AN ABSTRACT OF THE THESIS OF

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Abstract approved: _____ George N. Somero

Organismal tolerance to abiotic environmental stresses contributes significantly to setting the distribution limits of organisms, as demonstrated by vertical zonation patterns in the marine intertidal zone. In this thesis, the ultimate (evolutionary) and proximate (mechanistic) causes of tolerance to temperature and emersion stresses associated with the intertidal zone were examined using porcelain crabs, genus *Petrolisthes*. Species of *Petrolisthes* from intertidal and subtidal microhabitats of four biogeographic regions of the Eastern Pacific were used in phylogenetically-based comparative analyses of morphological, physiological, and biochemical adaptation to environmental stress. A phylogenetic tree based on the sequence of the 16sRNA gene was developed to facilitate these analyses. Organismal thermal tolerance limits are adapted to match maximal microhabitat temperatures. Acclimation of thermal tolerance limits suggests that temperate intertidal zone species are living close to their thermal maximum in nature. Respiratory responses to emersion vary among species from different vertical zones. Experimental examination of oxygen consumption rates and lactate accumulation during emersion suggests that intertidal species are able to respire in air using thin membranous

regions on the ventral meral segments of their legs (leg membranes). Leg membrane size is positively correlated with body size across species, but not within a single species. Evolutionary analyses indicate that leg membranes may not have evolved for purposes of aerial respiration, but their presence may have allowed intertidal and subtidal species to achieve larger body sizes and higher metabolic rates. The thermal stabilities of an enzyme, lactate dehydrogenase (LDH), from 22 species of *Petrolisthes* varied widely, but were not correlated with maximal habitat temperatures. Comparative analyses did not indicate any evolutionary relationship between LDH thermal stability and microhabitat conditions. Experimental evidence suggests that interspecific differences in LDH stability are genetically based, and are due both to intrinsic properties of the LDH molecules and extrinsic protein stabilizers. Elucidation of the mechanism(s) of LDH stabilization in *Petrolisthes* may provide novel insight to the field of protein stabilization. These results studies suggest that individual traits may be subjected to differing levels of selection, and thus the analysis of environmental adaptation requires careful consideration of the biological significance of the traits being examined.

A Comparative Analysis of Morphological, Physiological, and Biochemical Adaptation to Abiotic Stress in Intertidal Porcelain Crabs, Genus *Petrolisthes*

by

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Jonathon Harris Stillman, Author

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TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

TABLE OF CONTENTS (Continued)

TABLE OF CONTENTS (Continued)

List of Figures

List of Figures, Continued

List of Figures, Continued

List of Tables

List of Appendices

A Comparative Analysis of Morphological, Physiological, and Biochemical Adaptation to Abiotic Stress in Intertidal Porcelain Crabs, Genus *Petrolisthes*

Chapter 1

Introduction

Adaptation of ectothermic organisms to environmental temperature stress has been intensively studied during the past 50 years (reviews in Vernberg, 1962; Vernberg and Vernberg, 1972; Alexandrov, 1977; Hochachka and Somero, 1984; Cossins and Bowler, 1987; Feder *et al.*, 1987; Huey and Kingsolver, 1989; Hoffmann and Parsons, 1991; Somero, 1995, 1996; Johnston and Bennett, 1996). Studies of temperature adaptation have focused on organisms living in diverse habitats, from marine to terrestrial, and have examined a large diversity of organisms, including both vertebrates and invertebrates. Studies conducted within an explicit evolutionary framework, as defined by modern usage of the comparative method (see below), have been made most frequently on lizards (e.g. Huey and Bennett, 1987; Garland *et al.*, 1991) and insects, specifically fruit flies (e.g. Kimura, 1988; Gilchrist *et al.*, 1997). Similar comparative studies of evolutionary adaptation to environmental stress in marine organisms are lacking.

Several studies of temperature-adaptive differences in marine fishes (Fields and Somero, 1997; Holland *et al.*, 1997), and abalone (Dahlhoff and Somero, 1993a, 1993b) have in part satisfied requirements of the comparative method in that studies have been conducted on closely related, congeneric species. However, these studies were not made

within a phylogenetic context. No studies within the conceptual framework of the comparative method have been made of the evolutionary responses of intertidal marine organisms to environmental stress.

Organisms living in the intertidal zone experience a suite of physical stresses, including fluctuations in temperature, aerial exposure, salinity, and hydrodynamic forces (Vernberg and Vernberg, 1972; Newell, 1979; Denny, 1988). Thus, intertidal organisms make excellent candidates for evolutionary studies of the adaptation of biological systems to environmental stress (Bartholomew, 1987). Here, I present a brief overview of the conceptual and methodological bases of modern comparative studies of evolutionary adaptation. Then, I introduce the study system that I will use to test hypotheses regarding adaptation of morphological, physiological and biochemical traits of intertidal organisms to temperature and aerial exposure.

Conceptual framework of comparative organismal biology

Modern evolutionary theory is founded in the observation that all life is based on a shared set of molecules (e.g. nucleic acids, proteins, carbohydrates) and some shared fundamental molecular mechanisms (e.g. DNA replication) (Stryer, 1988; Bull and Hichman, 1998). Beyond these shared foundations, organisms accomplish physiological requirements in diverse ways, and adjust physiological processes to meet specific needs with greater diversity yet. The diversity of physiological processes seen represents the evolutionary responses to many different sets of selective pressures, including those imposed upon organisms by the environment in which they live. The study of the relationship between organismal biological diversity and environmental stress is known as environmental physiology (Feder *et al.*, 1987; Garland and Carter, 1994). Studies of

evolutionary environmental physiology traditionally employ the comparative method. The comparative method, simply stated, is the use of multi-species comparisons to establish general patterns of the evolutionary responses of organisms to environmental or biological selective forces (Bartholomew, 1987; Harvey and Pagel, 1991).

The objective of comparative biology is not to establish an encyclopedic listing of the relationship between individual organisms and their habitats. Rather the objective is in part to determine the general rules that dictate the ways in which organisms evolve in response to environmental stress (Bartholomew, 1987). Comparative organismal biology is based on the documentation of existing organismal diversity (Feder, 1987), and can provide an approach to answering questions regarding the proximate (mechanistic) and ultimate (evolutionary) causes of observed biological diversity (Huey, 1987). Examination of organismal diversity using the comparative method can suggest modes of convergence and divergence in physiological function, ways that closely related species survive in dissimilar environments, the generality of responses to environmental selective forces, and an understanding of the evolutionary constraints placed on physiological function (Bartholomew, 1987).

The conceptual development of the comparative method was formalized during a period known as the New Synthesis, which occurred during the 1940's and 1950's (Bartholomew, 1987). In the decades following this period, research reports documenting patterns of physiological correlation with environmental or with other biological parameters filled the volumes of many journals. The importance of these comparative studies in revealing fundamental physiological mechanisms cannot be overstated. When correlations between physiology and environment were found in these studies, many were quick to ascribe the term "adaptation" to describe the correlation. This labeling was made in accordance with the operating paradigm at that time, that natural

selection had optimized individual biological traits to their fullest extent. If a trait was not as optimal as deemed possible, then this was assumed to be because of some trade-off made for the optimization of a different trait. Thus observations of correlation between trait optimization and environment were taken to represent evolutionary adaptation (Gould and Lewontin, 1979). In a landmark paper, Gould and Lewontin (1979) pointed out that the adaptive explanations forwarded under the above-described paradigm, which they refer to as the "adaptationist programme," were biased due to the atomization of traits (i.e. the splitting of traits into units that did not represent those upon which natural selection acted), and likely were incorrect. Gould and Lewontin (1979) encouraged comparative biologists to take a pluralistic and historical approach to their studies. It was this suggestion that largely changed the comparative method, and spurred the development of methods to include historical information, by means of phylogenetic analyses, into comparative studies (e.g. Felsenstein, 1985). The point by Gould and Lewontin (1979) regarding the misuse of the term "adaptation" was used to generate a new set of vocabulary (Gould and Vrba, 1982, Appendix 1) so that comparative biologists and evolutionary biologists would precisely communicate the exact nature of an "adaptation." Not all scientists agreed with the suggestions of Gould and co-workers to make the term "adaptation" implicitly historical. For example, one widely accepted interpretation of this term is a general state of increased fitness as compared to other organisms when faced with an environmental stress (Mayr, 1988). However, the general consensus among evolutionary physiologists is to interpret the term within a historical context (Gould and Vrba, 1982, Appendix 1). The logical rationale for including historical reference in identification of traits as adaptations follows.

Observed organismal diversity has generally been accepted to represent, to a large extent, the product of the forces of natural selection. The theory of natural selection, first

forwarded by Charles Darwin (1859), has been widely incorporated in comparative studies that report correlations of physiological and environmental characteristics as the direct result of selective forces acting upon the organisms. Darwin's definition of natural selection, essentially "descent with modification", implicitly requires knowledge of both organismal diversity and of the evolutionary history of the organisms in question. The incorporation of the evolutionary history of the organisms being studied formalizes the modern comparative method (Felsenstein, 1985). Incorporation of the evolutionary history into any analysis follows from the "unarguable premise that species are not independent biological units that are devoid of history and genealogical affinities" (Huey, 1987). Without incorporation of evolutionary history, the conclusions drawn from any comparative study may be weakened; the adaptive significance of a trait in extant species may reflect phylogenetic heritage and not a recent evolutionary response to features of the environment (Harvey and Pagel, 1991; Miles and Dunham, 1993; Pagel, 1994).

In statistical analyses of comparative correlations between a biological trait and an environmental trait, or between two biological traits, evolutionary history must be incorporated in order to satisfy the assumptions of the analyses. Consider the hypothetical data in Figure 1.1A. This scatter plot of two continuous variables suggests that there is some weak, but positive correlation between the variables. Standard statistical procedures would be to use regression analysis to test the hypothesis that the relationship between these variables is different from the null hypothesis (no relationship). One assumption of regression analysis is that data points are independent (Sokal and Rohlf, 1995). If each point in Figure 1.1A represents a species, then the assumption of independence translates into the evolutionary history represented in Figure 1.1B, where all of the species radiated simultaneously from a common ancestor. This case, although possible, is not a likely descriptor of the actual evolutionary history

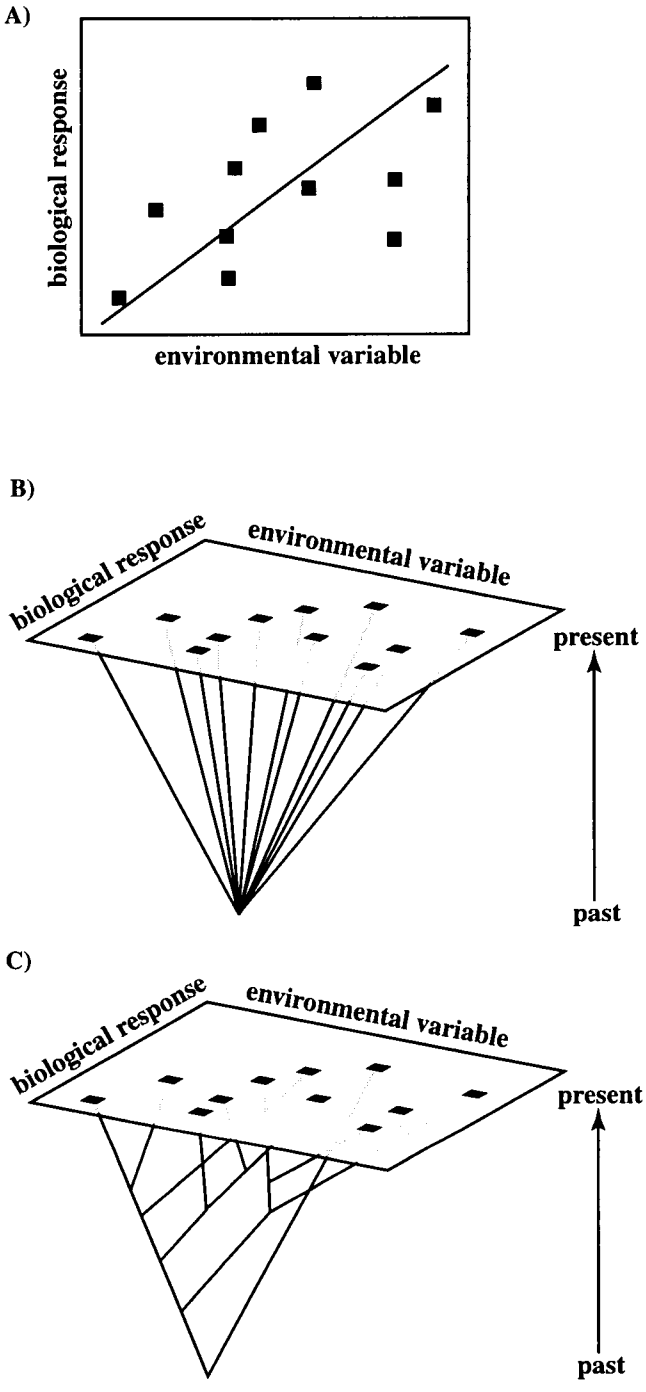

Figure 1.1. Hypothetical data illustrating the importance of phylogenetic analyses in comparative evolutionary biology. Regression of a biological variable against an environmental trait (A) assumes complete independence of each point, representing a simultaneous origin of each species, as depicted in (B). However, a more realistic model of the origin of the species is depicted in (C), and in this case data are not equivalently independent.

of a group of organisms. Figure 1.1C illustrates a more realistic evolutionary history with different occurrences of species divergences and an indication of the timing of those divergences. Biological data, as depicted in Figure 1.1C, thus do not meet the assumptions of regression analysis, and performing such an analysis, as in Figure 1.1A, is inappropriate. Methods of data transformation, such as phylogenetic independent contrasts, have been created to control for the effects of evolutionary history, by combining phylogenetic information with biological data to create a new, independent, set of data (Felsenstein, 1985; Harvey and Pagel, 1991; Miles and Dunham, 1993). Phylogenetic independent contrasts are generated from the values of any trait at the point of common ancestry of two taxa or groups of taxa and are standardized by the evolutionary time between those taxa. This standardization makes the phylogenetic independent contrasts independent, and thus these modified data can then be appropriately analyzed using standard statistical methods, such as regression analysis (see Appendix 2 for more details).

While one analytical method of comparative data uses phylogenetic information to remove the effect of phylogeny, as described above, another method uses phylogenetic tree topology to study the evolutionary history of specific structural or functional traits and test hypotheses concerning the origin and adaptive significance of those traits (Coddington, 1988; Lauder, 1990; Pagel, 1994). Such an analysis generally includes mapping traits and environmental features onto a phylogenetic tree, with examination of the co-occurrence of changes in environment with appearance or changes in traits. The mapping is either done using the characteristics of extant species, or with data from the fossil record. Use of fossil data is far superior to use of extant species in this regard because ancestral states can actually be documented. When no fossil record is available, traits are mapped based on the assumption that extant species are similar in biology and

habitat to their closest ancestor. In this way, inferences of ancestral states are based on the properties of extant organisms. Use of phylogenetic information in both historical and statistical ways provides the most comprehensive incorporation of the evolutionary history of a group of organisms in comparative analyses.

From a methodological standpoint, use of the comparative method addresses a number of issues of experimental design. One such issue is the selection of appropriate study species for the questions being asked. Study species must first and foremost be selected because they possess the specific organismal or environmental diversity that is of interest (Huey, 1987). Additionally, phylogenetic information can be used to select species that have diverged on appropriate time frames (Huey, 1987). Study of distantly related species may be more appropriate for elucidation of broad scale patterns, while specific patterns of adaptation to a particular environmental stress may be better studied using closely related species (Huey, 1987; Harvey and Pagel, 1991). The reason that use of closely related species can add to inferences of adaptation to specific environmental factors follows from the notion that a comparative study can be likened to "an experiment over historical time" (Huey, 1987). Like any experiment, attempts must be made to control all extraneous factors. The likelihood that differences between species strictly reflect independent evolutionary histories (i.e. extraneous factors) and not a specific response to an environmental factor (i.e. experimental treatment) increases with time (Huey, 1987).

Experiments must be designed to provide an appropriate amount of replication for statistical analyses. In most experiments, multiple sets of treatments and multiple sets of controls are created so that the effect of the treatment can be unambiguously and generally established (Sokal and Rohlf, 1995). Phylogenetic information can be used to

compare nested sets of species, thus providing a basis for the analysis of the generality of the observed patterns from comparative studies (Huey, 1987; Garland and Adolph, 1994).

From the above guidelines, an ideal group of species with which to conduct studies of evolutionary responses to the environmental stresses associated with life in the intertidal zone would possess the following characteristics. The group of study species would be one that was comprised of a large number of closely related species that occur over a large range of the environmental stress(es) of interest. The group of species would be comprised of multiple smaller groups of closely related species, each small group possessing members that live over the complete range of microhabitat conditions observed in all species. The organisms would be abundant, easy to collect, and tractable for study. Lastly, the group of species would possess members with a wide range of physiological responses to their particular microhabitat conditions. Not very many groups of organisms meet the above criteria, and as lamented by Huey (1987), practical considerations have unfortunately made such broad comparative studies rare.

Study system: porcelain crabs

A group of intertidal organisms that meets many of the above criteria for selection of a study system is the porcelain crabs, genus *Petrolisthes* (Crustacea: Decapoda: Anomura: Porcellanidae). There are over 100 species of *Petrolisthes* worldwide (Appendix 3), with 46 species found in the Eastern Pacific Ocean (Fig. 1.2) (Haig, 1960; Appendix 3). Latitudinal distribution boundaries of *Petrolisthes* in the Eastern Pacific create four geographic assemblages: North Temperate, Northern Gulf of California, Tropical (Southern Gulf of California to Ecuador), and South Temperate (Fig. 1.2) (Carvacho, 1980). With the exception of one species, *P. armatus*, species are only found

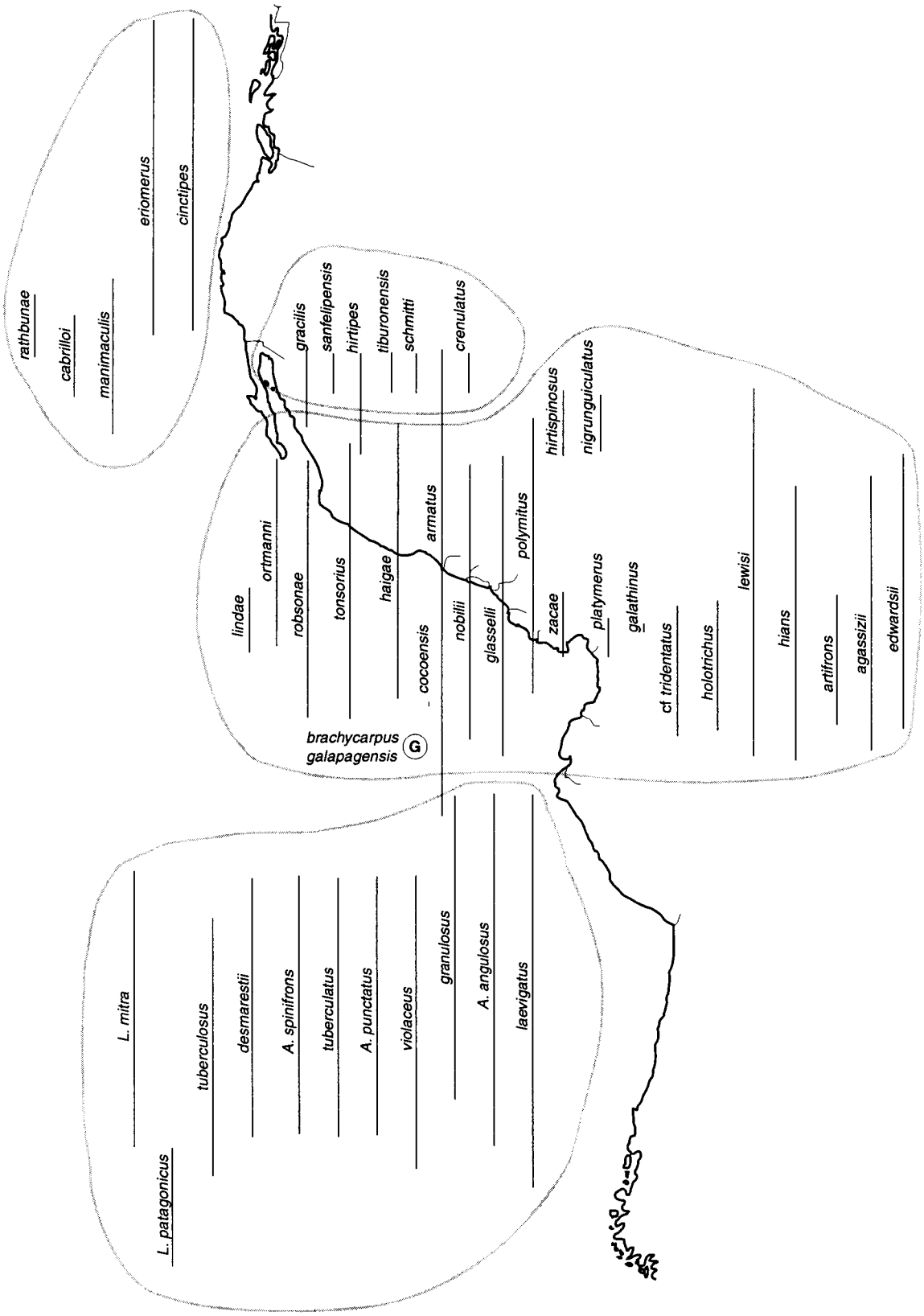

Figure 1.2. Distribution of Eastern Pacific *Petrolisthes*. Circled "G" indicates the Galapagos Islands. Species are grouped into four biogeographic regions.

within one geographic assemblage. Five species inhabit the North Temperate region, from the central region of the Pacific coast of the Baja Peninsula north into Canada. Within this region, there are two species, *P. cinctipes* and *P. eriomerus*, that are generally distributed to the north, and three species to the south, with a major faunal break around the region between Point Conception and Monterey Bay, California (Fig. 1.2) (Haig, 1960; Carvacho, 1980). Seven species occur in the Northern Gulf of California, four of which are endemic. Two occur throughout the Gulf of California, and one, *P. armatus*, occurs throughout the tropics, to Ecuador (Fig. 1.2) (Haig, 1960; Carvacho, 1980). Twenty-four species occur in tropical regions, from the Southern Gulf of California to Ecuador (Fig. 1.2) (Haig, 1960; Carvacho, 1980). Some of these species have broad geographic distributions, while others are endemic to narrow regions, such as *P. brachycarpus* and *P. galapagensis*, which are only found on the Galapagos Islands (Harvey, 1991), and *P. cocoensis*, only found on Cocos Island (Fig. 1.2) (Haig, 1960). In the Southern Temperate region, there are six species of *Petrolisthes* and five species previously described as *Petrolisthes* but now placed into genera *Allopetrolisthes* (three species) and *Liopetrolisthes* (two species) (Haig, 1960; Weber Urbina, 1991).

These four geographic regions comprise three different classes of water temperature. The temperate regions are generally cool (8-18°C), with cooler regions towards the poles (8-11°C). Throughout the tropics, water temperatures are warm (26-30°C) year-round (although in upwelling areas, temperatures can be as low as 18-20°C, B. Menge, pers. comm.), and in the Northern Gulf of California, water temperatures vary on a seasonal basis (15-30°C). In winter, water temperatures are similar to those in temperate regions, while in summer water temperatures can exceed those in tropical regions. Thus crabs have mean body temperatures in one of three classes: temperate, tropical or seasonally tropical.

