. Ghiretti, ed. Physiology and Biochemistry of Haemocyanins. Academic Press, New York.

Root, R. W. 1931. The respiratory function of the blood of marine fishes. —Biological Bulletin, 61: 427-456.

Sabourin, T. D. 1984. The relationship between fluctuating salinity and oxygen delivery in adult blue crabs. —Comparative Biochemistry and Physiology, 78A: 109-118.

Sanders, N. K., A. J. Arp, and J. J. Childress. 1988. Oxygen binding characteristics of the hemocyanins of two deep-sea hydrothermal vent crustaceans. —Respiration Physiology, 71: 57-68.

Sartoris, F. J. and H. O. Portner. 1997. Increased concentrations of haemolymph Mg^{++} protect intracellular pH and ATP levels during temperature stress and anoxia in the common shrimp *Crangon crangon*. —The Journal of Experimental Biology, 200: 785-792.

Schmidt-Nielsen, K. 1997. Animal physiology: adaptation and environment. Cambridge University Press, New York. 607 pp.

Scholander, P. F. and L. Van Dam. 1954. Secretion of gases against high pressures in the swimbladder of deep sea fishes. I. Oxygen dissociation in blood. —Biological Bulletin, 107: 247-259.

Sky-Peck, H. H. 1964. Determination of Mg^{++} in serum and urine. —Clinical Chemistry, 10: 391-398.

Smaldon, G. 1973. Osmoregulation in *Pisidia longicornis* (L.) and *Porcellana platycheles* (Pennant) (Decapoda, Anomura) subjected to reduced salinities. —Comparative Biochemistry and Physiology, 44A: 893-895.

Spicer, J. I. and A. C. Taylor. 1994. Oxygen-binding by haemocyanins from an ecological series of amphipod crustaceans. —Marine Biology, 120: 231-237.

Stillman, J. 1998. A comparative analysis of morphological, physiological, and biochemical adaptation to abiotic stress in intertidal porcelain crabs, genus *Petrolisthes*. —Ph.D. dissertation, Oregon State University, Corvallis. 184 pp.

——. 2000. Evolutionary history and adaptive significance of respiratory structures on the legs of intertidal porcelain crabs, genus *Petrolisthes*. —Physiological and Biochemical Zoology, 73: 86-96.

——, and C. Reeb. 2001. Molecular phylogeny of eastern Pacific porcelain crabs, genera *Petrolisthes* and *Pachycheles*, based on the mtDNA 16S rDNA sequence: phylogeographic and systematic implications. —Molecular Phylogenetics and Evolution, 19: 236-245.

——, and G. Somero. 1996. Adaptation to temperature stress and aerial exposure in congeneric species of intertidal porcelain crabs (genus *Petrolisthes*): correlation of physiology, biochemistry and morphology with vertical distribution. —Journal of Experimental Biology, 199: 1845-1855.

—— and ——. 2000. A comparative analysis of the upper thermal tolerance limits of eastern Pacific porcelain crabs, genus *Petrolisthes*: influences of latitude, vertical zonation, acclimation, and phylogeny. —Physiological and Biochemical Zoology, 73: 200-208.

Strathmann, M. F. 1987. Reproduction and Development of Marine Invertebrates of the Northern Pacific Coast. University of Washington Press, Seattle. 670 pp.

Svedberg, T. 1933. Sedimentation constants, molecular weights and isoelectric points of the respiratory proteins. —Journal of Biological Chemistry, 103: 311-325.

Taylor, A. C. 1988. The ecophysiology of decapods in the rock pool environment. —Symposium Zoological Society of London, 59: 227-261.

——, C. M. Astall, and R. J. Atkinson. 2000. A comparative study of the oxygen transporting properties of the haemocyanin of five species of thalassinidean mud-shrimps. —Journal of Experimental Marine Biology and Ecology, 244: 265-283.

——, S. Morris, and C. R. Bridges. 1985. Oxygen and carbon dioxide transporting properties of the blood of three sublittoral species of burrowing crab. —Journal of Comparative Physiology B, 155: 733-742.

——, and J. I. Spicer. 1988. Functional significance of a partial-emersion response in the intertidal prawn *Palaemon elegans* (Crustacea: Palaemonidae) during environmental hypoxia. —Marine Ecology Progress Series, 44: 141-147.

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. —Journal of Experimental Biology, 183: 1-13.

——, and R. C. Terwilliger. 1982. Changes in the subunit structure of *Cancer magister* hemocyanin during larval development. —Journal of Experimental Zoology, 221: 181-191.

Toulmond, A. 1992. Properties and functions of extracellular heme pigments. Pp. 231-256 *in* C. P. Mangum, ed. Blood and Tissue Oxygen Carriers, Advances in Comparative and Environmental Physiology. Springer-Verlag, Berlin.

Truchot, J. P. 1975. Factors controlling the *in vitro* and *in vivo* oxygen affinity of the hemocyanin in the crab *Carcinus maenas*. —Respiration Physiology, 24: 173-189.

——. 1980. Lactate increases the oxygen affinity of crab hemocyanin. —Journal of Experimental Zoology, 214: 205-208.

——. 1983. Regulation of acid-base balance. Pp. 431-457 *in* L. H. Mantel, ed. The Biology of Crustacea. Vol. V: Internal Anatomy and Physiological Regulation. Academic Press, New York.

——. 1988. Problems of acid-base balance in rapidly changing intertidal environments. —American Zoologist, 28: 55-64.

——. 1992. Respiratory function of arthropod hemocyanins. Pp. 377-410 *in* C. P. Mangum, ed. Blood and Tissue Oxygen Carriers, Advances in Comparative and Environmental Physiology. Springer-Verlag, Berlin.

——, and A. Duhamel-Jouve. 1980. Oxygen and carbon dioxide in the marine intertidal environment: diurnal and tidal changes in rockpools. —Respiration Physiology, 39: 241-254.

Weiland, A. L. and C. P. Mangum. 1975. The influence of environmental salinity on hemocyanin function in the blue crab *Callinectes sapidus*. —Journal of Experimental Zoology, 193: 265-274.

Wheatly, M. G. 1988. Integrated responses to salinity fluctuation. —American Zoologist, 28: 65-77.

Young, 1972. The physiological ecology of haemocyanin in some selected crabs. II. The characteristics of haemocyanin in relation to terrestrialness. —Journal of Experimental Marine Biology and Ecology, 10: 193-206.