In each of the four geographic regions, species are distributed across a vertical gradient in the intertidal and subtidal zones. Studies of *Petrolisthes* assemblages in the northern North Temperate (Washington state) (Jensen and Armstrong, 1991), the Northern Gulf of California (Romero, 1982), and the central South Temperate (Chile) region (Weber Urbina, 1986) have documented vertical zonation patterns in the intertidal zone. At each site, there are species found strictly in upper-intertidal zone microhabitats, and species that live in the low-intertidal zone or are strictly subtidal, and therefore never experience emersion during low tide. As a result, individual species of *Petrolisthes* experience microhabitat conditions generated both by differences in geographic distribution and vertical distribution. Because small differences in vertical distribution in the intertidal zone can create large differences in microhabitat conditions (Edney, 1961; Newell, 1979), patterns of intertidal distribution have the potential to create a much greater range of thermal microhabitat conditions than geographic distribution patterns alone. Therefore, *Petrolisthes* occur over a much larger range of microhabitat temperatures than indicated by the range of water temperatures among geographic regions. Additionally, differences in vertical intertidal distribution create differences in other environmental factors, such as time of emersion. Hence, organisms are exposed to desiccation stress, and aquatic respiratory systems are required to function in air.

If species living within one geographic region are all more closely related to one another than to species from other geographic regions, then these crabs provide a system for nested analyses of evolutionary adaptation to environmental stress. To determine whether this potential exists, the evolutionary history of these crabs must be determined through phylogenetic analyses. There has never been a phylogenetic analysis of the Eastern Pacific porcelain crabs. The only phylogenetic analysis of porcelain crabs that has been made was of the Western Pacific porcelain crabs of Taiwan (Hsieh, 1993).

Some species of *Petrolisthes* are extremely common. For example, densities of *P. cinctipes* have been estimated at nearly 4000 individuals m^{-2} in beds of the mussel *Mytilus californianus* (Jensen, 1990). In addition to living among mussels, most *Petrolisthes* are found living underneath small boulders, where densities can be lower, but still high enough so that a large number of individuals can easily be collected within a short amount of time (Jensen and Armstrong, 1991). Porcelain crabs are among the most common, abundant, and diverse groups of crustaceans found in wave-sheltered cobble or boulder intertidal habitats (Villalobos Hiriart *et al.*, 1992).

Compared to other abundant intertidal crabs, there are relatively few studies on the biology of porcelain crabs. *Petrolisthes* have been the subject of several studies of sensory biology (Eguchi *et al.*, 1982; Meyer-Rochow *et al.*, 1990; Ziedins and Meyer-Rochow, 1990; Meyer-Rochow and Meha, 1994; Meyer-Rochow and Reid, 1996), and ammonia excretion (Hunter and Kirschner, 1986). Behavioral studies made on *Petrolisthes* include examination of communication during courtship (Molenock, 1975), agonistic interactions (Molenock, 1976), and larval settlement (Jensen, 1989). Studies of the feeding physiology of porcelain crabs have shown that these crabs are principally suspension feeders (Nicol, 1932; Wicksten, 1973; Hartman and Hartman, 1977; Trager and Genin, 1993), although they will also scavenge or scrape food from the substratum (Gabaldon, 1970; Kropp, 1981). Filter feeders are easy to feed in a laboratory setting, and laboratory studies of survivorship (Jensen and Armstrong, 1991) and larval development (Gore, 1971, 1972a, 1972b, 1975; Yaqoob, 1974; Huni, 1979; Pellegrini and Gamba, 1985; Saelzer *et al.*, 1986; Wehrtmann *et al.*, 1996, 1997, among others), indicate that these crabs tolerate laboratory conditions well.

By far, the most common studies of porcelain crabs are taxonomic and biogeographic (Haig, 1960, 1962, 1966, 1981, 1983, 1987, 1988; Viviani, 1969; Gore,

1973, 1974, 1976; Bahamonde *et al.*, 1975; Scelzo, 1980; Werding, 1982, 1983, 1996; Kropp, 1986, 1994; Weber Urbina, 1986, 1991; Haig and Kropp, 1987; Harvey, 1991; Weber Urbina and Galleguillos, 1991; Hendrickx, 1993; Oliveira *et al.*, 1995; Osawa, 1997, among others). Studies of the environmental physiology of porcelain crabs include the examination of settlement patterns and substratum preferences (Jensen, 1989, 1990, 1991; Jensen and Armstrong, 1991), determination of the resistance to desiccation stress (Jones, 1976; Jones and Greenwood, 1982; Pellegrino, 1984) and salinity stress (Huni, 1979), and examination of survivorship during environmental stress (Jensen and Armstrong, 1991).

The following chapters address the question of evolutionary adaptation to environmental stress in *Petrolisthes*. In Chapter 2 differences in the physiology and morphology of two sympatric species, *P. cinctipes* and *P. eriomerus*, living in different vertical intertidal zones are examined. Jensen and Armstrong (1991) showed that *P. cinctipes*, which lives higher in the intertidal zone, survived in both water and air at 25°C. However, *P. eriomerus*, which lives in the low intertidal and subtidal zones, only survived in water at 25°C (Jensen and Armstrong, 1991). Survival times of *P. eriomerus* while in air were also found to be a function of body size (Jensen and Armstrong, 1991). In Chapter 2, the physiological bases for the observed differences in survival of *P. cinctipes* and *P. eriomerus* are examined. I show that these two species have differences in thermal tolerance that correlate with maximal microhabitat temperatures, and that *P. cinctipes* possesses secondary respiratory structures, membranous regions on the walking legs (leg membranes), which allow it to respire while in air.

The remaining chapters examine the entire suite of Eastern Pacific porcelain crabs as a study system for an evolutionary analysis of environmental physiology. In order to make evolutionary inferences from the results of my studies I generated a phylogenetic

tree for the Eastern Pacific *Petrolisthes* based on molecular sequence data from a mitochondrial ribosomal RNA gene. In chapter 3, I examine the diversity, evolutionary history, and adaptive significance of leg membranes in relation to emersion stress, with implications for increasing body size and metabolic rate. In chapter 4, I present a comparative analysis of organismal thermal tolerance limits as they relate to microhabitat temperature and phylogenetic affinity. Finally, in Chapter 5, I present a comparative study of the patterns and mechanisms of the thermal stability of a glycolytic enzyme, lactate dehydrogenase, in relationship to microhabitat thermal conditions.

In each of these chapters, I address issues on both ultimate (evolutionary) and proximate (mechanistic) levels. That is, I provide a treatment of the patterns of physiological diversity observed and provide experimental evidence in attempts to elucidate the mechanistic bases of the physiological diversity. In cases where the mechanistic bases are unresolved, I have attempted to forward hypotheses appropriate for further experimentation.

Overall, this thesis integrates observational and experimental approaches to evolutionary studies of environmental physiology. By consideration of the evolutionary response to environmental stress of traits on three biological levels (morphological, physiological, biochemical), the relative amount of selection acting upon these traits can be evaluated. Each of the chapters in this thesis includes a focused analysis of a particular trait. When considered in total the sum of their results may indicate an overall estimate of organismal adaptation to environmental stress (a pluralistic view), or indicate that some traits are more strongly selected than others, and that a trait-by-trait, or atomistic approach, is warranted in the determination of organismal adaptation to environmental stress.

Chapter 2

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

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Abstract

We examined physiological and biochemical responses to temperature and aerial exposure in two species of intertidal porcelain crabs (genus *Petrolisthes*) that inhabit discrete vertical zones. On the shores of the Northeastern Pacific, *P. cinctipes* (Randall), occurs under rocks and in mussel beds in the mid to high intertidal zone, and *P. eriomerus* (Stimpson) occurs under rocks in the low intertidal zone and subtidally to 80m. Because of their different vertical distributions, these two species experience very different levels of abiotic stress. Individuals of *P. cinctipes* can be emersed during every low tide, but *P. eriomerus* is only emersed during the lowest spring tides, and on most days is not emersed at all. Temperatures measured underneath rocks in the mid intertidal zone were as high as 31°C, 15°C higher than maximal temperatures measured under rocks in the low intertidal zone. In air, at 25°C, large specimens of *P. cinctipes* were able to maintain a higher respiration rate than similarly sized *P. eriomerus*. No interspecific differences in the respiratory response to emersion were seen in small specimens. Examination of the response of heart rate to temperature revealed that *P. cinctipes* has a 5°C higher Arrhenius break temperature (ABT - the temperature at which there is a discontinuity in the slope of an Arrhenius plot) than its congener (31.5°C vs. 26.6°C). The heart rate of *P. cinctipes* recovered fully after a cold exposure (1.5°C), but the heart rate of *P. eriomerus* did not recover after exposure to 2°C or cooler. The ABT of heart rate in *P. cinctipes* was very close to maximal microhabitat temperatures, thus individuals of this species may be living at or near their thermal tolerance limits. *P. cinctipes* were able to maintain aerobic metabolism during emersion, whereas *P. eriomerus* shifted to anaerobic metabolism. A pronounced 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 similarly treated. *P. cinctipes* possesses a membranous structure on the ventral merus of each walking leg, but this structure is not found in *P. eriomerus*. To test the function of the leg membrane, we measured the aerial respiration rates and the lactate accumulation of *P. cinctipes* with their leg membranes obscured. These individuals had significantly lower aerial respiration rates at 30°C than control crabs. Crabs with leg membranes obscured also had a large accumulation of lactate during a 5 h period of emersion at 28°C, but control crabs had no accumulation under the same conditions. These data suggest that the leg membrane functions as a respiratory structure. The results of this study illustrate that a suite of morphological, physiological, and biochemical features allows *P. cinctipes* to live higher in the intertidal zone than *P. eriomerus*.

Introduction

One predominant feature of the rocky intertidal zone is the distribution pattern of organisms in discrete vertical zones (Connell, 1961). Zonation patterns have been shown to be due to both biotic factors, such as competition and predation, and abiotic factors, such as temperature, wave exposure and desiccation stress (Connell, 1961; Edney, 1961). Studies of abiotic stress have shown that species living higher in the intertidal zone generally have a greater resistance to abiotic factors than do species living lower in the intertidal zone. Often, however, species found lower in the intertidal zone are competitively dominant, and exclude the upper species from lower zones (Connell, 1961). Like animals and plants living on the surfaces of rocks, animals found beneath rocks (rupestrine species) have been shown to live in discrete vertical zones (Jones, 1976; Willason, 1981; Pellegrino, 1984; Menendez, 1987; Gherardi, 1990; Jensen and

Armstrong, 1991). However, the levels of abiotic stress experienced by rupestrine fauna and the responses of those fauna to abiotic stress have not been well characterized.

Porcelain crabs (genus *Petrolisthes* (Anomura: Porcellanidae)) are a rupestrine taxon with a broad geographic distribution (Haig, 1960). Two species of porcelain crabs are common along rocky shores of the Northeastern Pacific, *P. cinctipes* and *P. eriomerus*. *P. cinctipes* is found in the mid to high intertidal zone, but *P. eriomerus* is found from the bottom of the *P. cinctipes* distribution range to a depth of approximately 80m (Morris *et al.*, 1980; Jensen and Armstrong, 1991). Both species live under stones and in crevices, and *P. cinctipes* is one of the most abundant members of the mussel (*Mytilus californianus*) bed fauna, where it has been reported in densities nearing 4000 individuals m^{-2} of mussel bed (Jensen and Armstrong, 1991).

Because of the difference in vertical distribution, these congeners may experience very different patterns of emersion during each tidal cycle (Fig. 2.1). Individuals of *Petrolisthes cinctipes*, living near the top of their vertical distribution range, experience emersion twice per day, every day, but *P. eriomerus* at the top of their distribution only experience emersion once per day during periods of low spring tides, and are not emersed during neap tides. The differences in frequency and duration of emersion are important in shaping the thermal characteristics of the vertical zones; initial studies described temperatures during low tide in *P. cinctipes* zones that were above the lethal limit of *P. eriomerus* (Jensen, 1989). Here we report a more detailed examination of the thermal microhabitats of *P. cinctipes* and *P. eriomerus*. Jensen and Armstrong (1991) showed differences in tolerance to high temperature between these two species under conditions of emersion. To investigate mechanisms that could account for the differences in thermal tolerance maxima between *P. cinctipes* and *P. eriomerus*, we examined the thermal

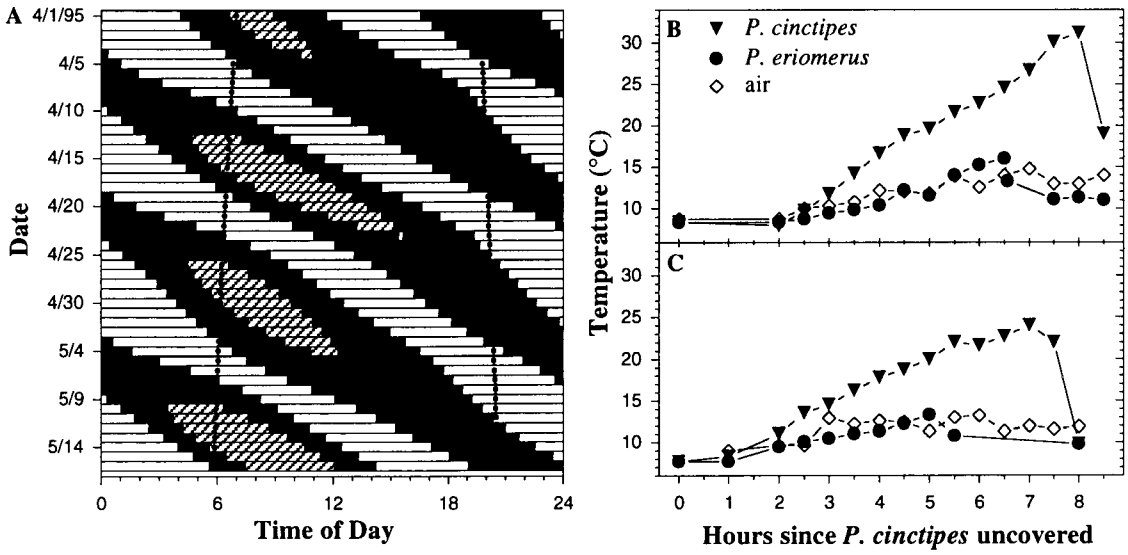

Figure 2.1. Microhabitat characterization. (A) Coverage patterns of *Petrolisthes cinctipes* and *P. eriomerus* living at the tops of their vertical distributions. Areas enclosed by black represent times during which *P. cinctipes* are emersed, and areas enclosed by hatched markings indicate times during which both *P. cinctipes* and *P. eriomerus* are emersed. White areas represent times when both species are immersed. Black dots represent the times of sunrise and sunset for each solar day. For example, on April 1, 1995, *P. eriomerus* were emersed from 6:00 to 8:30, and *P. cinctipes* were emersed from 4:00 to 11:30 and from 16:30 to 21:30. (B,C) Habitat temperatures during low tide on May 18, 1995 (B) and May 19, 1995 (C). Environmental conditions on May 18 were no clouds, no wind, no waves and a low tide of -0.61 meters at 9:50 AM. Conditions on May 19 were no clouds, but high winds, with a low tide of -0.43 meters at 10:45 AM. Maximal temperatures in the *P. cinctipes* zone were 31.2°C on May 18 (B) and 24°C on May 19 (C). Ambient seawater temperature was 8-9°C on both days.

sensitivity of heart beat rate during extreme, but environmentally realistic, temperature fluctuations.

Because *Petrolisthes cinctipes* is emersed for a much greater percentage of time than is *P. eriomerus*, it is possible that this species possesses adaptations for semi-terrestrial existence, such as those that have been shown for other terrestrial, and semi-terrestrial crabs (Burggren and McMahon, 1981; DeFur *et al.*, 1983; Burggren, 1992; Burnett, 1992; Wolcott, 1992). Terrestrial crabs can usually support higher rates of aerial respiration than can aquatic crabs (Wallace, 1972; Hawkins *et al.*, 1982; Houlihan and Innes, 1984; Santos and Costa, 1993). Adaptations at the morphological, physiological and biochemical levels are responsible for the increased ability for aerial gas exchange in land crabs (Burggren, 1992). Here we examined differences between species of *Petrolisthes* in their ability to aerially respire at each of these three levels.
Aerial and aquatic whole animal oxygen consumption rates at a range of temperatures were measured, and poise of metabolism between aerobic and anaerobic pathways was examined by measuring the amount of lactate accumulation during emersion.

Morphological adaptations of crabs that breathe air include a general reduction in gill number and gill surface area (Gray, 1957; Hawkins and Jones, 1982) as well as the formation of a functional lung by an increased vascularization of the inner lining of the branchiostegites (Farrelly and Greenaway, 1994). A different morphological strategy to facilitate aerial respiration has been described in two endemic Scopimerinae ghost crabs of Australia (*Scopimera inflata* and *Dotilla myctiroides* (Brachyura: Ocypodidae)). These crabs have no lungs and while emersed, they support their high metabolic rate using membranous gas exchange surfaces ('gas windows') on the dorsal surface of the meral segments of each walking leg (Maitland, 1986). *Petrolisthes cinctipes* possesses an analogous uncalcified membranous area on the ventral surface of the meral segments of

each walking leg, but every meral segment of *P. eriomerus* is fully calcified (Jensen and Armstrong, 1991). To assess the possible function of the leg membrane structures in *P. cinctipes* as respiratory structures, we have measured aerial and aquatic respiration rates and lactate accumulation (metabolic poise) of *P. cinctipes* with their leg membranes obscured.

The morphological, physiological and biochemical differences we describe between these two species suggest that *Petrolisthes cinctipes* possesses adaptations that allow it to live higher in the intertidal zone than its congener.

Materials and Methods

Habitat characteristics

Emersion patterns (Fig. 2.1A) were generated using Harbor Master software (Zihua, Monterey, CA). Heights of the top of the *Petrolisthes cinctipes* (1.24 m above mean low tide) and *P. eriomerus* (0.15 m above mean low tide) vertical distributions at Cape Arago, OR (43° 21' N; 124° 19' W) were empirically determined by noting the time when the tides covered and uncovered the tops of each zone. These times were used to determine the height of each zone using tidal prediction curves in Harbor Master. Measurements were made on 4 consecutive days, and the mean height of each zone was used for the generation of the emersion patterns (Fig. 2.1A). Tidal predictions for Bandon, OR (43° 07' N; 125° 25' W) were selected as an approximation for the tidal patterns at Cape Arago, OR.

Rocks that had the appropriate morphology to attain high temperatures (flat and thin, and with a horizontal surface fully exposed to sunlight), and that supported natural assemblages of *Petrolisthes* were selected in the upper and the lower intertidal zones.

Thermocouple probes (Omega Inst., K-type wire probes) were placed under the rocks just as they were uncovered by the receding tide, and left in place throughout the low tide period. At 30 min intervals, each probe was connected to a digital thermometer (Omega Inst., HH 82) and the temperature was recorded. Air and ocean water temperatures were also monitored. Cloud cover and wind speed were qualitatively determined. Temperatures were monitored during the lowest tide on consecutive days in May, 1995 at Cape Arago, OR (Figs. 2.1B,C).

Collection and maintenance of specimens

Specimens were collected from locations as specified for each experiment (below). All specimens were collected at low tide, and immediately transported to Oregon State University (OSU) in Corvallis, Oregon, where the crabs were held in temperature controlled, recirculating aquaria at the temperature of the water at the collection site. Every third day, crabs were fed a diet composed of a unicellular algal culture (Algal diet C, Coast Seafood, Inc., Bellevue WA) and a homogenized mixture of algae (mostly *Ulva* spp., *Laminaria* spp. and *Macrocystis* spp.), mussels and fish pellets. Crabs were not fed on any of the three days immediately preceding experimentation.

Respirometry

Specimens used for measurement of whole animal oxygen consumption ($\dot{V}O_2$) were collected on August 1, 1993 at Clallam Bay, Washington (48° 16' N; 124° 18' W). Crabs were held at the collection temperature, 14±1°C, for two weeks preceding measurement of oxygen consumption rate. Rates of aquatic and aerial $\dot{V}O_2$ were determined using a Gilson Differential Respirometer. For measurement of aquatic $\dot{V}O_2$ ($\dot{V}O_2{}^W$), crabs were placed in glass chambers containing 20 ml of sea water that had

been filtered to remove particulate matter, and a piece of Whatman filter paper was added for traction. The chambers were connected to the respirometer and adjusted to the experimental temperature. The change from the aquarium temperature to the experimental temperature was made at a rate intended to mimic the rate of temperature change in the natural environment during a low tide period. After the experimental temperature was reached, the chambers were allowed to equilibrate for 1 h before the respirometer was closed and measurements of oxygen consumption were initiated. Every measurement was conducted in triplicate 30-60 min periods, with 10 min between each period during which time the respirometer was opened to allow flushing of the system with fresh air; the water was not changed between replicates. Following measurement of $\dot{V}O_2{}^w$, each chamber was removed from the apparatus, the water was removed, and the chambers were re-connected to the respirometer. Then, triplicate measurements of aerial $\dot{V}O_2$ ($\dot{V}O_2{}^a$) were made on the same specimen in the same manner as for $\dot{V}O_2{}^w$. On a given day, $\dot{V}O_2$ for each animal was measured at only one temperature, first in water, and then in air. Following measurement of $\dot{V}O_2{}^a$, each crab was uniformly blotted and weighed to the nearest 0.01 g. On successive days, temperatures were increased. This protocol was used to minimize the likelihood of damage to the organisms from sub-lethal levels of thermal stress. $\dot{V}O_2$s were measured for large (carapace width (cw) 15- 20 mm, wet mass 3.5-5 g) and small (cw 5-8 mm, wet mass 0.5-1.2 g) specimens to examine the effect of size on response of $\dot{V}O_2$ at different temperatures.

To calculate $\dot{V}O_2$, the change in gas volume (corrected against a blank) was plotted against time. The consumption of oxygen was always linear with time, suggesting that the specimens did not experience PO_2's below the critical O_2

concentration (P_c). The slope of the linear relationship between O_2 and time was used to calculate weight specific O_2 consumption rate.

Thermal tolerance limits of heart rate

The thermal tolerance limits of both species were indexed by the response of heart rate to changes in temperature. Specimens were collected at Cape Arago, Oregon on October 4, 1994 and held at 11±1°C, for 4 weeks preceding experimentation. Heart rates of *Petrolisthes cinctipes* and *P. eriomerus* were monitored by impedance. Specimens were immobilized by lashing them to a piece of plastic mesh (Vexar) which was then secured to a small rock. The specimen was immersed in an aerated, temperature controlled water bath containing sea water at the acclimation temperature. Pinholes through the carapace were made laterally on either side of the heart, and an impedance electrode, 0.025 mm diameter ceramic coated copper wire, with the ceramic insulation removed over the last 1 mm of the tip, was inserted into each hole. Because the specimens were immobilized, the wires did not need to be secured to the carapace or held in place by any device other than bending the wire at the hole. Wires were connected to an impedance pneumograph (Narco Bio-systems, Houston, TX). The impedance signal was amplified and individual heart beats were monitored and recorded by a strip chart recorder (Gould, Inc., Cleveland, OH). The pinholes had no deleterious effects on crab survival.

Temperatures were either increased or decreased at a rate that was determined to be environmentally realistic (1°C every 15 min, see Fig. 2.1) for a thermally extreme day. Heart rate was monitored for 1-2 min intervals every 15 to 30 min. Heart beats were counted if the amplitude of a peak on the chart was at least three times as large as the background noise. Heart rates were expressed as beats min^{-1} or transformed to the natural

logarithm of beats min^{-1} for Arrhenius plots. Arrhenius break temperatures (ABTs) were determined using regression analyses to generate the best fit line on both sides of a putative break point (where there was an inflection in the slope of data points) on Arrhenius plots. The temperature at which these two lines intersected was taken as the ABT (see Dahlhoff *et al.*, 1991).

Lactate accumulation experiments

Specimens used for interspecific comparisons of lactate production were collected from Cape Arago, OR on January 29, 1995.

Lactate production was measured in crabs kept in air at two different temperatures (10° or 28°C) over a 5 hour period. Specimens were blotted and weighed to the nearest 0.1 g and then placed into individual containers containing a piece of sea water-soaked filter paper. The glass containers were partially immersed into a circulating water bath for temperature control, and the air was humidified by vigorous aeration of the water in the bath. Individuals were removed after incubation periods of 1, 3 and 5 h and immediately frozen by freeze clamping in liquid N_2. Specimens were stored at -70°C for subsequent analysis of lactate concentration.

To assay total body lactate, frozen crabs were ground with a mortar and pestle under liquid N_2 and immediately placed into 2 body mass equivalent volumes (2 ml g^{-1}) of 0.75 M $HClO_4$. The mixture was incubated on ice for 10 min and then centrifuged at 10 000 ***g*** for 10 min. The supernatant was neutralized to pH 7.0 by addition of 3 M KOH, incubated on ice for 10 min and then centrifuged at 10 000 ***g*** for 10 min to remove the $KClO_4$ precipitate. The resulting supernatant was held on ice, and used within 1-2 h for enzymatic determination of lactate concentration using the method described by (Noll, 1984), with a commercially purchased L-lactic acid kit (Boehringer Mannheim).

Leg membrane studies

Dissection microscopy was used to visualize the morphological features of the meral segment of walking legs. The legs were cut along the anterior-posterior axis (the thinnest axis), and the muscle tissue was removed from the inner surface of the ventral merus.

Examination of the physiological function of the leg membrane of *Petrolisthes cinctipes* was conducted by obscuring the leg membrane and measuring (in large and small specimens) the respiratory response to emersion (as defined above), and (in large specimens) the metabolic poise during emersion. Leg membranes were obscured by application of two thin coats of nail polish (Revlon creme). Only a few legs were painted at a time to minimize the time that the animals spent out of water. Crabs had either all of their legs painted, half of their legs painted (control for paint effects) or none of their legs painted. Crab legs were painted 2 d before experimentation began. Respirometry was conducted as described above. Specimens used for respirometry were collected at Cape Arago, OR on October 4, 1994 and held at 11±1°C for 2 weeks preceding experimentation. Specimens used to examine lactate production of *P. cinctipes* with their leg membranes obscured were collected from Cape Arago, OR on May 18, 1995, and held at 11±1°C for 2 weeks as above.

A taxonomic survey was completed at the Los Angeles County Museum of Natural History to determine the prevalence of the leg membrane in the genus *Petrolisthes*.

Results

Habitat characteristics

Differences in frequency and duration of emersion of crabs living at the vertical maxima of their distributions show that *Petrolisthes cinctipes* may be emersed during every low tide, but that *P. eriomerus* are emersed only during low spring tides (Fig. 2.1A). These emersion patterns indicate that individuals of *P. cinctipes* may spend as much as 50% of their time out of water. While some rocks have small puddles of water beneath them, most have only wet substratum or other rocks beneath, and thus the crabs cannot seek refuge from emersion (pers. obs.).

Measurement of habitat temperatures on 2 consecutive days during May, 1995, illustrates the importance of weather conditions to microhabitat temperatures as well as the large differences between the thermal microhabitats of *Petrolisthes cinctipes* and *P. eriomerus* on hot days (Figs. 2.1B,C). When low spring tides occur towards the middle of the solar day, as on May 18, 1995, and the weather is clear and calm (Fig. 2.1B), microhabitat temperatures increase rapidly, and dramatic differences can be seen between rocks in the upper intertidal zone (*P. cinctipes*, Figs. 2.1B,C) versus the lower intertidal zone (*P. eriomerus*, Figs. 2.1B,C). With an increase in wind, the maximal temperature achieved is decreased, and on May 19, 1995 (Fig. 2.1C), a windy but sunny day, the maximal temperature (24°C) was ~7°C lower than on May 18, 1995 (31.2°C), a calm and sunny day (Fig. 2.1B). Rocks in the upper intertidal zone are for the most part bare on the upper surface, and the temperature under flat thin rocks in this zone increases at a rate of approximately 4°C per hour during the most rapid heating conditions. Temperatures under rocks in the lower intertidal zone do not increase to the same degree as those in the upper intertidal zone (Figs. 2.1B,C). In the spring and summer, rocks in the lower

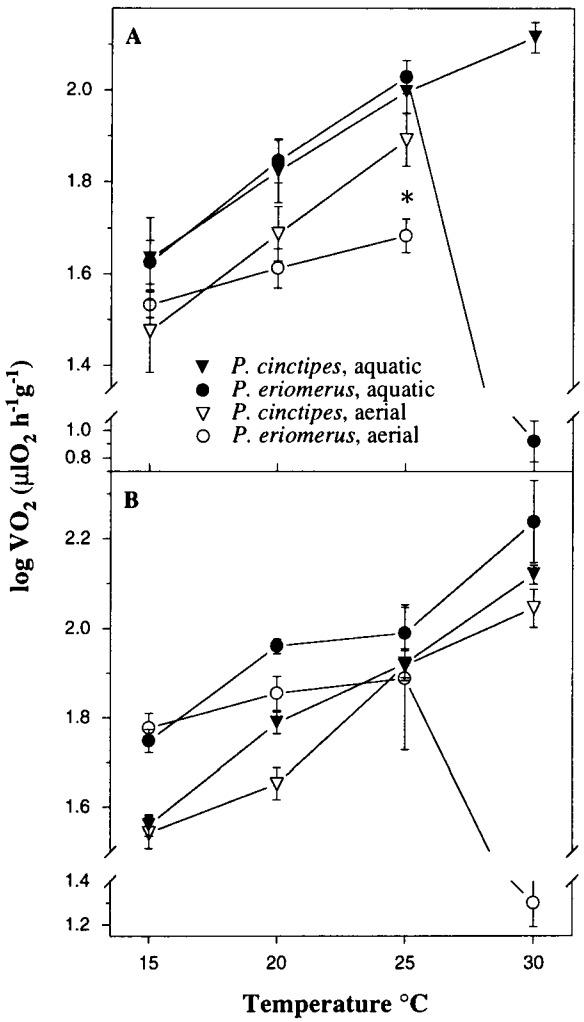

Figure 2.2. Whole animal oxygen consumption rates of crabs at different temperatures and aerial exposures in (A) large and (B) small specimens. Error bars are 1 S.E.M. Points in A are all n=6 individuals. In (B), n=6 for all points except the following: n=12 for immersed and emersed *P. cinctipes* at 15°C , n=11 for immersed *P. eriomerus* at 15°C, n=10 for emersed *P. eriomerus* at 15°C, n=5 for immersed and emersed *P. eriomerus* at 20 and 25°C, n=4 for immersed *P. eriomerus* at 30°C, and n=3 for emersed *P. eriomerus* at 30°C. * denotes a significant difference between aerial $\dot{V}O_2$ of *P. cinctipes* and *P. eriomerus* at 25°C (ANOVA; $p<0.05$).

intertidal zone usually harbor an abundance of algae. The algae minimize increases in the under-rock temperatures by insulating the rock, and by providing a source of water for evaporative cooling, which removes thermal energy from the rock.

Respiratory responses to abiotic stress

Respiratory responses to temperature and emersion differed as functions of species and body size. The ability to aerially respire was different between species, as well as between large and small individuals within a species. Large specimens showed little interspecific difference in $\dot{V}O_2{}^W$ at 15, 20 and 25°C (Fig. 2.2A). However, $\dot{V}O_2{}^a$ of *P. eriomerus* was 40% lower than that of *P. cinctipes* at 25°C (81.7 ± 29.1 vs. 49.0 ± 10.3 µl O_2 $h^{-1}g^{-1}$; ANOVA of ln transformed data; p=0.012) (Fig. 2.2A). In addition, all large specimens of *P. eriomerus* died during measurement of $\dot{V}O_2{}^W$ at 30°C (Fig. 2.2A). For small specimens, there were no differences between $\dot{V}O_2{}^W$ and $\dot{V}O_2{}^a$ within species, except at 30°C for *P. eriomerus*; most of the variation was interspecific and not dependent on emersion state (Fig. 2.2B). Small specimens of *P. eriomerus* had very high $\dot{V}O_2{}^W$ at 30°C (Fig. 2.2B), however all specimens were found to be moribund at the end of the experiment and did not recover after they were returned to ambient temperatures. The only mortality observed during the experiment involved *P. eriomerus* at 30°C.

Thermal tolerance limits of heart rate

The thermal tolerance limits of *Petrolisthes cinctipes* and *P. eriomerus*, as indexed by the ABT and post cold recovery of heart rate are shown in Figure 2.3. ABTs of heart rate in *P. cinctipes* were ~5°C higher than in *P. eriomerus* (31.5 ± 0.5°C vs. 26.6 ± 0.3°C, ANOVA; p<0.0001, n=6 for each species) (Fig. 2.3A). Differences were also

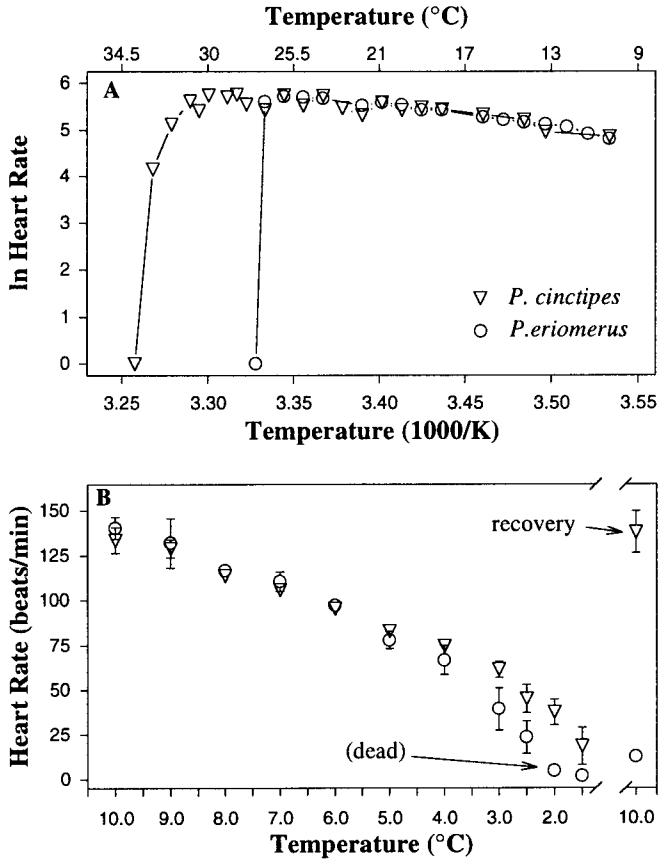

Figure 2.3. Thermal limits of *in vivo* heart rate during (A) heating and (B) cooling. Error bars in (B) are 1 S.E.M. Data in (A) are representative individuals; Interspecific comparisons were made using the Arrhenius break temperatures derived from the figures.

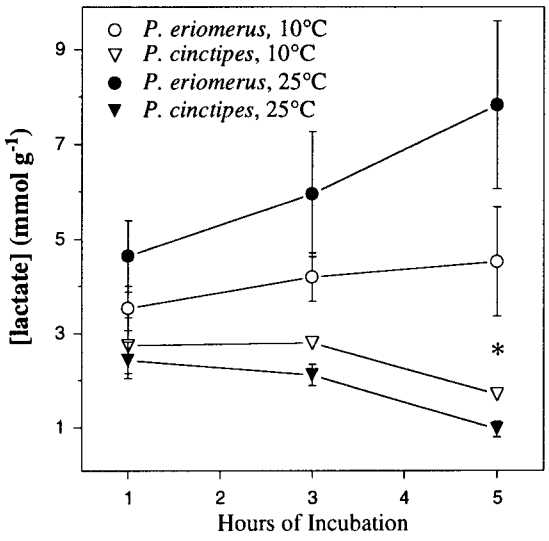

Figure 2.4. Lactic acid accumulation of large crabs during emersion. Error bars are 1 S.E.M., n=5 for each point, and * denotes a significant difference in lactate concentration between *Petrolisthes cinctipes* and *P. eriomerus* at 25°C (ANOVA; $p<0.0005$).

seen after cold exposure (Fig. 2.3B). The heart beat of *P. cinctipes* exhibited complete recovery after exposure to 1.5°C, whereas the lower critical temperature of *P. eriomerus* was 2°C, and recovery after exposure to this temperature was never observed (n=6 for each species). It is worth noting that the Arrhenius plots of *P. cinctipes* and *P. eriomerus* heart rate are qualitatively different. The heart rate of *P. cinctipes* decreased more gradually above the ABT, than did the heart rate of *P. eriomerus.*

Lactate Accumulation

Total body lactate accumulation shows that large specimens of *Petrolisthes eriomerus* in air at 25°C undergo a shift in metabolic poise towards anaerobiosis (Figs. 2.2A, 2.4). Over a 5 h incubation period at 25°C, the lactate concentrations in whole crabs increased for *P. eriomerus* and decreased in *P. cinctipes* (Fig. 2.4). *P. eriomerus* incubated in air for 5 h at 25°C had 808% higher concentrations of lactate (7.827 ± 1.58 vs. 0.968 ± 0.15 mmol g^{-1}) than *P. cinctipes* at the same temperature, a significantly higher level (ANOVA; $P<0.0005$, n=5 for each species) (Fig. 2.4). In addition, *P. eriomerus* held at 25°C for 5 h accumulated 173% more lactate than conspecifics held at 10°C (4.52 ± 1.04 mmol g^{-1}), but the difference was not significant (ANOVA; $p=0.083$, n=5 for each group). *P. cinctipes* at 10 and 25°C did not accumulate significantly different levels of lactate at any individual time point (Fig. 2.4). In *P. cinctipes* incubated at 10 and 25°C, lactate levels decreased between hours 1 and 5 (one-tailed t-test; $p<0.05$), but there was no effect of temperature on lactate levels (Fig. 3.4).

Leg membrane studies

Photographs of the ventral merus with muscle fibers removed indicate that there is a gross morphological difference between the exoskeletons of *Petrolisthes cinctipes* and

P. eriomerus (Figs. 2.5A,B). *P. cinctipes* possesses an uncalcified exoskeleton over a portion of the ventral surface of the meral segment (Fig. 2.5A), but *P. eriomerus* has a solid, thick exoskeleton over the entire meral segment (Fig. 2.5B).

Measurement of $\dot{V}O_2{}^a$ in large specimens of *Petrolisthes cinctipes* with their leg membrane obscured revealed a response to aerial exposure similar to that seen in *P. eriomerus* (Fig. 2.6A; compare with Fig. 2.2A). Crabs with their leg membranes obscured had a 28% lower $\dot{V}O_2{}^a$ at 30°C (44.0 ± 7.47 vs. 61.1 ± 1.39 µl O_2 $h^{-1}g^{-1}$; ANOVA of ln transformed data; p=0.004, n=8 for obscured crabs and n=10 for control crabs), but rates were not significantly different at 15, 20 or 25°C. Control crabs with half of their leg membranes obscured did not have different $\dot{V}O_2{}^a$ than crabs with no leg membranes obscured (Figs. 2.6A,B). Small specimens of *P. cinctipes* with their leg membranes obscured did not show a reduction in $\dot{V}O_2{}^a$ at 30°C, and rates were similar for all three groups of crabs at each temperature measured (Fig. 2.6B). Consistent with the effects of obscuring the leg membranes on respiration rate, *P. cinctipes* with their leg membranes obscured had a 188% higher lactate concentration after incubation for 5 hours at 28°C than did control crabs (12.54 ± 2.06 vs. 6.64 ± 1.43 mmol g^{-1}; ANOVA; p=0.003, n=6 for each group) (Fig. 2.7).

Sixteen out of 79 species of *Petrolisthes* surveyed possessed a leg membrane structure similar to that found on *P. cinctipes* (data not shown). From the collection data and the data given in Haig (1960) we could find no statistically significant relationship between the species' maximal size and leg membrane occurrence. Due to inadequate collection records, we are unable to ascertain if the leg membrane appeared predominantly in species that occur higher in the intertidal zone.

A)

B)

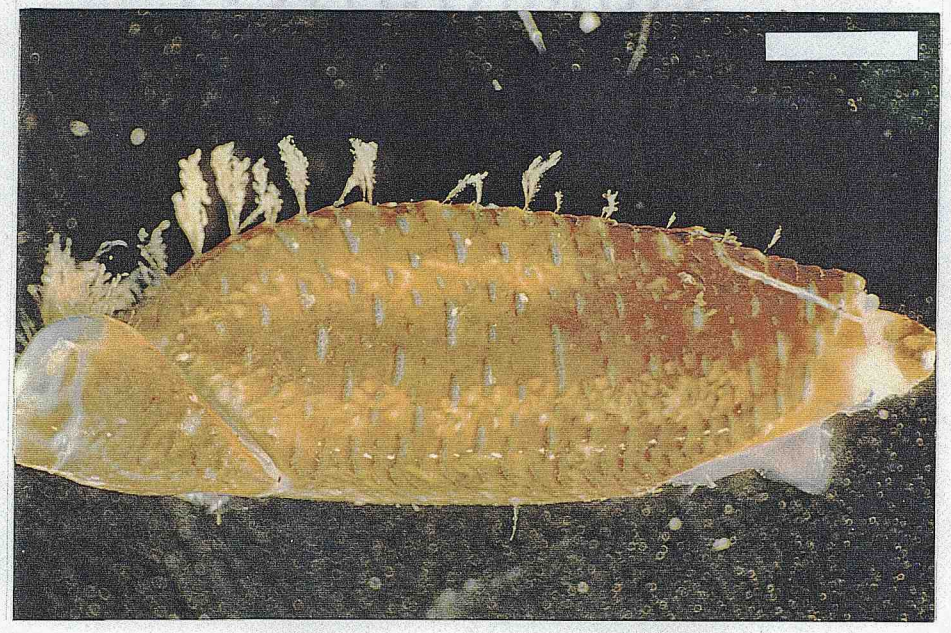

Figure 2.5. Light microscope images of the ventral surfaces of meral segments of the second walking leg in (A) *Petrolisthes cinctipes* and (B) *P. eriomerus*. White scale bar represents 1mm.

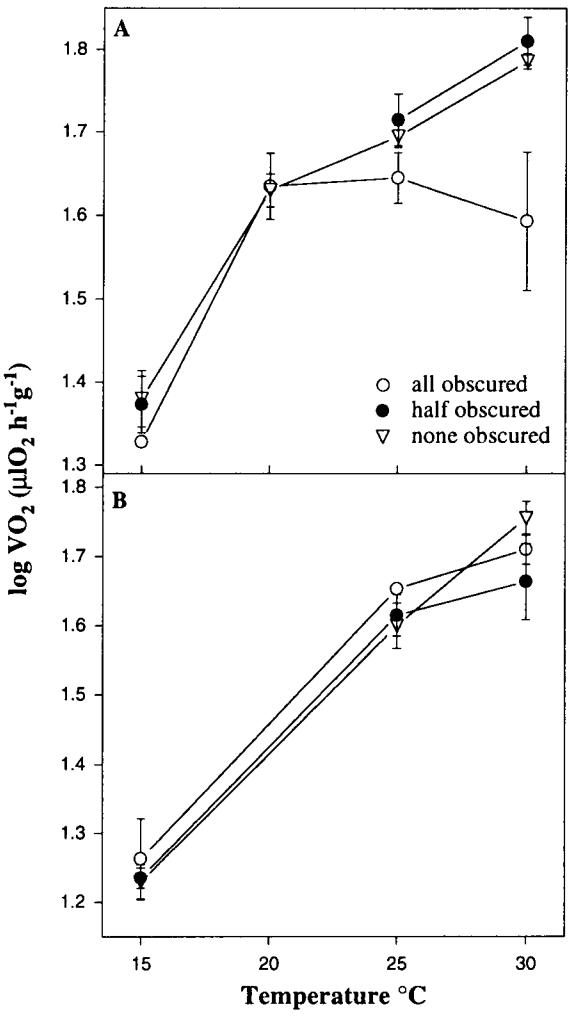

Figure 2.6. Whole animal aerial oxygen consumption of *Petrolisthes cinctipes* with their leg membranes obscured. Points represent mean ± 1 S.E.M. (A) Large specimens, n=4 for all points except for the following: n=6 for no membranes obscured at 20°C, n=8 for all leg membranes obscured at 30°C, and n=10 for no membranes obscured at 30°C. (B) Small specimens n=4 for all groups except for the following: n=3 for all leg membranes obscured at 25 and 30°C and for half leg membranes obscured at 30°C, and n=2 for no leg membranes obscured at 30°C. * denotes a significant difference between the aerial oxygen consumption rates of large specimens of *P. cinctipes* with and without their leg membranes obscured at 30°C (ANOVA; p=0.004).

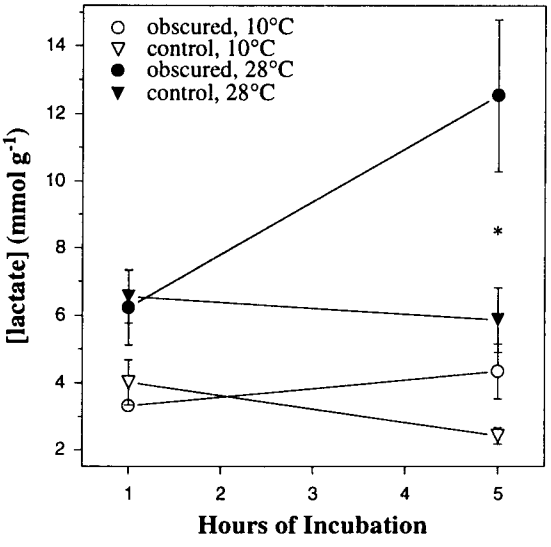

Figure 2.7. Lactic acid accumulation in large specimens of *Petrolisthes cinctipes* with their leg membranes obscured after emersion at 10 and 28°C for 1, 3 or 5 h. Each point is the mean ± 1 S.E.M. for 6 individuals. * denotes a significant difference between crabs with leg membranes obscured and control crabs at 28°C (ANOVA; p=0.003)

Discussion

Our results indicate that *Petrolisthes cinctipes* experiences much longer times of emersion, as well as much higher microhabitat temperatures than does *P. eriomerus*. *P. cinctipes* possesses physiological, biochemical and morphological characteristics that may facilitate its survival in the mid to high intertidal zone, and by the same token, the lack of these characteristics may play a role in restricting *P. eriomerus* to the lower intertidal and subtidal zones. These characteristics include the ability of *P. cinctipes* to respire effectively in air, the higher thermotolerance of heart beat of *P. cinctipes*, and the morphological differences that confer the ability for aerial respiration in *P. cinctipes*.

Habitat differences

Differences in duration and frequency of emersion (Fig. 2.1A) and vertical distribution of macrophytes contribute to the large differences in thermal microhabitats of *Petrolisthes cinctipes* and *P. eriomerus* (Figs. 2.1B,C). At the extremes, *P. cinctipes* can experience emersion for a total of over 12 h per day divided between two low tide periods (Fig. 2.1A), and can experience temperature fluctuations of over 20°C during a low tide (Fig. 2.1B), or, as shown for mussel beds, over 30°C annually (Elvin and Gonor, 1979). Although the temperatures that we measured were not crab body temperatures, because the air underneath rocks is likely to be saturated with water, crab body temperatures and air temperatures are likely to be equivalent (Weinstein, 1995). It is worth noting that the temperatures seen on May 18, 1995 (Figs. 2.1B,C) represent extreme, infrequently occurring temperatures, as the prevailing weather conditions on the Oregon coast are not conducive to creating these extreme temperatures. The infrequency of thermally stressful days, however, does not negate their selective importance; only one day such as the one

we observed on May 18, 1995 would be necessary to subject *P. eriomerus* living above their normal distribution limit to a lethal thermal stress. In addition, although all individuals of *P. cinctipes* are subjected to varying degrees of thermal fluctuation, subtidal *P. eriomerus* never experience emersion, and only experience a 5°C annual temperature fluctuation, associated with upwelling (Barry *et al.*, 1995). The rocks in the low intertidal zone where *P. eriomerus* are found usually harbor large amounts of foliose algae. The algae act as a thermal buffer for rupestrine fauna due to the algae's thermal inertia, as well as the large amount of algae-associated water (both intracellular and extraorganismal) that can support evaporative cooling, hence removing thermal energy from the rock. On May 18, 1995 (the day on which the data in Fig. 2.1B were collected), the algae dried out extensively during low tide, turning black and brittle (pers. obs.).

Rock morphology affects the under-rock temperature, as massive stones, stones without air spaces underneath, or stones with flowing water or air circulation, all do not reach under-rock temperatures as high as flat, thin stones with a small dead air space below. Thus, because most rocks do not meet the above criteria for the thermally maximal microhabitat, not all of the individuals of *Petrolisthes cinctipes* experience temperatures as high as those shown in Figure 2.1B. However, as some individuals do experience thermally stressful microhabitats, the potential selective importance of a high thermal tolerance is maintained. The ability of crabs to select rocks with certain morphology is not known, but observation that large individuals are more common under large stones, suggests that selection of, and competition for shelter may occur. We have never observed *Petrolisthes* moving between rocks when emersed, so they may have a limited ability to behaviorally thermoregulate. Where *P. cinctipes* occur in beds of *Mytilus californianus*, the thermal microhabitat may be more uniform, and potentially as

thermally stressful, as *M. californianus* body temperatures have been measured as high as 33°C, and estimated as high as 37°C (Elvin and Gonor, 1979).

Respiratory responses to abiotic stress

Large differences in respiration rate were observed between conspecifics of different size. The interspecific difference in $\dot{V}O_2{}^a$ of large specimens at 25°C was not seen in small specimens (Figs. 2.2A,B). Hawkins *et al.* (1982) showed a similar size dependence in respiratory responses to emersion in intertidal crabs. Large differences in $\dot{V}O_2{}^a$ between large specimens of *Helice crassa* (an inhabitant of the high intertidal and supratidal zones) and *Macrophthalmus hirtipes* (an inhabitant of the low intertidal and subtidal zones) were apparent at higher temperatures, but there were much smaller differences between small specimens of the same two species. Thus, adaptations facilitating aerial respiration may be most pronounced in terrestrial or semi-terrestrial species that attain a large body size.

Thermal tolerance ranges

Studies of the thermal tolerance limits of heart rate reveal that *Petrolisthes cinctipes* is able to tolerate both higher and lower temperatures than is *P. eriomerus*, and is therefore more eurythermal. This finding, along with the description of the thermal microhabitat that these crabs can experience (above), in part can explain the differential survival of these species when reciprocally transplanted to different vertical intertidal zones. Such transplant experiments revealed that *P. eriomerus* did not survive transplantation to the *P. cinctipes* zone, but that *P. cinctipes* survived when transplanted to the *P. eriomerus* zone (Jensen and Armstrong, 1991).

Our studies of thermal tolerance ranges also reveal that *Petrolisthes cinctipes* may be living at or near the edge of its physiological tolerance limits, as field temperatures measured (Fig. 2.1B) during very hot days were very close to the ABT of heart rate in this species (Fig. 2.2A). Temperatures measured at the top of *Petrolisthes eriomerus* zone never exceeded 25°C, and temperatures measured at the top of *P. cinctipes* zone never exceeded 32°C. Thus, small increases in sea surface temperature, which might translate into small increases in habitat temperature, might profoundly affect species' distribution patterns at the latitudinal and vertical limits of the species' range. A recent comparison of species assemblages in 1931-1933 (Hewatt, 1937) and 1993-1994 (Barry *et al.*, 1995) has shown a decrease in the abundance of *P. cinctipes* from the intertidal zone in Pacific Grove, CA, at the Hopkins Marine station of Stanford University (Barry *et al.*, 1995). Barry *et al.* (1995) also showed an increase in mean sea surface temperature and in maximal sea surface temperature between 1933 and 1993. Whether or not the change in abundance of *P. cinctipes* over this 60 year period is a direct result of increases in water temperature or is a result of other factors, such as microhabitat rearrangement and the settlement characteristics of *Petrolisthes* (Jensen, 1989) remains to be tested. Although thermally induced mortality of *Petrolisthes* has not been observed in the field, we have observed large amounts of mortality of *Hemigrapsus oregonensis* during low tide near Seattle, in the Puget Sound on days when under-the-rock temperatures exceeded 33°C. Mass mortality of *Mytilus edulis* have been reported for mussels during extremely stressful low tide periods (Tsuchiya, 1983).

Petrolisthes eriomerus did not survive temperatures at or below 2°C (Fig. 2.3B), but *P. cinctipes* showed complete recovery from 1.5°C (Fig. 2.3B). Tissue temperatures of *Mytilus californianus* that were as low as -10°C have been estimated (Elvin and Gonor, 1979), and it is likely that during these periods *P. cinctipes* living among mussels

would benefit from having hearts that could remain functional during exposure to low temperatures. Thus, tolerance to cold temperatures could have a strong selective advantage in *P. cinctipes.*

Lactate accumulation

Compared to its congener, *Petrolisthes eriomerus* exhibited an elevated dependence on anaerobic glycolysis when emersed at 25°C, as shown by a significant rise in total body lactate concentration (Fig. 2.4) and a smaller rise in $\dot{V}O_2{}^a$ with rising temperature than seen in *P. cinctipes* (Fig. 2.2). The latter species showed a decrease in total body lactate concentration with time at both 10 and 25°C, but no effect of temperature on lactate concentration (Fig. 2.4). Levels of lactate seen in *P. eriomerus* after aerial incubation were of the same magnitude as those found in the crab *Leptograpsus variegatus* (Forster *et al.*, 1989; Greenaway *et al.*, 1992). Although porcelain crabs are for the most part quiescent during periods of emersion, because they are ectothermic, rising body temperatures will elevate metabolic rates. Unlike its congener, *P. cinctipes* appears to rely largely on aerobic ATP generating pathways during heating, so it is better able to withstand emersion at high temperatures without suffering losses in metabolic efficiency due to shifts from aerobic to anaerobic generation of ATP.

Leg membrane studies

The data presented here support the hypothesis that the leg membrane structures in *Petrolisthes cinctipes* are functional respiratory structures, and are utilized during periods of high metabolic demand while emersed (Figs. 2.6, 2.7). *P. cinctipes* with their leg membranes obscured had a significantly lower $\dot{V}O_2{}^a$ at 30°C and a significantly higher lactate accumulation at 28°C than control crabs (Figs. 2.6, 2.7).

Although leg membranes are not found in the majority of species of *Petrolisthes*, 16 of 79 species examined possessed such structures. Whether the leg membrane structures play a respiratory role in other species of *Petrolisthes*, including subtidal as well as intertidal species, is not known. Although we do not know the vertical distribution for many of the other species of *Petrolisthes* possessing the leg membrane, at least one species, *P. rathbunae*, is mainly subtidal (Haig, 1960). This suggests that totally aquatic species having very high metabolic rates might also utilize this structure for aquatic respiration, although this conjecture remains to be investigated.

The genus *Petrolisthes* is composed of species with widely differing morphological features (claw size, setae distribution, exoskeleton texture), and leg membranes were found on species with morphological features both similar to and very different from *P. cinctipes*. In addition, in two species (*P. tiburonensis*, *P. gracilis*), the leg membrane was only present on the second and third walking legs (middle and most posterior); the first pair had a complete exoskeleton, and in one species (*P. hians*), the leg membrane was only found on the second leg. Without a phylogenetic tree for the genus, and knowledge of the distribution and physiology of other species possessing the leg membrane, it is impossible to infer the function of the leg membrane and its adaptive significance in species besides *P. cinctipes*. The examination of the function of the leg membrane in additional species, along with the development of a phylogeny for the genus, is necessary to elucidate the evolutionary history of the leg membrane as an adaptive feature.

Leg membranes have evolved independently in at least two infraorders of decapod crustaceans: family Ocypodidae (infraorder Brachyura) and family Porcellanidae (infraorder Anomura). Maitland (1986) reports that the gas windows are typical in Scopimerinae, of the family Ocypodidae. The leg membrane of *Scopimera inflata* occurs

on the dorsal side of the merus (Maitland, 1986), but the membrane in *Petrolisthes* spp. are all on the ventral side of the merus. This suggests that the leg membranes have evolved independently in the two groups of crabs.

In summary, temperature stress and aerial exposure are two predominant abiotic factors that influence distribution patterns in the intertidal zone. We have shown that *Petrolisthes cinctipes*, a mid to high intertidal zone inhabitant, has a wider thermal tolerance range than its congener, *P. eriomerus* , which lives in the low intertidal and subtidal zones, and *P. cinctipes* has additional respiratory structures which allow it to maintain an aerobic metabolic state while emersed. One very important finding was that *P. cinctipes* may encounter temperatures near or at their physiological tolerance limits. Increases in sea surface temperature or in air temperature that are associated with global warming could cause lethal temperatures in the current microhabitat of *P. cinctipes*, the long term effects of which might result in changes in the distribution and abundance of the species. Due to the gregarious nature of settlement in *Petrolisthes* (Jensen, 1989), a reduction in population size during one season could lessen recruitment and settlement in successive seasons, and thus affect population size over a long time span. In addition to the potential importance of this species as an indicator of the effects of global warming on intertidal organisms, the different tolerances to temperature in *P. cinctipes* and *P. eriomerus* present a model system for study of temperature adaptation in crustaceans. The genus is highly speciose and species are found from the temperate regions to tropical regions, in the intertidal and subtidal zones, some species having wide distributions, and others narrow ones. These congeneric species provide an excellent study system for addressing the roles that morphological, physiological and biochemical adaptations play in establishing and maintaining species distribution patterns, and in addition provide a group of organisms to study temperature adaptation and the evolution of eurythermality.

Chapter 3

Evolutionary History and Adaptive Significance of Respiratory Structures on the Legs of Porcelain Crabs, Genus *Petrolisthes*

Abstract

Organisms that live in the marine intertidal zone face multiple physiological challenges from the abiotic stresses associated with the transition from water to land. One such physiological challenge is the ability of respiratory structures to function while in air. Crabs are one group of organisms that have evolved multiple strategies for aerial respiration. Most frequently, terrestrial and semi-terrestrial crabs have evolved different gill morphology or developed functional lungs, thereby improving their capability for aerial respiration. In at least two groups of crabs, ghost crabs and porcelain crabs, a third strategy for aerial respiration is present. In these crabs, decalcified areas on the meral segments of the walking legs, termed "leg membranes," are used as respiratory structures. Here, we examine the evolutionary history and adaptive significance of leg membranes in porcelain crabs, genus *Petrolisthes*. Approximately 25% of *Petrolisthes* species worldwide have leg membranes on the ventral surface of the meral segment. However, leg membranes are only found in Eastern Pacific species, of which about 50% possess leg membranes. Interspecific variation in leg membrane size is from 0 to 60% of the surface area of the meral segment. Intraspecific variation is approximately 5 to 10% of the surface area. Leg membrane size is positively correlated with body size across species, but not within one species, *P. cinctipes*. However, in *P. cinctipes*, leg membrane size on

one leg is positively correlated with leg membrane sizes on other legs. In large bodied species that live in the intertidal zone, whole animal lactate accumulation during aerial incubation at elevated body temperatures is 200-300% higher in specimens with their leg membranes obscured, indicating that the leg membranes are functional respiratory structures in these species. However, in some very large or fast subtidal species, leg membranes are inadequate to prevent the need for anaerobic fermentative pathways. Phylogenetic analysis based on sequence data from the 16sRNA gene suggests that the leg membrane phenotype is ancestral to one of two Eastern Pacific *Petrolisthes* clades, and that early leg membranes were small in size. Comparative analyses using phylogenetic independent contrasts indicate a relationship between leg membrane size and body size that is independent of phylogenetic inertia. Thus, it is possible that leg membranes have facilitated the evolution of larger body sizes or increased locomotory activity by providing additional respiratory surfaces to accommodate the higher metabolic demands associated with those traits.

Introduction

Intertidal organisms are routinely exposed to a suite of rapidly fluctuating physical factors as a result of the low-tide-period-generated shift from a marine to a terrestrial habitat. Temperature can increase by over 20°C during a low tide period (Stillman and Somero, 1996), or decrease during winter low tides to below freezing (Elvin and Gonor, 1979). Salinity can decrease significantly when low tide occurs during rainy periods or when freshwater runoff is great (D'Inaco *et al.*, 1992). Additionally, intertidal organisms that do not occur in tide pools are emersed during low tide and thus are bathed in air rather than water (Newell, 1979). The three above-mentioned stresses,

as well as additional stresses such as wave force (Denny, 1988) all pervasively affect aspects of the physiology of intertidal organisms (Newell, 1979). Intertidal organisms have evolved differing levels of physiological tolerance to these stresses, and tolerance limits define the maximum potential vertical distribution in the intertidal zone. Actual patterns of vertical distribution and intertidal zonation, however, reflect the summation of a complex set of factors including physiological tolerance limits, microhabitat preferences, life history characteristics and biotic interactions between species (Connell, 1961; Edney, 1961; Jensen and Armstrong, 1991; Stillman and Somero, 1996).

Organisms that live in the mid-to upper-intertidal zones can spend over 50% of their lives emersed (Stillman and Somero, 1996). Emersion, which results in the loss of the ocean water buffer for temperature and salinity, also creates a large physiological problem to most aquatically respiring organisms (Newell, 1979). Aquatically respiring organisms generally have respiratory structures that are well suited for gas exchange while immersed, but not when emersed. Most aquatic organisms use gills as the main site for gas exchange, and while gills function very well in water, they do not perform well when in air. The drop in gas exchange performance is primarily due to problems involved with preventing the collapse of the gills' fine lammellar structures while emersed (Copeland, 1968). Thus, aquatic-intertidal organisms have two main respiratory strategies while emersed: live with less oxygen, or evolve new ways to aerially respire.

Many intertidal organisms undergo a metabolic suppression to reduce energetic (oxygen) demands or switch to anaerobic fermentation pathways while emersed (Vernberg and Vernberg, 1972). For example, mussels in the genus *Mytilus* close their valves during emersion and switch to very efficient anaerobic pathways (de Zwann, 1977). Some sublittoral crabs that venture out of the water, such as *Cancer productus* and *Carcinus maenas*, retain branchial water, and are thus able to keep their gills in an

"aquatic" environment during periods of emersion (DeFur *et al.*, 1983; Depledge, 1984). However, this branchial water can become quite hypoxic and serves more as a CO_2 sink than an O_2 source (DeFur *et al.*, 1983). These crabs still suffer hypoxia and undergo metabolic suppression during emersion (Depledge, 1984).

The second strategy is to maintain a similar level of oxygen consumption, but evolve new structures with which to obtain the needed oxygen. Such structures are greatly varied among intertidal and semi-terrestrial taxa (Newell, 1979). Some intertidal crabs that retain branchial water remain aerobic by recirculation, through specialized structures, of the water over the outer surface of the carapace, where gas exchange with the atmosphere can occur, thereby providing reoxygenated water to the gills (DeFur, 1988). In terrestrial and semi-terrestrial crabs, changes in gill structure and function have been well documented. Air breathing crabs generally exhibit a reduction in gill number and in gill surface area (Gray, 1957; Hawkins and Jones, 1982). Additionally, the gills of air breathing crabs can be structurally reinforced by thicker epithelial and chitin layers to prevent collapse while emersed (Copeland, 1968). In some cases, gills are replaced with a functional lung, created by enlarging the carapace and adding increased vascularization to the inner lining of the branchiostegites (Farrelly and Greenaway, 1994).

Recently, an additional morphological character facilitating aerial gas exchange in intertidal crabs has been described. This character is a decalcified area on the meral segment of the walking legs, and has been described in two distantly related groups of crabs: brachyuran Ocypodid ghost crabs in the genera *Scopimera* and *Dotilla*, which are endemic to Australia, (Maitland, 1986) and anomuran Porcelain crabs in the genus *Petrolisthes* (Jensen and Armstrong, 1991; Stillman and Somero, 1996). In both groups, these decalcified areas (termed 'leg membranes' by Stillman and Somero, 1996) serve as

secondary respiratory structures during periods of increased metabolic demands during emersion, allowing the crabs to remain aerobic during the emersion period.

The genus *Petrolisthes* is relatively large, with more than 100 species worldwide (Appendix 3). Unlike most genera of crabs, where members specialize on only one type of microhabitat (be it terrestrial, intertidal, or subtidal), species of *Petrolisthes* are found throughout the intertidal and subtidal zones (Haig, 1960; Romero, 1982; Weber Urbina, 1986; Jensen and Armstrong, 1991). In the Eastern Pacific, there are 4 major biogeographic assemblages of *Petrolisthes*: North and South Temperate zones, the Northern Gulf of California, and a tropical fauna, which ranges from Mexico to Ecuador (Carvacho, 1980). In each of these biogeographic regions, there are multiple species living in discrete vertical zones, such that at each location there are a different set of intertidal species and a different set of subtidal species (Haig, 1960; Romero, 1982; Weber Urbina, 1986; Jensen and Armstrong, 1991). Thus, there are four groups of congeners that experience differential amounts of emersion stress. Because of the large number of species, as well as the repeated habitat clines, these crabs provide an excellent study system for addressing fine scale adaptation to emersion stress using the comparative method (Harvey and Pagel, 1991; Garland and Adolph, 1994).

Here, the occurrence, diversity, and functional and evolutionary significance of leg membranes throughout the genus *Petrolisthes* is presented. Both interspecific and intraspecific variation in leg membrane size is analyzed with respect to distribution and body size. Leg membrane function is examined by measuring the respiratory status, as indexed by lactic acid accumulation, during emersion of crabs with their leg membranes obscured (following Stillman and Somero, 1996; Chapter 2).

The evolutionary origins and adaptive significance of leg membranes are examined using a phylogenetic tree for *Petrolisthes* based on molecular sequence data

from the mitochondrial gene for the large subunit ribosomal RNA (16sRNA). This gene has been shown to provide an appropriate amount of sequence divergence for species-level tree construction in other anomuran crabs (e.g. Cunningham *et al.*, 1992; Levinton *et al.*, 1996) and other arthropods (e.g. Fang *et al.*, 1993). Phylogenetically independent contrasts were generated to correct for the effect of phylogenetic inertia in analyses comparing leg membrane size to body size and distribution. Stillman and Somero (1996) showed that in small specimens, the physiological need for leg membranes as aerial respiratory structures was not high. Maximum body sizes among *Petrolisthes* range from ~4 to >30 mm carapace length (a much greater range than used in intraspecific comparisons by Stillman and Somero (1996)), thus questions regarding the proximate and ultimate relationship between leg membrane size and body size can be addressed.

By default, constructing a phylogenetic tree for purposes of comparative studies also resulted in a first time analysis of the relationships among Eastern Pacific porcelain crabs. The phylogenetic relationships among Eastern Pacific taxa, as well as the relationships between Eastern Pacific taxa and some Western Pacific taxa, are presented.

Materials and Methods

Specimen collection and preparation

Specimens used for this study were either part of the archival collections of the Los Angeles County Museum and the National Museum of Natural History, or were hand collected by the author. Because of the presence of thinning of the exoskeleton due to improper fixation or storage in some specimens, only those with very solid carapaces were used. Specimens collected by the author were either dried or were fixed in formalin and stored in 70% ethanol. Before photographic documentation, the carapace length and

width of the specimen was measured, and then walking legs were removed and placed under a dissecting microscope equipped with a photographic extension tube. For at least one specimen of each species, legs were dissected along the thin axis of the leg (anterior-posterior), and the muscle tissue was removed (as in Fig. 2.5). This preparation gave the most striking photographic documentation of leg membrane morphometrics, but non-dissected preparations were equally sufficient for data collection. Color photographs were made of the ventral surface of each leg, and glossy prints were used in subsequent analyses.

Leg membrane size analysis

To quantify the size of the leg membrane, expressed as a percentage of surface area of the ventral side of the merus, the meral segment and the leg membrane portion of the meral segment were excised from the photograph with a razor blade. Both segments were weighed on an analytical balance to the nearest ten-thousandth of a gram (0.1 mg). Percent coverage of the meral surface area by the leg membrane was calculated as the weight of the leg membrane segment divided by the weight of the leg membrane segment plus the non-leg membrane segment of the meral surface. The three pairs of walking legs are referred to as leg 1 to leg 3 (anterior to posterior).

Leg membrane function

For analysis of leg membrane function, specimens were collected and placed into flow through aquaria at an on-site laboratory, where they were continuously immersed. Specimens were allowed the maximal time available to acclimate to similar conditions (see below). *Petrolisthes granulosus, P. violaceus, P. laevigatus* and *P. tuberculatus* were collected at Las Cruces, Chile (33°33'S, 71°36'W), from a bouldery intertidal zone

habitat adjacent to Universidad Católica's marine laboratory, the Estación Costera del Investigaciónes Marínas. These four species were collected on October 12, 1997 and used in experiments on October 17-20, 1997. *P. hirtipes* were collected August 15, 1997 at Pelican Point, Cholla Bay, Puerto Peñasco, Mexico (31°39'N, 113°15'W) and used in experiments on August 18, 1997. The largest specimens available from each species were used in the experiments.

The functional properties of leg membranes from a number of species were examined following the procedure in Stillman and Somero (1996). Leg membranes were obscured by application of two thin coats of nail polish (Revlon creme). Only a few legs were painted at a time to minimize the time that the animals spent out of water. Crabs had either all of their legs painted, or none of their legs painted (a control for the effect of the painting, where half of the legs were painted, was omitted because no effect was seen in previous studies (Stillman and Somero, 1996) (Fig. 2.6A)). Crab legs were painted 2 days before experimentation began. Crabs were incubated in air at elevated body temperatures selected to represent warm, but not extreme, temperatures during low tide (25-28°C for temperate species and 35°C for tropical species). Following a 5 hour incubation period, crabs were frozen immediately by freeze clamping in liquid nitrogen and stored at cryogenic temperatures.

To assay total body lactate, frozen crabs were ground with a mortar and pestle under liquid N_2 and immediately placed into 2 body mass equivalent volumes (2 ml g^{-1}) of 0.75 M $HClO_4$. The mixture was incubated on ice for 10 min and then centrifuged at 10 000 ***g*** for 10 min. The supernatant was neutralized to pH 7.0 by addition of 3 M KOH, incubated on ice for 10 min, and then centrifuged at 10 000 ***g*** for 10 min to remove the $KClO_4$ precipitate. The resulting supernatant was held on ice and used within 1-2 h

for enzymatic determination of lactate concentration using the method described by Noll (1984), with a commercial L-lactic acid kit (Boehringer Mannheim).

Molecular phylogenetics

DNA EXTRACTION, AMPLIFICATION, AND SEQUENCING

Whole genomic DNA from muscle tissue was extracted using the QIAGEN QIAamp tissue kit. DNA extractions were made from at least three different individuals of each species, unless fewer specimens were available, in which case multiple extractions were made from one or two individuals such that a total of three DNA extracts were available. Whole genomic DNA from each extract was used in polymerase chain reactions (PCR (Mullis and Faloona, 1987; Saiki *et al.*, 1988)) to amplify a 550 base pair region of the mitochondrial 16s ribosomal gene with primers 16SAR (5'-CGCCTGTTTATCAAAAACAT -3') and 16SBR (3'-CCGGTCTGAACTCAGATCACGT -5') (Palumbi *et al.*, 1991; Cunningham *et al.*, 1992). Triplicate reactions were performed from each DNA extract in order to minimize the effects of any mistakes made by the *Taq* DNA polymerase enzymes. Reactions consisted of 67 mM Tris-HCl, 6.7 mM $MgCl_2$, 16.6 mM $(NH_4)_2SO_4$, 0.07% βME, 0.25 mM each (dATP, dGTP, dCTP and dTTP), 2 μM 16SAR and 16SBR primers, 0.5 Unit *Taq* and 2 μl of a 1:50 dilution of the whole genomic DNA extraction, in a total reaction volume of 50 μl. Thermal cycling conditions were 30 cycles of 94°C for 40 seconds, 50°C for 90 seconds and 72°C for 120 seconds, followed by a 10 min incubation at 72°C. Reactions were immediately stored at 4°C. Thermal cycling was performed in a MJ-Research PTC-100 Hot Bonnet thermal cycler in 200μl thin walled tubes. No oil was added to overlay the reactions.

The entire reaction volumes were electrophoresed through a 0.5X TAE buffered (20 mM Tris-HCl, 0.057% glacial acetic acid, 0.5 mM EDTA, pH 7.8) 1% agarose gel pre-stained with ethidium bromide. Following photography under UV light, bands of about 550 base pairs were excised. Glass wool filters (a 700 µl microfuge tube with a small hole in the bottom over which was placed a small wad of glass wool) were used to separate PCR products from the agarose matrix. The gel slices were placed on top of the glass wool and the filters were placed into a larger microcentrifuge tube (1.7 ml) to collect the filtrate and centrifuged at 325 ***g*** for 2-5 min, or until no more liquid passed through the filter. Filtrates from separate reactions of the same DNA extract were pooled and DNA was precipitated from the filtrate by addition of 0.1 volumes of 3 M sodium acetate, pH 5.2 and 2 volumes of 100% ethanol followed by overnight incubation at -20°C. Precipitated DNA was pelleted by a 35 min centrifugation at 16,600 ***g***, the pellet was washed twice with 70% ethanol, aspirating the wash alcohol with a drawn pipette, and the pellet was resuspended in 25 µl water. After resuspension, 1 µl of DNA was electrophoresed (as above) with a molecular size and quantification standard (Promega pGEM Markers) placed into an adjacent lane. Band intensity under UV light of the cleaned thermal cycle product and the molecular weight and quantity standard was compared, and the standard with intensity closest to the product was selected. Based on the standard, the quantity of product was calculated as follows: (standard band quantity(ng))•(standard band size(bp)/product size(bp))=product quantity(ng), and since one µl of the product was electrophoresed, the product quantity represents ng/µl.

Sequencing was performed using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit. To 8µl of the terminator mix, 50-100 ng of DNA and 3.2 pmoles primer were added in a final reaction volume of 20 µl. Cycle sequencing was performed following the manufacturers protocols: 25 cycles of 96°C for 30 seconds,

50°C for 15 seconds and 60°C for 4 min followed by storage at 4°C. Following the thermal cycling, extension products were purified by ethanol precipitation by adding the entire 20 µl reaction to a microfuge tube containing 2 µl of 3 M sodium acetate, pH 5.2 and 50 µl of 100% ethanol and placing this on ice for at least 30 min. Following the ice incubation, the microfuge tubes were centrifuged at 16,600 ***g*** for 30 min and the DNA extension product pellets were cleaned as above and vacuum dried. After pellets were completely dried, they were stored in the dark at 4°C until electrophoresis. Extension products were electrophoresed through an acrylamide-urea gel (Owl Scientific) (6% Acrylamide/bis acrylamide (19:1), 8.3 M Urea, 1X TBE (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA, pH 8.3)) using a Perkin Elmer ABI 373 Automated sequencer. Sequence data were analyzed by eye, ambiguous nucleotides appropriately coded, and one consensus sequence was generated for each species.

PHYLOGENETIC ANALYSES

16sRNA sequences were aligned using Clustal-W, and adjusted by eye using a map of the secondary structure of 16sRNA as a guide (Gutell, 1993). For phylogenetic analyses, only unambiguously aligned sequence data were used. Data removed occurred in loop regions of the secondary structure, especially in a loop region from 210 to 250 base pairs from the 5' end of the segment (Appendix 4).

Phylogenetic analyses were performed using several programs from the suite of phylogenetic software known as PHYLIP version 3.5c (Felsenstein, 1989). Phylogenetic trees were generated using three different methods: parsimony, maximum likelihood, and distance using the software programs DNAPARS, DNAML, and DNADIST, respectively. (General descriptions of the logical framework of these methods, as well as the generation of independent contrasts (see below) are provided in Appendix 2).

Parsimony and Distance analyses were each performed with 100 bootstrapped data sets for evaluation of the statistical robustness of phylogenetic nodes. During tree construction, species were added to the trees in random order. Randomization was performed to minimize the amount of sampling error encountered during branch and bound tree construction, where once a basic tree morphology is selected, exploring alternate basic tree topologies is restricted (Swofford *et al.*, 1996). Distance matrices were created using a maximum likelihood model because this model allows for unequal base composition and unequal numbers of transition (purine↔purine or pyrimidine↔pyrimidine) and transversion (purine↔pyrimidine) mutations. Since mitochondrial DNA is very rich in adenine (A) and thymine (T) nucleotides, and because transitions tend to greatly outnumber transversions, the maximum likelihood model should produce the most conservative pair-wise distances (Swofford *et al.*, 1996). Nodes (points of common ancestry) on consensus trees that were supported by bootstrap values of >60%, or that were given distances significantly ($p<0.01$) greater than zero by maximum likelihood analyses were accepted as real – all other nodes were collapsed.

Independent contrasts analyses

Independent contrasts (Felsenstein, 1985) were generated using the CAIC software package (Purvis and Rambaut, 1995) and the results of PHYLIP analyses. Contrasts of leg membrane size, body size (mm carapace length) and vertical intertidal position were generated. These contrasts were used in linear regression analyses where the regression was forced through the origin, as is required for analyses of independent contrasts (Purvis and Rambaut, 1995).

Results

Occurrence and size variation of leg membranes

A total of 101 species that are currently, or were formally, classified within the genus *Petrolisthes* (including 95 species of *Petrolisthes*, three species of *Allopetrolisthes*, one species of *Liopetrolisthes*, one species of *Neopetrolisthes*, and one species of *Parapetrolisthes*) were examined for the presence of leg membranes (Appendix 3). Leg membranes were only present in Eastern Pacific and Western Atlantic species, where they are found on 22 out of a total of 55 species of *Petrolisthes* (20:42 Eastern Pacific and 3:13 Western Atlantic), and 1 out of 3 *Allopetrolisthes*. The leg membranes vary in size (where present) from 1% to 60% of the meral surface area, and are sometimes present on only two of three pairs of walking legs (Fig. 3.1, Table 3.1).

For two species, I found sufficient variation to report two different morphotypes: *Petrolisthes gracilis* and *P. tridentatus*. In *P. gracilis*, one morphotype has no leg membranes on leg one and small leg membranes on the other legs (8-9% surface area). The other morphotype of *P. gracilis* has leg membranes on all three legs (15-33% surface area) (Table 3.1). In *P. tridentatus*, Pacific specimens (*P.* cf *tridentatus*) have a membrane on all three legs (20-30% surface area), whereas in the Atlantic specimens, there are no membranes on the first legs and small membranes on legs two and three (13-19% surface area) (Table 3.1).

Intraspecific variation of leg membrane size was examined in *Petrolisthes cinctipes*. Leg membranes from 88 individuals were measured and the leg membrane sizes were plotted as a function of body size (Figs. 3.2A-C). Plots were made comparing leg membrane size on one leg to leg membrane size on a second leg (Figs. 3.2D-F). Regression analyses indicate that while leg membrane size is not correlated with

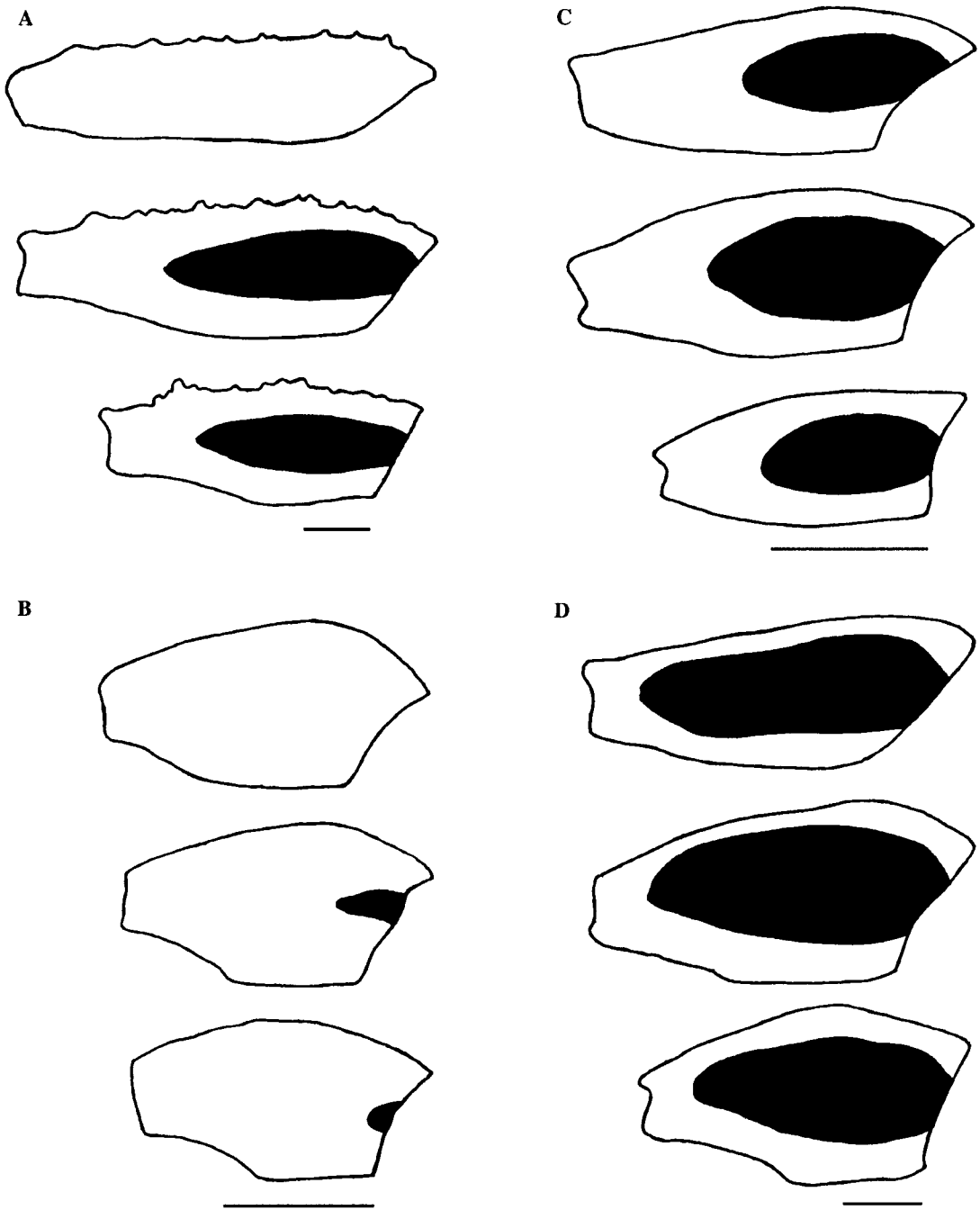

Figure 3.1. Tracings of photographs of the ventral merus surface from four species, showing occurrence of leg membranes (areas shaded by black). Legs are arranged leg 1 to leg 3, top to bottom, for each species. Species are A) *Petrolisthes tiburonensis* B) *P. granulosus* C) *P. cabrilloi* and D) *P. tuberculosus*. Scale bars represent 4 mm.

Table 3.1. Leg membrane size variation throughout the genus *Petrolisthes*.

species[1]	n	max size[2]	vert dist[3]	leg 1[4]	leg 2[4]	leg 3[4]
brachycarpus	1	6.0	n/a	0.0±n/a	6.3±n/a	5.4±n/a
cabrilloi	1	15.4	4	28.1±n/a	38.2±n/a	33.3±n/a
cinctipes	88	21.1	4	38.8±3.1	40.5±3.4	31.0±3.6
galapagensis	1	10.1	n/a	34.3±n/a	40.7±n/a	39.7±n/a
gertrudae	1	4.4	4	18.5±n/a	12.8±n/a	23.6±n/a
gracilis-1[5]	4	10.4	5	0.0±0.0	8.1±0.7	9.1±0.8
gracilis-2[5]	4	10.4	5	14.8±2.4	29.9±4.2	33.4±6.0
granulosus	5	14.5	5	0.0±0.0	4.7±1.5	1.1±0.0
hians	1	5.6	n/a	2.1±n/a	12.5±n/a	7.3±n/a
hirtipes	16	11.1	3	50.3±5.0	54.5±3.6	51.6±3.9
laevigatus	25	23.8	4	59.3±4.6	60.0±4.5	56.1±6.4
lewisi austrinsus	15	6.0	3	7.0±0.0	12.5±8.1	5.1±1.3
lewisi lewisi	1	6.0	3	6.9±n/a	11.8±n/a	5.1±n/a
nigrunguiculatus	1	9.6	n/a	40.0±n/a	44.9±n/a	47.3±n/a
quadratus	1	7.0	6	0.0±n/a	22.9±n/a	14.2±n/a
rathbunae	1	18.6	2	43.4±n/a	41.7±n/a	34.5±n/a
schmitti	1	6.0	2	12.5±n/a	22.5±n/a	25.1±n/a
tiburonensis	1	11.8	3	0.0±n/a	27.0±n/a	33.4±n/a
tonsorius	1	11.6	n/a	59.5±n/a	56.1±n/a	53.2±n/a
tridentatus[6]	2	6.0	5	0.0±0.0	18.4±2.8	12.8±3.1
cf *tridentatus*[6]	17	6.3	5	19.4±n/a	28.2±4.7	29.1±5.2
tuberculatus	21	22.0	2	49.8±5.0	52.8±4.8	50.0±8.4
tuberculosus	10	30.0	1	53.2±4.5	57.1±6.4	55.9±4.5
violaceus	23	27.0	3	50.5±5.4	47.5±3.8	39.0±3.5
A. spinifrons	1	20.3	2	36.7±n/a	36.3±n/a	44.6±n/a

1. Species listed are all of those examined that possessed leg membranes, and all fall within the genus *Petrolisthes* except for one species, *Allopetrolisthes spinifrons*. Species surveyed include those in the collections of the Los Angeles County Natural History Museum and the National Museum of Natural History.
2. Maximum body size in mm carapace width. Data are from Haig (1960) and for *P. gertrudae*, from Werding (1996).
3. Vertical distribution (highest for each species) in the intertidal zone coded as 6=splash zone to 1=subtidal. Data are from (Chace and Hobbs, 1969; Romero, 1982; Weber Urbina, 1986; Jensen and Armstrong, 1991; Werding, 1996), (Fig. 4.1).
4. Data represent mean percentage of meral surface area occupied by leg membrane ± 1 standard deviation, or n/a where n=1.
5. Distinction between two types of *P. gracilis* made only on the basis of leg membrane phenotype.
6. Distinction between two types of *P. tridentatus* made by geographic separation and color in life. *P. tridentatus* is the Caribbean form, *P.* cf *tridentatus* the Pacific form.

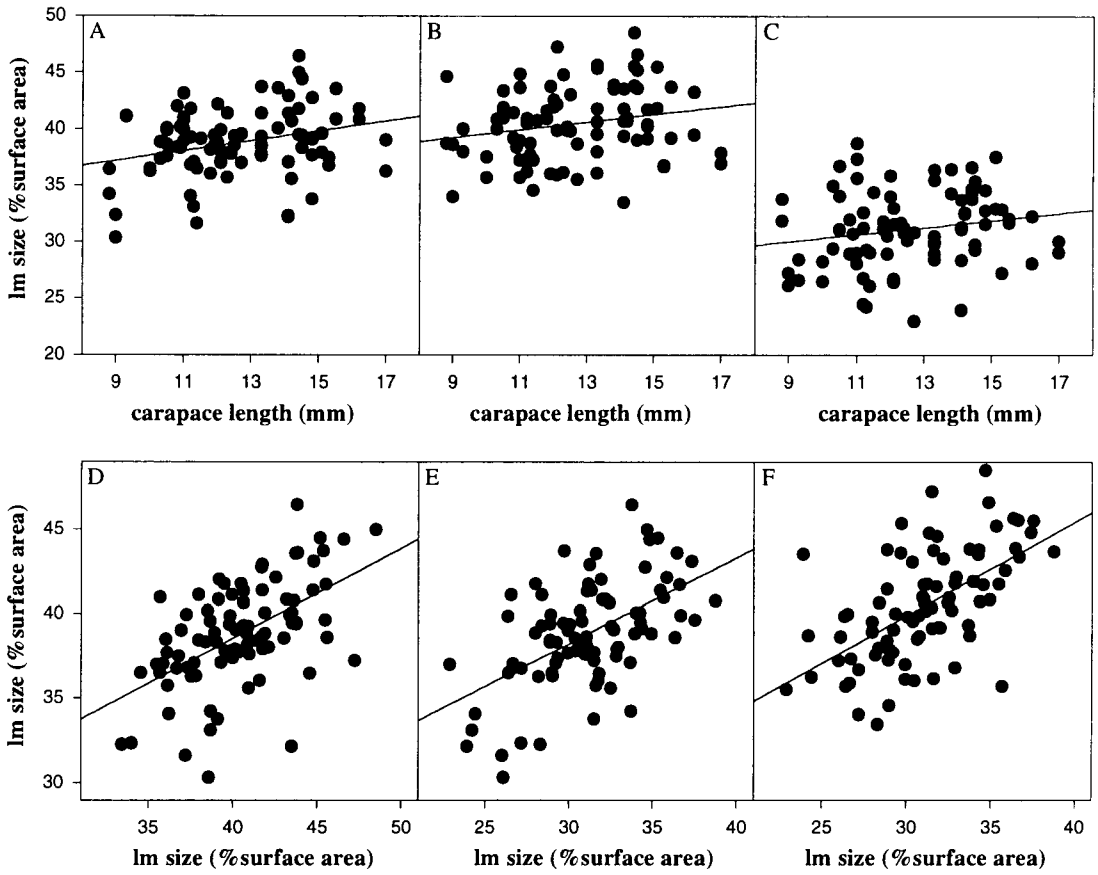

Figure 3.2. Variation of leg membrane size in *Petrolisthes cinctipes*. Leg membrane size as a function of body size on leg 1 (A), leg 2 (B) and leg 3 (C). Leg membrane size on one leg as a function of leg membrane size on another leg for comparisons of leg 1(y-axis) vs. leg 2(x-axis) (D), leg 1(y) vs leg 3(x) (E) and leg 2(y) vs leg 3(x) (F). Regression coefficients for each plot are in Table 3.2.

body size, individuals with large leg membranes on one leg tend to have large membranes on the other two legs as well (Table 3.2). Inter-individual genetic or ontogenetic differences explain about 30% ($r^2 = 0.30$ to 0.34) of the variation in leg membrane size in *P. cinctipes* (Table 3.2).

Interspecific variation in leg membrane size is positively correlated with each species' maximum body size in all legs, and for total leg surface area covered by leg membrane (Figs. 3.3A-D, Table 3.2). Body size explains as much as 50% ($r^2 = 0.35$ to 0.50) of the variation in leg membrane size (Table 3.2)

Leg membrane function

Obscuring the leg membrane resulted in large differences in whole animal lactate accumulation in intertidal species that have large leg membranes (i.e. *Petrolisthes hirtipes*, *P. laevigatus* and *P. cinctipes* (Fig. 3.4) (t-test, $p<0.05$). Obscuring the leg membranes, however, did not have an effect in *P. granulosus*, a high intertidal species with very small membranes (Figs. 3.1, 3.4, Table 3.1). Obscuring the leg membranes of *P. violaceus*, a low intertidal, large bodied crab and *P. tuberculatus*, a subtidal, extremely active crab, did not result in lactate accumulation levels that were different from control levels (Fig. 3.4). However, in *P. violaceus* and *P. tuberculatus*, both treatment and control lactate accumulation were high (Fig. 3.4) unlike *P. granulosus*, where both treatment and control lactate accumulation were low. Lactate accumulation of control specimens was highest in *P. violaceus* and *P. tuberculatus*, and lowest in *P. granulosus* and *P. hirtipes*, two small-bodied crabs (Fig. 3.4, Table 3.1).

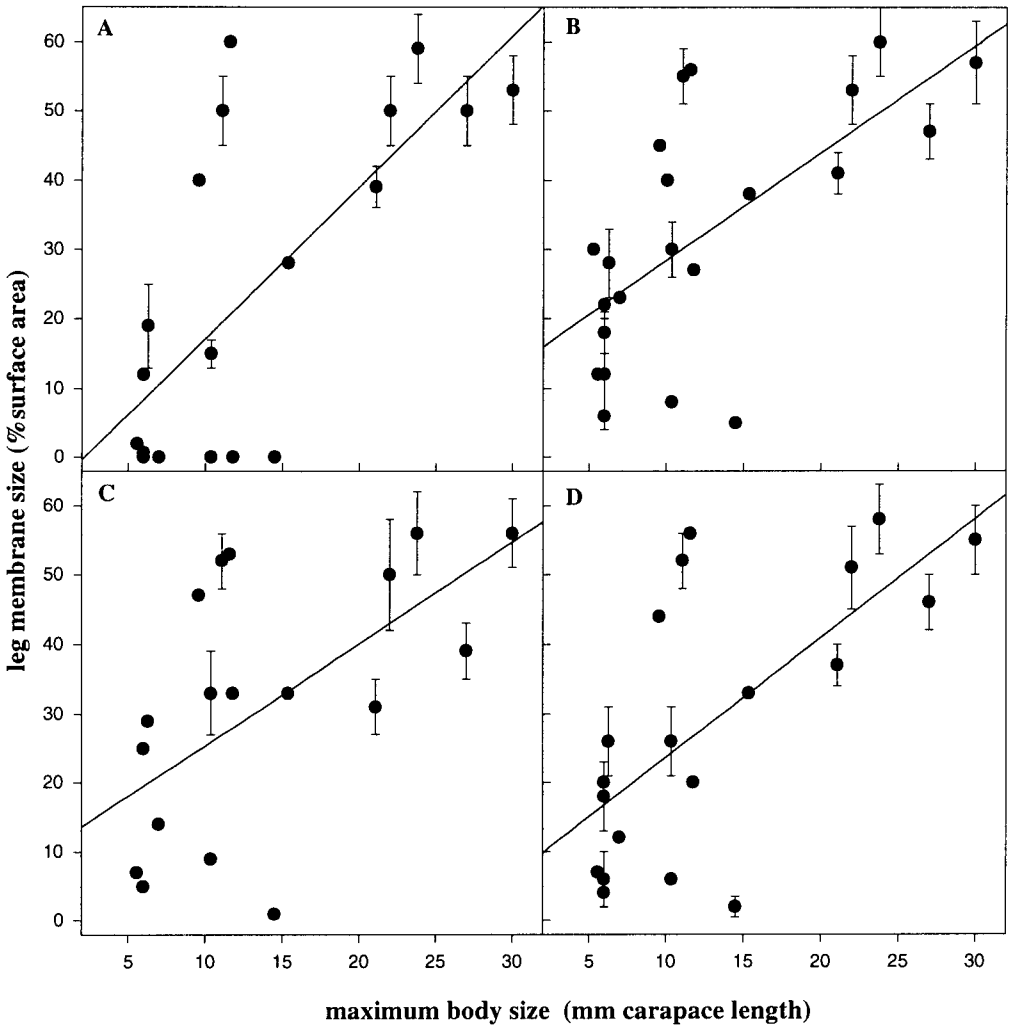

Figure 3.3. Interspecific comparison of the relationship of leg membrane size to body size. Plots are of: leg 1 (A), leg 2 (B), leg 3 (C), total leg surface area (D). Each point represents a different species, and error bars are 1 S.D. Data are from Table 4.1. Regression coefficients for each plot are in Table 3.2.

Table 3.2. Results of regression analyses from Figures 3.2 and 3.3.

Figure:	Regression Parameters:[1]				
	b	m	r^2	F	P
3.2					
A. leg 1 vs. body size	33.33	0.43	0.077	7.1	0.0093
B. leg 2 vs. body size	36.20	0.34	0.045	4.1	0.0474
C. leg 3 vs. body size	27.15	0.31	0.034	3.1	0.0858
D. leg 1 vs. leg 2	17.25	0.53	0.309	38.5	1.9×10^{-8}
E. leg 1 vs. leg 3	23.03	0.51	0.301	37.1	3.1×10^{-8}
F. leg 2 vs. leg 3	23.10	0.57	0.336	42.1	0.5×10^{-8}
3.3					
A. Leg 1 vs. body size	-4.71	2.19	0.504	18.31	0.0005
B. Leg 2 vs. body size	12.84	1.55	0.425	14.76	0.0010
C. Leg 3 vs. body size	10.68	1.47	0.354	9.30	0.0073
D. Total vs. body size	6.38	1.73	0.454	14.98	0.0011

1. Linear regression parameters from y=b+mx.
2. F and P are results of regression ANOVA (1, 86 degrees of freedom for Figures 3.2A-F and 1,17, 1,20, 1,18 and 1,18 for Figures 3.3A-D).

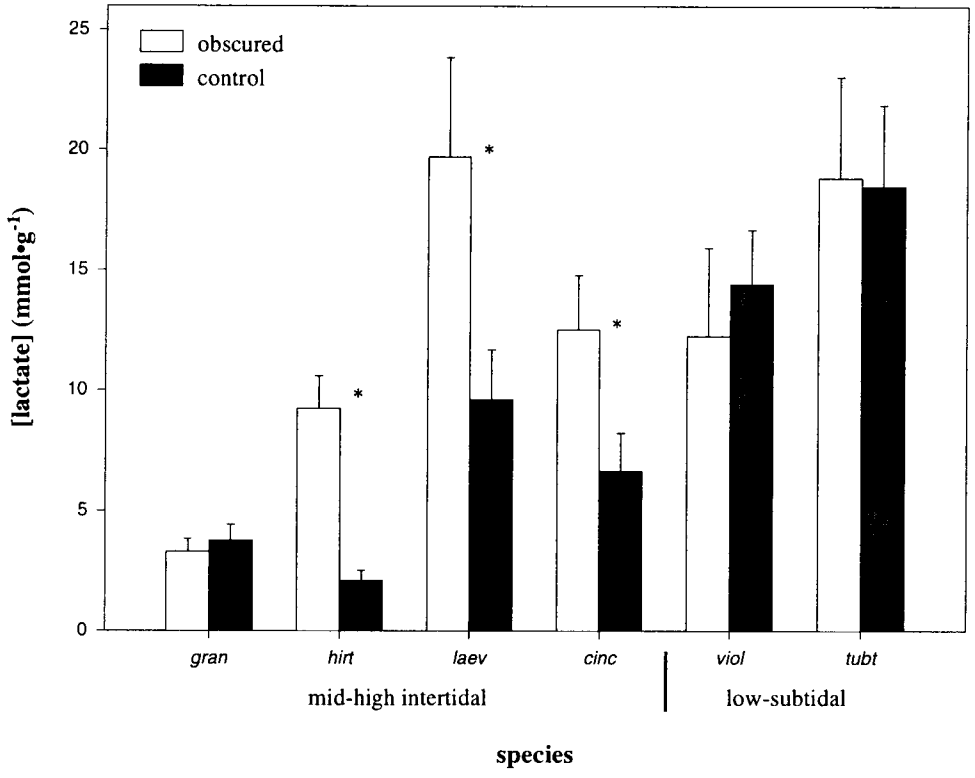

Figure 3.4. Whole animal lactate accumulation following 5 hour emersion periods at moderately high temperatures in 6 species of *Petrolisthes*. Species are coded as follows: *gran - P. granulosus, hirt - P. hirtipes, laev - P. laevigatus, cinc - P. cinctipes, viol - P. violaceus, tubt - P. tuberculatus*. Error bars are 1 S.E.M., and n=5 for all species, except *P. laevigatus*, where n=4. Asterisks denote a statistically significant difference between lactate accumulation of specimens with leg membranes obscured and controls (ANOVA, $p<0.05$). Species are arranged by microhabitat and by metabolic demands, as indexed by size and activity level; high intertidal zone, low metabolic rate is towards the left, and low intertidal zone, high metabolic rate towards the right. Data for *P. cinctipes* are from Stillman and Somero (1996), Figure 2.7.

Phylogenetic analyses

DNA sequences for the 16sRNA gene were usually invariant among conspecifics, although occasionally there were polymorphisms at a single site (Appendix 4). It is uncertain if the discrepancies were due to experimental error in sequence determination or are due to real genetic polymorphism within the population. The region of the 16sRNA gene amplified from different species were sometimes different in length, some containing more sequence in the 3'- or 5'-end regions than others. Following alignment, sequences from all species were truncated such that all of the sequences were set to the same start and end codon by deleting extra 3'- or 5'-end data. Loop regions also were sometimes different in length, and gaps were inserted as necessary for proper sequence alignment (Appendix 4). Sequence data from additional species of *Pachycheles*, from different populations of *Petrolisthes armatus* (Northern Gulf of California, Pacific Panama, Atlantic Panama), and from several color-morphs of *P. galathinus* are also presented in Appendix 4. Data from only one of each of the three above species groups are used in the phylogenetic analyses presented here.

Phylogenetic trees are presented here in a condensed form (Fig. 3.5). Distance and parsimony analyses (Fig. 3.5, left tree) resulted in identical tree topologies, although bootstrap values varied between the two methods at some nodes (e.g. the node at the separation of *Petrolisthes novaezelandiae* and *P. elongatus* is supported by a bootstrap value of 62 by the distance analysis vs. 85 by parsimony analysis). Maximum likelihood analysis resulted in trees that had different topologies in some regions (Fig. 3.5, right tree). Usually, the maximum likelihood tree topology varied in regions with short branch lengths (but notice the rearrangement of the position of *P. haigae*) (Fig. 3.5).

The phylogenetic tree developed for the generation of phylogenetic independent contrasts (Fig. 3.6; PIC tree) was constructed using the distance method.

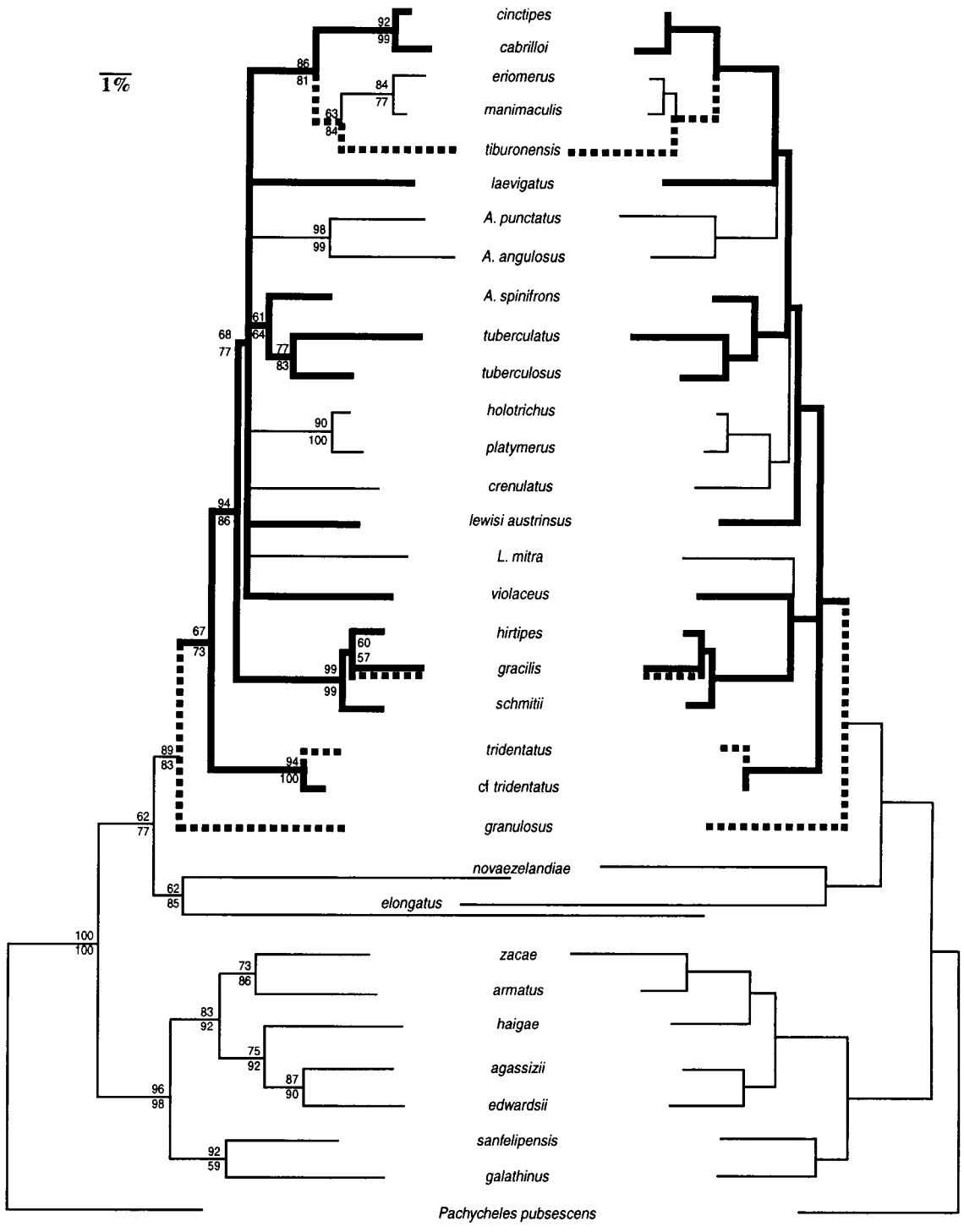

Figure 3.5. Phylogenetic trees of porcelain crabs. The left tree represents the results of distance and parsimony analyses, and the right tree the results of maximum likelihood analyses. Bootstrap values for distance (top) and parsimony (bottom) analyses are shown on the left tree. Species are in the genus *Petrolisthes* except those with generic initial A (*Allopetrolisthes*), L (*Liopetrolisthes*), or *Pachycheles*. Thickened lines indicate presence of the leg membrane phenotype; Black lines represent those species with all legs possessing membranes and dashed lines those species without membranes on leg 1.

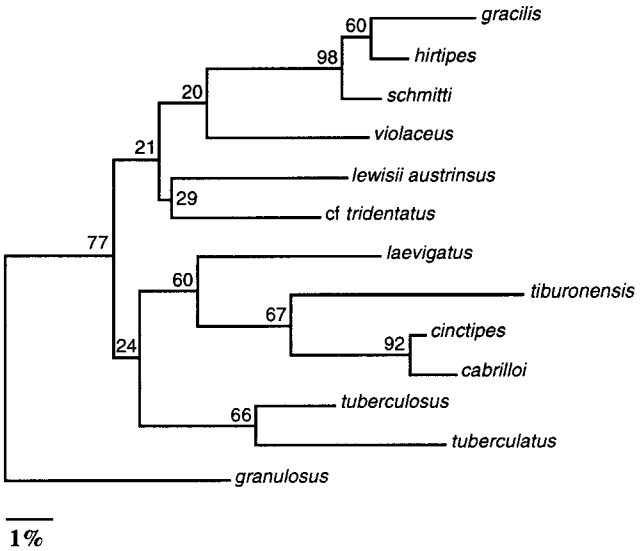

Figure 3.6. Phylogenetic tree used for generation of independent contrasts. These 13 species of *Petrolisthes* are those that possess leg membranes and for which data are available on leg membrane size, intertidal zone distribution, and 16sRNA sequence.

The PIC tree, which contains a subset of the species for which sequence data and leg membrane data were available, retained the same basic topology as the tree including all the taxa (Fig. 3.5). Bootstrap values for some of the nodes were greater in the PIC tree (Fig. 3.6). Specifically, *Petrolisthes laevigatus* is grouped with the North Temperate species, supported by a bootstrap value of 60 (Fig. 3.6), whereas this grouping was not supported by bootstrap analyses when all the taxa were included (Fig. 3.5, left tree).

The four weakly supported nodes (i.e. bootstrap values < 60) in the PIC tree have been retained. The retention of these nodes was made on the basis of their presence in the maximum likelihood analysis of all of the taxa (Fig. 3.5). In this tree (Fig. 3.5, right tree), *P. violaceus* is grouped with the same group of species as in the PIC tree (Fig. 3.6). Additionally, the relative position of *P. laevigatus* grouped with *P. cinctipes*, *P. cabrilloi* and *P. tiburonensis* in the PIC tree (Fig. 3.6) is concordant with the maximum likelihood tree (Fig. 3.5, right hand tree). One discordant grouping is that of *P.* cf *tridentatus* together with *P. lewisi austrinsus* in the PIC tree (Fig. 3.6), while *P.* cf *tridentatus* is ancestral to *P. lewisi austrinsus* in the maximum likelihood analysis (Fig. 3.5, right tree).

Independent contrasts analyses

Independent contrasts of the total percentage of meral surface area covered by leg membranes were generated and plotted against contrasts of maximum body sizes and vertical intertidal zone distributions (Fig. 3.7). Results of regression analyses indicate that there is a positive correlation between leg membrane size contrasts and body size contrasts (Fig. 3.7, filled circles, regression line shown) but not between leg membrane size contrasts and vertical position contrasts (Fig. 3.7, open circles, no regression shown). The slope of the regression line is 0.017 (Fig. 3.7), identical to the slope found by regression analyses of non-phylogenetically corrected data (Fig. 3.3D, Table 3.2).

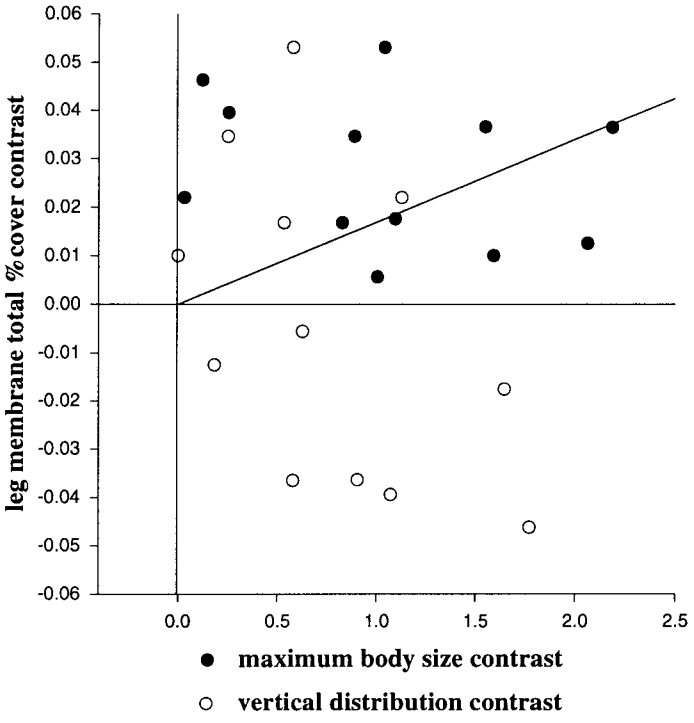

Figure 3.7. Independent contrasts of leg membrane total percent coverage plotted against independent contrasts of maximum body size and vertical distribution. Recall that when a contrast is plotted vs. a contrast, data can fall anywhere to the right of the x=0 line (but they can have any y-value). Also recall that regression of a contrast vs. contrast plot must be forced through the origin (see Appendix 2 for more details). Regression analysis of leg membrane coverage contrast vs. maximum body size contrast indicated a positive correlation between these variables (solid line, y=0.017x), as all of the data points fell above the y=0 line. Regression analyses of leg membrane coverage contrast vs. vertical position contrast did not indicate a significant relationship between the two variables, and thus no line is shown.

Discussion

Leg membrane size variation

The size of leg membranes varies from 0 to 60% of the surface area of the ventral side of the meral segment of the walking legs (Fig. 3.3, Table 3.1). Intraspecific variation in leg membrane size is less than the amount of interspecific variation (Figs. 3.2A-C, 3.3A-D, Table 3.1). The correlation of leg membrane size between legs of individual specimens (Figs. 3.2D-F, Table 3.2) suggests that genetic or ontogenetic effects may account for about 30% of the leg membrane size variation (Table 3.2). To demonstrate that the intraspecific variation in leg membrane size is caused by ontogenetic effects, it would be necessary to be able to define differences within the microhabitat of each species, or identify differences between individuals during development. Although studies of intraspecific ontogenetic variation have not been performed, variation in leg membrane size does not appear to be related to body size, with larger animals having larger variances, as one might expect if there were environmentally induced differences in leg membrane size (Figs. 3.2A-C). Assuming that the variation is genetic and not created by environmentally-induced ontogenetic differences, then the potential for natural selection to produce variation in leg membrane size exists (Feder, 1987).

Maximum body size in species that possess leg membranes ranges from about 5 to about 30 mm carapace length. This size range represents almost the entire size range for all of the species within this genus, and for almost all of the species within the entire family Porcellanidae. While this size range is small when compared to classical studies of physiological scaling (e.g. the "shrew to elephant" curve of size specific oxygen consumption rates (Schmidt-Nielsen, 1991)), it is sufficient to see a positive relationship between leg membrane size and body size (Figs. 3.3A-D).

Functional significance of leg membranes

Measurement of whole-organism lactate levels following a 5-hour aerial incubation of six species of *Petrolisthes* (Fig. 3.4) indicated that possessing leg membranes allowed intertidal species (*P. hirtipes*, *P. cinctipes*, and *P. laevigatus*) to remain aerobic while emersed, but that low-intertidal and subtidal species (*P. violaceus* and *P. tuberculatus*) were unable to remain aerobic (Fig. 3.4). Additionally, in the small-bodied *P. granulosus* (Table 3.1), whose leg membranes are small (Fig. 3.1B, Table 3.1), the obstruction of the leg membranes had no effect on lactate accumulation (Fig. 3.4). Thus, the functional capabilities of the leg membranes of intertidal species appear to be adequate in supporting aerial respiration during low tide periods. In the low intertidal and subtidal species, which do not normally experience long periods of emersion, the functional significance of the leg membranes as respiratory structures remains uncertain. More studies of the function of leg membranes of low intertidal and subtidal species (e.g. *P. violaceus*, *P. tuberculatus*, and *P. tuberculosus* (Table 3.1)) as a function of time, temperature, and emersion (as in Stillman and Somero (1996), Figs. 2.4, 2.7) may provide a greater understanding of the respiratory function of leg membranes in those species. In *P. cinctipes*, obstruction of leg membranes did not alter aquatic oxygen consumption rates (Chapter 2). However, the role of leg membranes as aquatic respiratory structures may be different in the above mentioned species.

Phylogenetic analyses

From the phylogenetic trees developed in this study (Fig. 3.5), the following observations regarding the evolutionary history of Eastern Pacific *Petrolisthes*, and the evolutionary history of leg membranes can be made:

1. **Overall tree topology:** The genus *Petrolisthes* can be subdivided into two main clades, one of which contains only "spiny" species (*P. galathinus, P. sanfelipensis, P. edwardsii, P. agassizii, P. haigae, P. armatus* and *P. zacae* (Haig, 1960)). These "spiny" species possess regular serrate teeth on the anterior margin of the carpal segment of each claw and can have many small spines on various parts of the carapace, walking legs and claws. Species in this clade can also have striated carapaces, as is seen in *P. agassizii, P. edwardsii, P. galathinus* and *P. sanfelipensis* (Haig, 1960).

The second clade contains crabs that generally have "smooth" carapaces, and do not possess spines on the carapace or other parts of the body. If members of this group have teeth on the anterior margin of the carpal segment of the claws, the teeth are irregular and non-serrate (Haig, 1960). This split is an easy one to make on the basis of morphological characters alone, but confirmation by molecular data is reassuring in assessment of the appropriateness of the 16sRNA gene for the phylogenetic analyses.

The position of two "smooth" Western Pacific species, *Petrolisthes elongatus* and *P. novaezelandiae*, with the Eastern Pacific "smooth" species (Fig. 3.5) suggests that the genus *Petrolisthes* may have split into the "spiny" and "smooth" clades prior to trans-Pacific migration.

The overall tree topology also suggests that there are some taxonomic issues that need further attention within the family Porcellanidae. Species in the genera *Liopetrolisthes* and *Allopetrolisthes* fall well in the "smooth" clade of the genus *Petrolisthes*, suggesting that their placement in separate genera may not be warranted.

2. **Topology of "smooth" clade:** While the placement of *Petrolisthes granulosus* as an ancestral outgroup to the "smooth" Eastern Pacific *Petrolisthes* is consistent among distance, parsimony, and maximum likelihood analyses, relationships within the remainder of the "smooth" species are more difficult to resolve. Distance and parsimony

analyses (Fig. 3.5, left tree) place the two *P. tridentatus* types as the next most ancestral, and the clade consisting of *P. hirtipes*, *P. gracilis* and *P. schmitti* as the third most ancestral. The remainder of the species have unresolved roots, although there are four bootstrap supported clades within this group of species (e.g. *A. spinifrons*, *P. tuberculatus*, and *P. tuberculosus* are one of the four clades) (Fig. 3.5, left tree).

Maximum likelihood analysis (Fig. 3.5, right tree) subdivides the remainder of the species into three equally ancestral clades: one containing the two *Petrolisthes tridentatus* species, the second containing *P. hirtipes*, *P. gracilis* and *P. schmitti* as well as *P. violaceus* and *Liopetrolisthes mitra. P. lewisi austrinsus* is ancestral to the remainder of the crabs, which are subdivided into three clades. One clade comprises the North Temperate species, *P. cinctipes*, *P. cabrilloi*, *P. eriomerus*, *P. manimaculis* and *P. tiburonensis*, which is endemic to the Northern Gulf of California, and the South temperate species, *P. laevigatus*, *Allopetrolisthes punctatus* and *A. angulosus*. A second clade contains three South Temperate species, *A. spinifrons*, *P. tuberculatus* and *P. tuberculosus*. The third clade comprises the tropical or seasonally tropical species, *P. holotrichus*, *P. platymerus* and *P. crenulatus* (Fig. 3.5, right tree).

3. **Evolutionary history of leg membranes:** The leg membrane phenotype is contained entirely within the clade of "smooth" *Petrolisthes*, and it appears to be the ancestral condition for Eastern Pacific "smooth" species (Fig. 3.5, thick solid and dashed lines). Additionally, the leg membrane may have first arisen only on legs 2 and 3 and have been small in size, if the phenotype of *P. granulosus* is similar to the ancestral condition (Fig. 3.5 thick dashed lines, Table 3.1). The phenotype of absence of leg membranes on leg 1 in other species (*P. tiburonensis*, *P. gracilis* and *P. tridentatus*) represents a reversion back to ancestral conditions (Fig. 3.5, thick dashed lines).

The relationship between leg membranes and vertical intertidal zone distribution is somewhat clarified by these phylogenetic analyses. *Petrolisthes granulosus* lives high in the intertidal zone (Table 3.1), suggesting that semi-terrestrial microhabitat conditions may be ancestral to the Eastern Pacific "smooth" species. Loss of leg membranes has occurred in some species that are primarily subtidal, such as *P. eriomerus*, *P. manimaculis*, *P. crenulatus*, *Allopetrolisthes punctatus*, and *Liopetrolisthes mitra* (Table 3.1). Loss of leg membranes has also occurred in some mid-intertidal species, such as *A. angulosus*, *P. holotrichus*, and *P. platymerus*, the latter two of which are small in size (Table 3.1). Leg membranes have been retained in some species that live in the low intertidal or subtidal zones, such as *P. violaceus*, *A. spinifrons*, *P. tuberculatus*, and *P. tuberculosus* (Table 3.1).

Adaptive significance of leg membranes

The language of adaptation in the context of evolutionary biology is complex (Appendix 1 contains a list of definitions). Within an historical context, a trait is only considered an adaptation if it arose, via natural selection, to allow similar functional advantages when faced with similar challenges as in contemporary species (Amundson, 1996). Stillman and Somero (1996) were conservative in their statements on the adaptive significance as an aerial respiratory structure of the leg membranes in *Petrolisthes cinctipes*. Assuming that *P. granulosus* is similar in form and physiology to the ancestral species in which leg membranes first arose, then it is difficult to ascribe the term "adaptation" for aerial respiration to the leg membrane phenotype. *P. granulosus* lives high in the intertidal zone, but has small leg membranes that do not play a large functional role during aerial respiration (Figs. 3.1, 3.4, Table 3.1). Thus, in *P. granulosus*, leg membranes might be "nonadaptations", structures providing no (known)

functional advantage (Gould and Vrba (1982) (Appendix 1)). In the larger-bodied intertidal species (e.g. *P. cinctipes*, *P. laevigatus*) leg membranes might most accurately labeled "exaptations" for aerial respiration, as the leg membranes are functionally significant for aerial respiration in these species, but did not evolve specifically as aerial respiratory structures (Appendix 1). Independent contrasts analysis does not indicate any evolutionary relationship between leg membrane size and vertical position in the intertidal (Fig. 3.7, open symbols), further reducing the argument that leg membranes are "adaptations" for aerial respiration.

Independent contrasts analysis suggests that there is an evolutionary relationship between leg membrane size and body size (Fig. 3.7, filled symbols). Assuming that the ancestral condition of "smooth" *Petrolisthes* includes small body sizes and small leg membrane sizes (i.e. similar to *P. granulosus*) (Figs. 3.5, 3.6, Table 3.1), the presence of leg membranes may have facilitated the evolution of species with the higher metabolic demands associated with increased body sizes. In this context, leg membranes may be appropriately termed "exaptations" that provide the additional respiratory surface needed for the increasing metabolic demands associated with larger body sizes or higher locomotor activity. This argument is developed further in the following paragraph.

Some of the largest (in terms of carapace dimensions) *Petrolisthes*, such as *P. violaceus* (Table 3.1), have a more robust or thicker body, thereby increasing their body volume by a disproportionate amount to the increase in body surface area, thus creating a smaller surface area to volume ratio. The increased whole-organism oxygen demands that results from possessing additional body tissue, requires a compensatory change in the ability of respiratory structures to acquire sufficient oxygen in order to prevent a state of metabolic hypoxia. The presence of leg membranes may provide sufficient additional respiratory surface area to allow these species to remain aerobic. In *P. violaceus*, the leg

membranes in this species may provide enough of an accessory respiratory surface to offset the increases in volume, allowing larger body volumes to evolve. Two species of *Petrolisthes*, *P. tuberculatus* and *P. tuberculosus* are extremely active, with locomotor activities that are more similar to grapsid crabs such as *Grapsus grapsus* or *Pachygrapsus crassipes* than to other porcelain crabs (pers. obs.). These species are both large (in terms of carapace dimensions) (Table 3.1), but unlike *P. violaceus*, are more dorso-ventrally flattened than most other species of *Petrolisthes* (pers. obs.). The large leg membranes of these species may provide the necessary accessory respiratory surface to support the higher metabolic demands of increased locomotor activity, allowing these crabs to evolve more rapid movement.

One caveat to these arguments of the adaptive significance of leg membranes stems from the assumption that ancestral character states are similar to those observed in extant species. *Petrolisthes granulosus*, although being most similar to the ancestral species in genotype, may not be similar in phenotype. The leg membrane phenotype has been lost several times within the genus, where the phenotype of species having large leg membranes on all legs changes to species with no leg membranes (Fig. 3.5). The amount of genetic divergence from *P. granulosus* to its closest ancestor is ~7% (Figs. 3.5, 3.6). This is a much larger value than for the amount of genetic divergence (~1.5%) from the common ancestor of *P. eriomerus* and *P. manimaculis* (two species that lack leg membranes) to its nearest ancestor that possesses membranes on all legs (Fig. 3.5). Assuming that genetic mutations accumulate at an equal rate among taxa, *P. granulosus* would have had 3-4 times as long to reduce their leg membranes than it took for the common ancestor of *P. eriomerus* and *P. manimaculis* to completely lose their leg membranes. Thus, the ancestral condition might have been large leg membranes. Only fossil evidence, which has not been reported for *Petrolisthes*, can resolve this issue.

Leg membranes - convergent evolution

Leg membranes have been described from two distantly related groups of crabs: the brachyuran Ocypodid ghost crabs, in the genera *Scopimera* and *Dotilla*, that are endemic to Australia (Maitland, 1986), and the anomuran Porcellanid porcelain crabs of the Eastern Pacific (Jensen and Armstrong, 1991; Stillman and Somero, 1996; Chapter 2). In addition to the previously reported cases, membranous regions can be found on the limbs of additional brachyuran crabs, including other Ocypodid crabs, such as *Scopimera bitympana* (photo in Wang and Liu, 1996), where the membranes are located on both sides of the meral segment of walking legs. The Portunid crab *Callinectes sapidus* (G. Jensen, pers. comm; pers. obs.) may have leg membranes on every segment of their legs, although comparison with other Portunid crabs is necessary to confirm the thin exoskeleton as a leg membrane. Leg membranes also have been observed in other anomuran crabs, such as the Hippid mole crab, *Emerita analoga* (pers. obs.). Additionally, membranous regions have been described on other regions of other anomuran crabs, such as the hydrothermal vent Galatheid crab, *Munidopsis lentigo*, where on the dorsal surface of the manus of each cheliped there is a small decalcified spot (Williams and Van Dover, 1983). Further studies to examine the functional significance of the membranous regions of these crabs, as well as to determine their evolutionary histories may advance our understanding of the adaptive significance of membranous decalcified areas in decapod crustaceans.

Chapter 4

A Comparative Analysis of Whole Animal Physiological Responses to Temperature Stress in Intertidal Crabs, Genus *Petrolisthes*

Abstract

Intertidal organisms are routinely subjected to a variety of physical stresses, including thermal extremes, fluctuating salinity, and large hydrodynamic acceleration forces. Temperature, because it impacts nearly all biological systems, is potentially one of the most important physical stresses in influencing the distribution patterns of intertidal organisms. Most studies of the thermal stress tolerances of intertidal organisms have shown that organisms living higher on the shore are more thermally tolerant than organisms living low in the intertidal zone or subtidally. Additionally, previous work has shown that tropical species have higher thermal tolerance limits than do temperate zone species. These studies, however, have not been made within the conceptual framework of the modern comparative method, comparing closely related species within a phylogenetic context. Here, research is presented on the thermal tolerance limits of congeneric species of Porcelain crabs, genus *Petrolisthes*, from intertidal and subtidal habitats throughout the Eastern Pacific. Thermal tolerance limits are positively correlated with water temperature at each site as well as with maximal microhabitat temperature, which is correlated with vertical position in the intertidal zone at each site. Phylogenetically independent contrasts were generated for thermal tolerance limits and maximal habitat temperature from a phylogenetic tree based on the 16sRNA gene

sequence. There was a strong positive correlation between independent contrasts of thermal tolerance limits and microhabitat temperature, suggesting that thermal tolerance limits have evolved in response to maximal microhabitat temperatures. Acclimation of *Petrolisthes* resulted in increased thermal tolerance at increased acclimation temperature, and this effect was seen more profoundly in temperate zone subtidal species than for temperate intertidal species. This result agrees with previous studies of acclimation of temperate and tropical crabs, which showed that tropical crabs were unable to increase their thermal tolerance limits with warm acclimation. Potentially, the results of acclimation studies indicate that the thermal tolerance limits of some species may be near current habitat temperature maxima. Global warming thus may affect the distribution limits of intertidal species to a greater extent than in subtidal species.

Introduction

Intertidal organisms are routinely exposed to a suite of rapidly fluctuating physical factors as a result of the low-tide-caused shift from a marine to a terrestrial habitat. The temperatures of intertidal organisms may change substantially when low tide occurs during periods of hot or cold weather. Likewise, salinity can fall rapidly when low tide occurs during periods of heavy precipitation. Additionally, intertidal organisms that do not occur in tide pools are emersed during low tide and thus are bathed in air rather than water (Newell, 1979). The three above-mentioned stresses, as well as additional stresses such as wave force (Denny, 1988) all pervasively affect aspects of the physiology of intertidal organisms. Intertidal organisms have evolved differing levels of physiological tolerance to these stresses, and tolerance limits define the maximum potential vertical distribution in the intertidal zone. Actual patterns of vertical

distribution and intertidal zonation, however, reflect the summation of a complex set of factors including physiological tolerance limits, microhabitat preferences, life history characteristics and biotic interactions between species (Connell, 1961; Edney, 1961; Jensen and Armstrong, 1991; Stillman and Somero, 1996).

Temperature is arguably the most important abiotic stress that ectothermal poikilothermic organisms experience because it pervasively affects biological processes at many levels of biological organization, from the whole organism to molecular processes (Hochachka and Somero, 1984; Somero, 1997). An organism's distribution pattern will to a large extent determine the mean and extreme temperatures that it encounters. Thus, species living in the mid-intertidal zone at one site experience a wider temperature range than a subtidal species at the same site, but the mean habitat temperature of the two species is similar. Organisms living in intertidal habitats may be exposed to varying amounts of terrestrial conditions, and thus experience a wider range of environmental stresses than low intertidal or subtidal zone organisms (Vernberg and Vernberg, 1972). Compared with studies of temperature adaptation of organisms from different latitudes (e.g. Vernberg, 1959a, 1959b, 1962; Vernberg and Tashian, 1959; Vernberg and Costlow, 1966; Graves and Somero, 1982), little work has been devoted to the evolutionary adaptation of organisms to temperature on local thermal gradients, such as are found in the intertidal zone (but see Stillman and Somero, 1996; Chapter 2).

Thermal sensitivity of organisms in the natural environment can be adjusted on two scales of biological time: short term acclimatization that occurs within the lifetime of an organism, and genetic adaptation occurring through processes of natural selection. Acclimatory responses to thermal extremes can include adjustments at behavioral, physiological, and biochemical levels (Hochachka and Somero, 1984). Behavioral modification of thermal sensitivity usually involves behavioral thermoregulation by

moving to a microhabitat with the desired thermal conditions. Physiological adjustments in thermal tolerance to higher temperatures can include the lowering of metabolic rate, and increased evaporative cooling. Biochemical adjustments can include changes in enzyme concentration, kinetic properties (by expressing different isozymes), substrate or cofactor concentrations, membrane lipid properties (Hochachka and Somero, 1984), or expression of heat shock proteins that act to repair proteins damaged by thermal stress (Lindquist, 1986; McLennan and Miller, 1990).

Adaptation to different temperatures can occur by alteration of any or all of the biological systems important in short-term temperature responses. Such changes could include the properties of macromolecules such as enzymes or other macromolecular assemblages such as membranes, changes in the ability to express different genes during thermal stress, or changes in the cellular machinery of the heat shock response. The mechanistic bases for thermal adaptation are a current source of intense research using both comparative and experimental methods (e.g. Feder *et al.*, 1996; Feder and Krebs, 1997).

Comparative studies of evolutionary adaptation have the most power when the study species are closely related and can be examined within the context of a known phylogenetic relationship (Harvey and Pagel, 1991). Use of closely related species allows inferences to be more directly made from correlated biological responses and environmental stresses. This is primarily because of the similarities in the biology of the organisms and a smaller amount of time for random (i.e. non-selected) genetic mutations to accumulate if those species have recently diverged from a common ancestor. Congeneric species are likely to be closely enough related so that differences between species may be directly compared, but divergent enough to have had sufficient time to evolve in response to environmental factors.

One group of congeneric species that is well suited for comparative, evolutionary studies of biological responses to environmental stress is the porcelain crabs of the genus *Petrolisthes*. The genus *Petrolisthes* is large, with over 100 species worldwide. In the Eastern Pacific, there are about 45 species distributed across both latitudinal and intertidal gradients (Haig, 1960; Carvacho, 1980; Romero, 1982; Weber Urbina, 1986; Jensen and Armstrong, 1991). Species are grouped in four main biogeographic regions: North and South Temperate, the Northern Gulf of California, and throughout the tropics (Figs. 1.2, 4.1) (Carvacho, 1980). Within each of these locations, species are distributed on a vertical gradient, such that there are species living solely in the mid to high intertidal zones, and others living in low intertidal and subtidal zones (Fig. 4.1).

The distributions of Eastern Pacific *Petrolisthes* result in a wide range of average and maximal body temperatures, as well as a variety of daily and seasonal body temperature fluctuations. Temperate low intertidal species (such as *P. eriomerus* (Figs. 4.1, 2.1B,C)) likely experience an annual temperature range of 8-16°C. In contrast, temperate high intertidal species (such as *P. cinctipes* (Figs. 4.1, 2.1B,C)) can have annual temperature ranges from 0-32°C. Like temperate subtidal species, tropical subtidal species have a narrow annual temperature range, but the temperatures experienced may range from 26-30°C. Although tropical intertidal species such as *P.* cf *tridentatus* can experience body temperatures as high as 40°C (pers. obs.), their annual temperature ranges are smaller, from 26-40°C. Perhaps the largest annual thermal range is experienced by the seasonally tropical fauna of the Northern Gulf of California, where water temperatures range from 28-30°C in late summer to 10-15°C in the winter (Robinson, 1973). Body temperatures of intertidal species living in the Northern Gulf of California, such as *P. gracilis* (Fig. 4.1), can be at least 40°C during summer months (pers. obs.), and may reach temperatures below 10°C during winter low tide periods.

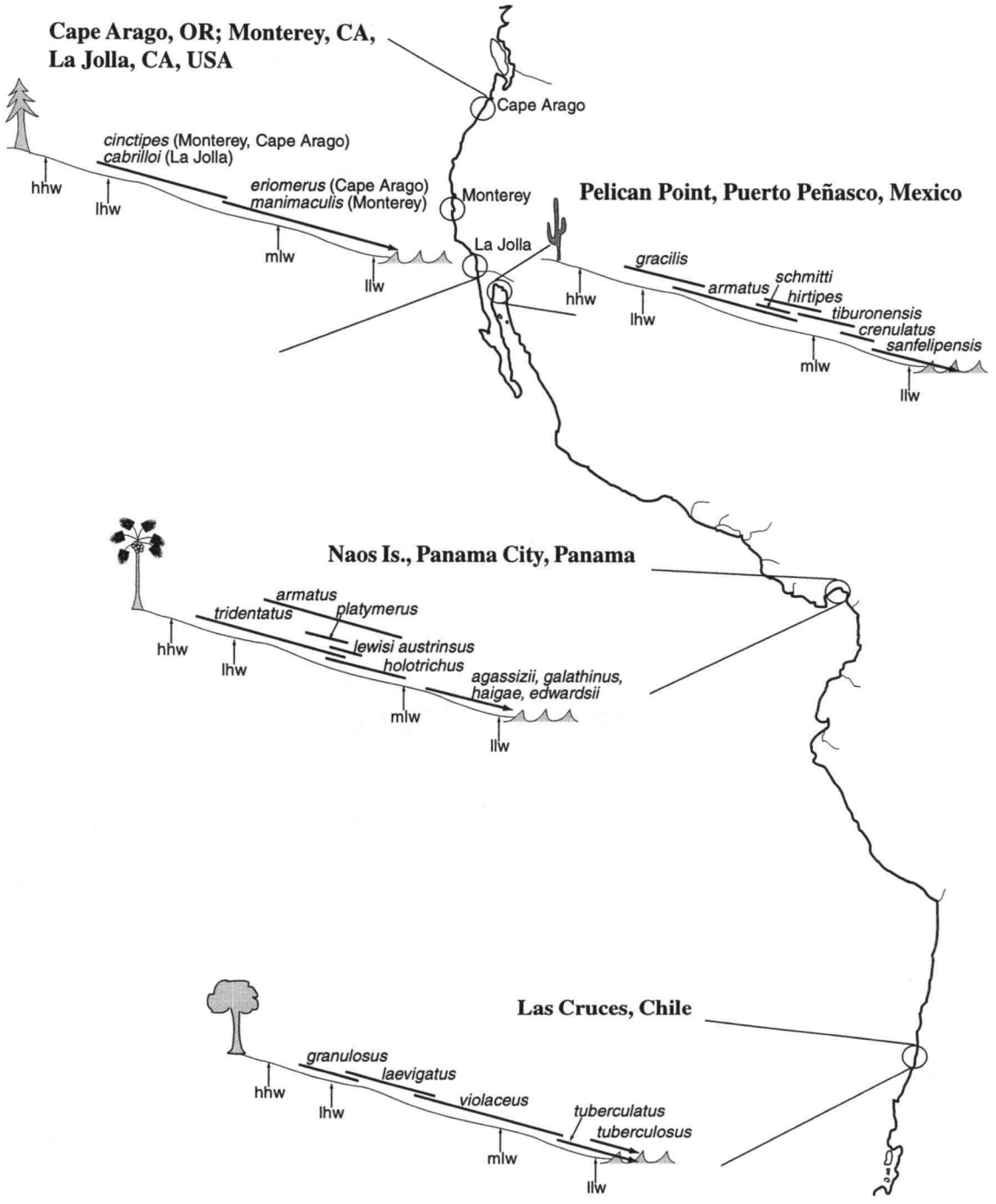

Figure 4.1. Latitudinal and vertical distribution patterns of Eastern Pacific *Petrolisthes*. Vertical distribution patterns are given for sites (circled) in 4 main biogeographic regions: North and South Temperate, Northern Gulf of California, and Panama. Species are arranged according to vertical position in the intertidal zone, as indicated by solid bars for each species. Listing of more than one species next to a bar indicates common distribution. Abbreviations for water height are: hhw: highest spring tides, lhw: lowest high tide, mlw: mean low tide height (generally set to 0 for each site), llw: lowest spring tides. Distribution data are from personal observations and literature cited in the text.

Measurement of the thermal limits of heart rate in two species of *Petrolisthes*, *P. cinctipes* and *P. eriomerus*, indicates that species have thermal tolerance limits that correspond to their respective microhabitat conditions. *P. cinctipes* is distributed in the upper intertidal zone and *P. eriomerus* in the low intertidal to subtidal zones throughout their distribution ranges from central California to northern British Columbia (Fig. 4.1) (Jensen and Armstrong, 1991). Measurement of the thermal limits of heart function in these two species indicates that *P. cinctipes* is able to tolerate both higher and lower temperatures than *P. eriomerus* (Fig. 2.3) (Stillman and Somero, 1996). Additionally, the upper thermal tolerance limit of *P. cinctipes* is very similar to the maximal microhabitat temperature for this species (Stillman and Somero, 1996; Chapter 2).

Here, a comprehensive survey of upper thermal tolerance limits for species of *Petrolisthes* from the four above biogeographic regions is presented. Results indicate that the thermal tolerance limits of species are closely correlated with microhabitat temperature maxima. The extent to which the thermal tolerance limits reflect evolutionary adaptation to microhabitat temperature is examined by analyzing the results of these studies within a phylogenetic context by application of independent contrasts analyses. The phylogenetic tree used to generate independent contrasts was constructed based on molecular sequence data for the 16sRNA gene (Appendix 4) following the methodology described in Chapter 3.

The extent to which thermal tolerance limits are phenotypically plastic is also investigated by examination of laboratory acclimation to different temperatures. Results indicate that acclimation can affect thermal tolerance limits and that intertidal species, such as *P. cinctipes*, may have acclimatized their thermal tolerance limits closer to the maximum achieved by acclimation than have subtidally living species, such as *P. manimaculis* (Fig. 4.1).

Materials and Methods

Specimen collection and maintenance

Specimens used in these studies were collected from intertidal locations listed below and held submerged at ambient water temperatures until the time of study. Adult crabs of similar size were selected for each species, and no freshly-moulted specimens were used. For examination of thermal tolerance limits of field specimens, 19 species were collected and held under constant conditions for less than 1 week. Collection locations, date of collection, and date of thermal tolerance assay are as follows: *Petrolisthes cinctipes*, *P. manimaculis*, Monterey Bay, California (36°36'N, 121°53'W), Jan. 21, 1996 coll., Jan. 25-26, 1996 assayed. *P. cabrilloi*, La Jolla, California (32°51'N, 117°16'W), Jan. 19, 1996 coll., Jan. 26, 1996 assayed. *P. armatus*, *P. gracilis, P. sanfelipensis*, *P. hirtipes*, *P. crenulatus*, Puerto Peñasco, Sonora, Mexico (31°39'N, 113°15'W), Aug. 15-16, 1997 coll., Aug. 17-18, 1997 assayed. *P. granulosus*, *P. laevigatus*, *P. violaceus, P. tuberculatus*, *P. tuberculosus*, Las Cruces, Chile (33°33'S, 71°36'W), Oct. 14-15, 1997 coll., Oct. 21-22, 1997 assayed. *P. tridentatus*, *P. armatus*, *P. galathinus*, *P. platymerus*, *P. agassizii, P. holotrichus*, *P. haigae*, Naos Island, Pacific Panama (8°50'N, 79°8'W), March 25-29, 1998 coll., April 2-6, 1998 assayed.

The response of thermal tolerance limits to thermal acclimation was examined in three species. Specimens of *Petrolisthes cinctipes*, *P. manimaculis* (collected at Monterey Bay, California, Sept. 3, 1997) and *P. eriomerus* (collected at Cape Arago, Oregon (43° 21' N; 124° 19' W), July 22, 1997) were held submerged at a common temperature in ambient water from Monterey Bay in flow-through aquaria following collection. On Sept. 20, 1997, healthy looking specimens of similar sizes were divided into three groups. Specimens in one group were used for immediate determinations of

thermal tolerance limits (see below). Specimens in the other two groups were acclimated at 8 or 18°C for a period of 10 weeks, and then used for immediate determinations of thermal tolerance limits. During the acclimation period, crabs were held in recirculating temperature controlled aquaria, and fed on alternate days with pulverized fish pellets. Two thirds of the water in each aquarium was changed once a week with fresh filtered seawater at ambient temperature from Monterey Bay (12-14°C). Acclimation temperatures were only altered for a period of approximately 60 minutes during the water change process.

Microhabitat characterization

To define the microhabitat conditions for each species studied, temperature measurements were made of the sea water at low tide, and thermal transects of crab microhabitats were made by flipping over stones and immediately measuring the temperature of crabs, the underside of the rocks, and the substratum. All temperature measurements were made using thermocouple probes (Omega Inst., K-type wire probes) connected to a digital thermometer (Omega Inst., HH 82), which are accurate to 0.1°C and were calibrated against a mercury thermometer. Transects throughout the intertidal were made with the intent to assess the maximal temperatures that crabs experienced. Temperatures were measured from underneath 15-20 rocks in each vertical zone shortly before the incoming tide submerged the rocks. Rocks that were likely to have high underside temperatures (Chapter 2) were preferentially selected, because the goal was to assess maximal temperatures, not average temperatures, in the under-rock microhabitat. All temperature measurements were made during late spring or summer and during periods of low spring tides that occurred during the middle of the day. Measurements were made on days with minimal cloud cover and wind (see Fig. 2.1).

Measurement of vertical distribution patterns was conducted using tide-table data and a simple device constructed from two meter-sticks and a length of aquarium tubing. Briefly, the tubing was attached to the meter sticks such that it ran along the length of each stick, with about 15 feet of slack between the sticks, forming an elongate "u" shape. The device was used as follows: While positioning one stick at the waters edge at the time of low tide, and the other stick at a rock somewhere up towards shore, the placement of each water meniscus was recorded using the ruling on the meter-stick. The difference in height of the menisci of the water in the tubing represented roughly the difference in height of the bottom of the sticks. Calibration of the base height was done using the sea-level height from published tide tables (NOAA, 1996-1998). By measuring the difference in height between rocks during a vertical transect from the low tide mark to the high tide line while noting species composition beneath each rock, it was possible to make a fairly accurate estimate of the vertical distributions of each species.

Measurement of thermal tolerance limits

The upper thermal tolerance limit of each species was determined by the following protocol, adapted from the procedure used for measurement of upper thermal tolerance limits of heart rate in *Petrolisthes cinctipes* and *P. eriomerus* (Stillman and Somero, 1996; Chapter 2). Individual crabs were placed into small plastic containers, each containing approximately 100 ml of seawater from the aquarium that the crabs had been acclimated in. These plastic containers were suspended in a water bath whose temperature was controlled to the nearest 0.1°C. The water bath was initially set at the ambient temperature of the aquarium that the crabs had been removed from, which was always within 1°C of the water temperature at collection locations for each species. Following placement of the crabs in the plastic containers, they were held for 30-60

minutes at the acclimation temperature. Thereafter, the temperature of the water in the containers was increased at a rate of 1°C/15 minutes with an immersion water heater. Every 15 minutes, the water bathing the crabs was aerated by vigorously bubbling with an air stone, the temperature in each container was checked (with thermocouple probes), and the sensory antennule activity of each crab was visually monitored. If no sensory antennule activity was noted, the mouth-parts of the crab were gently prodded with the thermocouple probe. If no responsiveness to the prodding was noticed, the specimen was considered to be dead.

Between 10 to 30 specimens of each species were simultaneously incubated in the above conditions, and the percentage of specimens alive at each temperature was calculated. This percentage was transformed by the function ($\sin^{-1}$(percentage alive$^{0.5}$)) and expressed in radians to make the proportion of surviving crabs linear with respect to temperature. Linear regression analysis was then used to find the slope of the line, and the temperature at which 50% of the crabs had died (= 0.785 radians) was calculated. This temperature is used as the measure for upper thermal tolerance limits, and is referred to as the LT_{50}.

The LT_{50} datum for each species does not have an associated variance because the LT_{50} is calculated from the proportion of specimens alive at each temperature. Due to constraints of time and specimen availability, it was impossible to repeat LT_{50} measurements for every species, but in two species, *Petrolisthes cinctipes* and *P. manimaculis*, there was less than a 0.5°C variation among three separate determinations of the LT_{50} for each species. Thus, while the measurement of LT_{50} only once for each species does not allow for parametric statistical analyses to be used, regression analysis of the relationship between LT_{50} and an environmental variable, however, can be performed with LT_{50}s of multiple species.

Independent contrasts analyses

The phylogenetic tree used to generate phylogenetic independent contrasts was generated as described in Chapter 3 of this thesis, but using species for which LT_{50} data were collected. Briefly, the phylogenetic tree was generated from a distance matrix based on a maximum likelihood model, with neighbor-joining used to construct the tree.

Phylogenetic analyses were performed using several programs from the suite of phylogenetic software known as PHYLIP, version 3.5c (Felsenstein, 1989). Independent contrasts (Felsenstein, 1985) were generated using the CAIC software package (Purvis and Rambaut, 1995). Phylogenetic trees and branch lengths were input according to the results of PHYLIP analyses. Contrasts of LT_{50} and maximal habitat temperatures were generated. These contrasts were used in linear regression analyses where the regression was forced through the origin, as is required for analyses of independent contrasts (Purvis and Rambaut, 1995).

Results

The phylogenetic tree developed for phylogenetic independent contrasts analysis is shown in Figure 4.2. The topology of this tree is similar to the tree in Figure 3.5.

Microhabitat characteristics

Petrolisthes in every biogeographic region of the Eastern Pacific are distributed on a vertical gradient in the intertidal zone (Fig. 4.1). Previously collected data on the vertical distribution of *Petrolisthes* in the North Temperate zone in California, Oregon and Washington (Jensen and Armstrong, 1991; Jensen, 1995), for the Northern Gulf of California (Romero, 1982), and for the South Temperate zone in Central Chile

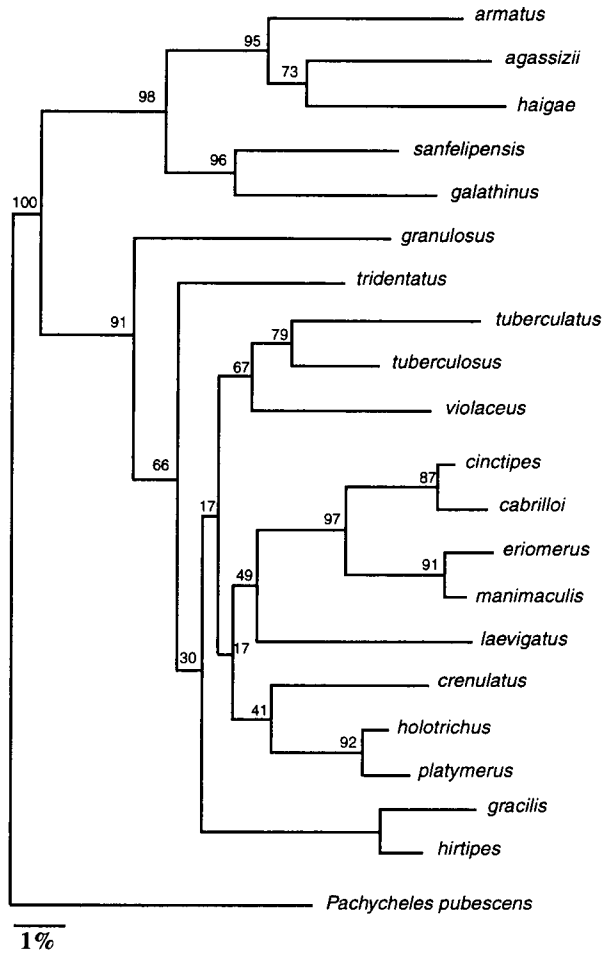

Figure 4.2. Phylogenetic tree based on 16sRNA gene sequence data from species of *Petrolisthes* for which LT_{50} data were collected for use in generation of independent contrasts. This tree was generated from a matrix of distances generated with a maximum likelihood model and constructed by neighbor-joining analysis. Values on the nodes are bootstrap values out of a total of 100 replicate data sets. The tree was rooted with the outgroup species *Pachycheles pubescens*, although this terminal node was not used to generate an independent contrast.

(Weber Urbina, 1986), as well as data reported here for Panama, are summarized in Figure 4.1. For simplification, vertical positions of each species, based on the upper vertical limit of each species, were categorized as follows: 5=high intertidal, below the splash zone to 1=subtidal, or intertidal only during the lowest spring tides.

Sea water temperatures during the times of temperature measurements ranged from 8-12°C in California and the Pacific Northwest, from 14-16°C in Chile, from 28-29°C in the Northern Gulf of California and from 26-28°C in Panama. Maximal microhabitat temperatures for low intertidal species never were more than 4°C above ambient water temperature at tropical field sites or 6-8°C above ambient temperature at temperate field sites.

Maximal microhabitat temperatures for high intertidal species ranged from 40-43°C in the Northern Gulf of California during summer (mid August) and in Panama during the hot, dry season (late March, early April). In the temperate regions, maximal temperatures were as high as 31.2°C (Stillman and Somero, 1996) (Fig. 2.1B) in Oregon during spring, and as high as 28°C in Chile during late spring. Maximal temperatures recorded in Chile may not reflect absolute maximal temperatures as measurements were not made on cloudless calm days, as those weather conditions never occurred during my studies there. Thus the approximate range of temperatures encountered during low tide in temperate zone species is about 18-20°C above ambient sea water temperature, while tropical species only encounter temperatures of 10-12°C above ambient sea water temperature.

Thermal tolerance limits

During thermal tolerance experiments, specimens exhibited a series of consistent behavioral traits. With increasing temperature, in sequence the specimens became

Table 4.1. Thermal tolerance limits, expressed as LT_{50} values, for field collected and acclimated porcelain crabs of the genus *Petrolisthes*.

species	n	LT_{50}[1]	collection location or acclimation temperature	vert dist[2]	T_{max}[3]
agassizii	16	37.0	Panama	1	33
armatus	16	40.2	Panama	4	41
armatus	24	40.6	N. Gulf of California	4	41
cabrilloi	18	33.5	La Jolla, California	4	33
cinctipes	48[4]	32.3	Monterey, California	4	33
crenulatus	18	39.4	N. Gulf of California	2	33
eriomerus	24	27.5	Oregon	2	16
galathinus	16	37.0	Panama	1	33
gracilis	18	41.1	N. Gulf of California	5	41
granulosus	18	34.8	Chile	5	33
haigae	16	36.2	Panama	1	33
hirtipes	10	39.3	N. Gulf of California	3	37
holotrichus	16	39.4	Panama	3	36
laevigatus	16	31.6	Chile	4	28
manimaculis	48[4]	28.5	Monterey, California	2	18
platymerus	16	39.6	Panama	3	36
sanfelipensis	16	37.4	N. Gulf of California	1	33
cf *tridentatus*	16	40.5	Panama	5	43
tuberculatus	18	28.4	Chile	2	18
tuberculosus	10	27.0	Chile	1	14
violaceus	18	30.1	Chile	3	18
cinctipes	16	32.1	8°C	4	33
cinctipes	16	34.0	18°C	4	33
eriomerus	16	27.9	8°C	2	16
eriomerus	16	31.4	18°C	2	16
manimaculis	16	27.6	8°C	2	18
manimaculis	16	31.6	18°C	2	18

1. LT_{50} values are in units of °C.
2. Vertical distribution (highest for each species) in the intertidal coded as 5=high intertidal to 1=subtidal. Data are from (Chace and Hobbs, 1969; Romero, 1982; Weber Urbina, 1986; Jensen and Armstrong, 1991), pers. obs.
3. Approximate maximal microhabitat temperatures, in °C. Values set using collected temperature data and adjusted according to weather conditions.
4. Numbers represent 3 determinations using 16 specimens for each determination.

increasingly active, exhibited loss of balance from which they could recover (as observed from them falling on their dorsal surface and then righting themselves), exhibited irreversible loss of balance, and lastly, ceased all movement. During periods of loss of equilibrium, spasmodic, jerky movements were often observed. Specimens generally died in a clenched contortion, where the appendages were held beneath the body. LT_{50} values determined for each species and treatment are summarized in Table 4.1.

Thermal tolerance limits of field-collected specimens were strongly correlated with maximal vertical position in the intertidal zone (Fig. 4.3). Comparison of species within each field site showed that LT_{50}s were linear with respect to maximal vertical intertidal position. Additionally, species from the two tropical habitats had similar LT_{50}s at similar vertical positions, as did species from the two temperate zone habitats. The differences between the highest and lowest LT_{50}s within a field site were greater in temperate zone species than in tropical species (Fig. 4.3), reflecting the larger differences in microhabitat temperature extremes in temperate microhabitats. Regression analyses indicated that the difference in LT_{50} relative to vertical position of temperate zone species (slopes of 2.38,1.88) was about twice that of tropical species (slopes of 0.85, 0.99) (Fig. 4.3).

Across species, LT_{50} is strongly and positively correlated with maximal habitat temperature (Fig. 4.4A). However, the LT_{50} of tropical low intertidal species is greater than the LT_{50} of temperate high intertidal species, even though microhabitat temperatures are similar in both groups (Figs. 4.3, 4.4A, Table 4.1)

Independent contrasts analyses

There was a strong, positive correlation between independent contrasts of LT_{50} and maximal microhabitat temperature (Fig. 4.4B). Contrasts were small in most cases,

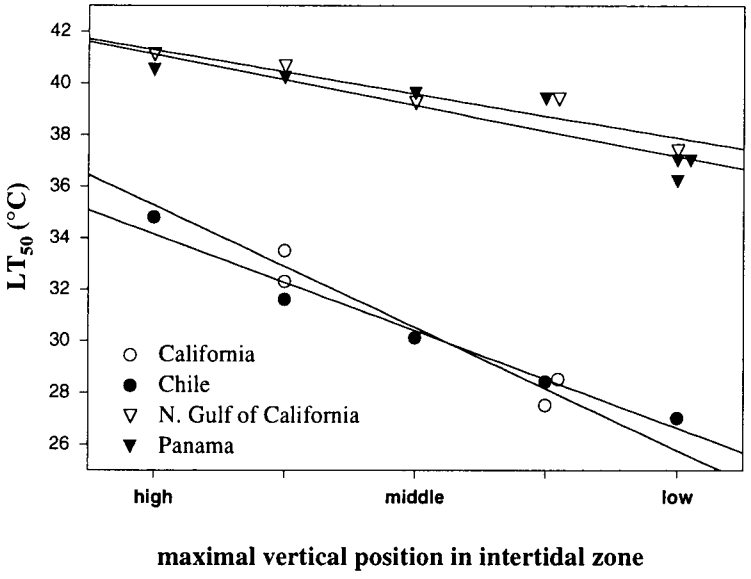

Figure 4.3. Thermal tolerance limits of *Petrolisthes* from four different locations with respect to maximal vertical distribution of each species. Vertical distributions of each species have been coded as 5=high intertidal zone to 1=low intertidal zone as described in the text. Each point represents the LT_{50} of one species. Regression lines have the following parameters: Northern Gulf of California, $y=42.12-0.85x$, $r^2=0.88$; Panama, $y=42.09-0.99x$, $r^2=0.83$; California, $y=37.64-2.38x$, $r^2=0.94$; Chile, $y=36.02-1.88x$, $r^2=0.97$. Several symbols have been jittered for purposes of data display only.

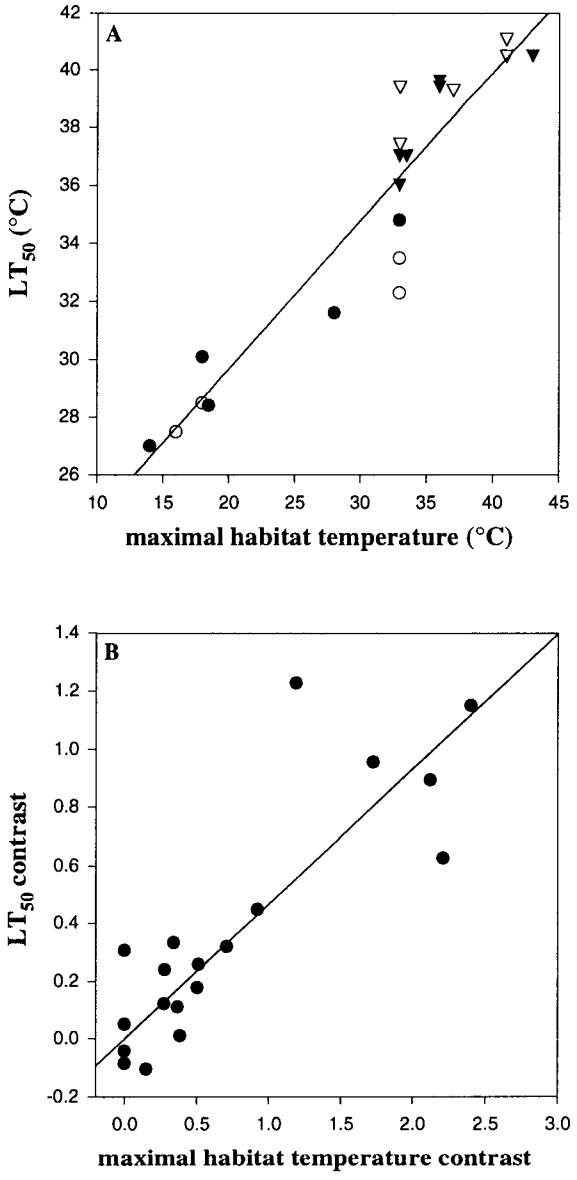

Figure 4.4. The relationship between LT_{50} and maximal habitat temperature for 20 species of *Petrolisthes* plotted as raw data (A) and as phylogenetically independent contrasts (B). Each point represents the LT_{50} from one species. Regression coefficients are (A) y=19.41+0.51x, r^2=0.88 and (B) y=0.4655x, r^2=0.72. Symbols in (A) are as in Figure 4.3. Overlapping symbols have been jittered for display purposes.

but for 5 different nodes, there were large differences in LT_{50} and maximal habitat temperature contrasts (Fig. 4.4B). This independent contrast analysis suggests that there has been a strong evolutionary adaptive response of LT_{50} with respect to microhabitat temperature.

Acclimation of thermal tolerance limits

The LT_{50}s of *Petrolisthes cinctipes*, *P. eriomerus* and *P. manimaculis* were all higher after acclimation at 18°C than they were after acclimation at 8°C. LT_{50} values for *P. cinctipes* were 32.1 and 34.0°C at 8 and 18°C, respectively, the effects of acclimation increasing the LT_{50} by 1.9°C (Fig. 4.5, Table 4.1). In contrast, the LT_{50}s of *P. manimaculis* and *P. eriomerus* changed by about twice as much following acclimation, as LT_{50}s between 8 and 18°C acclimations differed by 4 and 3.5°C, respectively (Fig. 4.5, Table 4.1).

Discussion

Measurement of thermal tolerance limits

Assessment of thermal tolerance limits by determining the LT_{50} produces similar results to those seen when the thermal tolerance limits of heart function were measured (Stillman and Somero, 1996). Arrhenius break temperatures (ABT) of heart rate were 31.5°C for *Petrolisthes cinctipes* and 26.6°C for *P. eriomerus* (Stillman and Somero, 1996), and these species had LT_{50}s of 32.3°C and 27.5°C, respectively. The slight increase in the LT_{50} temperature over the ABT of heart rate is not surprising since the heart can still be beating at temperatures greater than the ABT. Although the specimen is

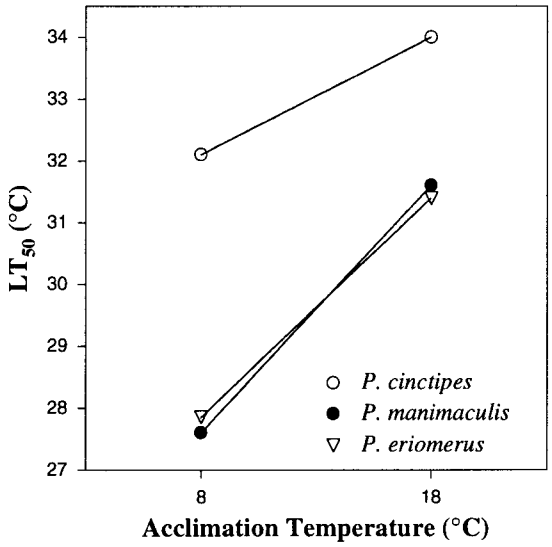

Figure 4.5. Acclimation of thermal tolerance limits in *Petrolisthes cinctipes, P. eriomerus* and *P. manimaculis*. Acclimation to 8 or 18°C was for a period of 10 weeks.

suffering, probably irrevocably, from heat stress at temperatures above the ABT, it may have still been "alive" by methods used to determine LT_{50}s.

Analyses of thermal tolerance limits of congeneric species from different biogeographic regions, and different vertical positions in the intertidal zone within each region, indicate that species have adapted their upper thermal tolerance limits to coincide with microhabitat conditions. In 19 species of *Petrolisthes*, maximal thermal tolerance limits reflected microhabitat conditions in terms of both vertical distribution and maximal microhabitat temperatures (Figs. 4.3, 4.4A).

One potentially confounding factor in the measurements of thermal tolerance limits in this paper is that these animals were used for experiments as soon as 48 hours after collection. Previous studies have shown that crustaceans can have dramatically different thermal tolerance limits depending on immediate acclimation conditions before measurement of thermal tolerance limits. For example, in *Artemia franciscana*, a brief heat shock (similar to what an intertidal crab might experience during a low tide period) can induce an increased thermal tolerance during subsequent heat shock (Miller and McLennan, 1988). The protective effects of the first heat shock wear off, however, and after less than 19 hours following the initial heat shock, protection decreased by 60% in *A. franciscana* (Miller and McLennan, 1988). Assuming that *Petrolisthes* have a similar duration of protective effects following heat shock as in *A. franciscana*, acclimation to identical conditions for the 48 hours prior to experimentation should have been long enough to ensure that the measured thermal tolerance limits reflect genetic differences between species, and not environmentally induced transient adjustment of thermal tolerance limits. However, if the heat shock response of *Petrolisthes* requires greater than 48 hours for the protective effects take to wear off, the LT_{50}s measured in this study may not entirely reflect genetic differences between species.

Comparative analysis of thermal tolerance limits

Phylogenetic analysis reveals that thermal tolerance limits, vertical distribution limits and thermal microhabitat characteristics are relatively similar in most species of the "spiny" *Petrolisthes* clade of *P. agassizii*, *P. galathinus*, and others (Figs. 3.5, 4.2, 4.3A). However, two species of this clade have different thermal habitats from the rest of the species in this clade. One of those species is *P. armatus*, found in mid-high intertidal zones and possessing thermal tolerance limits that are about 3-4°C higher than the next closest subtidal living relatives, *P. agassizii* and *P. haigae* (LT_{50} of 40.5 vs. 36-37°C). The other "spiny" *Petrolisthes* to deviate from the most common habitat of this clade is *P. desmarestii*, which is a subtidal South Temperate species.

While the "spiny" *Petrolisthes* are mostly similar in their thermal tolerance limits and microhabitat characteristics, the same can not be said for the remainder of the *Petrolisthes*. There is a large range of LT_{50} values within this group, from 27.5 to 41.1°C. Thermal tolerance limits, as well as variation in microhabitat preference, do not seem to be conserved within the "smooth" *Petrolisthes* species (Fig. 4.2). Rather it appears that thermal tolerance limits are set to match the present microhabitat conditions within this group, and that sister species do not necessarily occupy similar thermal microhabitats (Fig. 4.2).

Phylogenetically independent contrasts analyses indicate that there has been an adaptive response of thermal tolerance limits to microhabitat temperature. There is a strong positive correlation between the independent contrasts of LT_{50} and maximal microhabitat temperature (Fig. 4.4B). The adaptive response to microhabitat temperature indicates that species of *Petrolisthes* have repeatedly invaded upper intertidal zone habitats, and have increased their thermal tolerance limits to do so.

Increasing thermal tolerance limits does not come without a cost, however. Costs involved with increasing thermal tolerance, especially costs in synthesis of stress proteins, and the energetic requirements associated with their actions (Lindquist, 1986), could have a very large selective effect in the event of mass mortality (e.g. Tsuchiya, 1983). The benefits of living higher in the intertidal could be great as well. Although there have not been many studies of predation upon *Petrolisthes*, both fish and subtidal crabs are known to prey on porcelain crabs (Cerda and Wolff, 1993; Norton, 1995). Thus, escaping predation pressure by living high in the intertidal zone may be beneficial enough to overcome the costs of having an increased thermal tolerance limit.

Rapidity of evolutionary adaptation to temperature can be easily understood in light of the strong selective force of temperature: if organisms experience lethal microhabitat temperatures only once during their pre-reproductive lifetime (which may be one to two years), their genes are removed from the population. Life history characteristics of *Petrolisthes* can also augment the selective forces of abiotic factors. Larval settlement in *Petrolisthes* is gregarious, that is larvae only settle where adults are living (Jensen, 1989), and post-larvae are routinely encountered living on and among adults during post-settlement periods. Thus, tolerance to abiotic stresses, such as temperature, can greatly increase survival and intraspecific competitive advantage throughout the post larval developmental and pre-sexual maturation periods.

Acclimation of thermal tolerance limits

Acclimation of thermal tolerance limits in three species of temperate *Petrolisthes* (Fig. 4.5) suggests that the more "warm-adapted" *P. cinctipes* may not be able to adjust its upper thermal tolerance limits to the same extent as the "cold-adapted", subtidal *P. eriomerus* and *P. manimaculis*. This result is concordant with what was found in studies

of the acclimation of thermal tolerance limits in temperate and tropical fiddler crabs, genus *Uca*, where only temperate zone species were able to adjust their thermal tolerance limits during acclimation (Vernberg and Tashian, 1959). The tropical species, because of the high microhabitat temperatures routinely experienced, were hypothesized to have had already "set" their thermal tolerance limits to their maxima; no further acclimation was possible. Acclimation of thermal tolerance limits in *Petrolisthes* suggests that the same phenomenon may be occurring; *P. cinctipes* may set its thermal tolerance limit closer to the maximal level than have *P. eriomerus* or *P. manimaculis*. This result suggests that in the event of global warming, the distributions of temperate, and perhaps tropical intertidal *Petrolisthes* may be more greatly impacted than those of subtidal species, who will be able to acclimatize their tolerance limits to a greater extent.

Mechanistic bases of thermal tolerance limits

Preliminary investigations of the thermal limits of nerve function in *Petrolisthes* suggest that the thermal dependence of action potential transmission reflect whole animal thermal tolerance limits (Jessica Knape, pers. comm.). The differences seen in nerve function implicate membranes, such as those at nerve synapses, in setting thermal tolerance limits. Membrane fluidity is very temperature sensitive, and specific membrane fluidity is required for appropriate cellular function (Cossins and Bowler, 1987; Hazel and Williams, 1990; Hazel, 1995). Behavioral changes of *Petrolisthes* noted during thermal stress experiments (i.e. loss of equilibrium) are similar to observations of behavioral changes in goldfish during heat death which were shown to be correlated with nerve membrane properties (Cossins *et al.*, 1977). Alterations in membrane lipid composition have been shown to be a common mechanism enabling organisms to adjust

membrane fluidity to withstand thermal stress (review in Hazel and Williams, 1990, Williams and Somero, 1996).

In addition to membrane differences, species may also possess different heat-shock responses, which have been shown to alter thermal tolerance limits at the cellular (Li and Laszlo, 1985), and organismal (Feder *et al.*, 1996; Feder and Krebs, 1997) levels. Further studies of the cellular differences among species of *Petrolisthes* may provide new insights into the cellular mechanisms of heat tolerance and eurythermality.

Chapter 5

Patterns and Mechanisms of Lactate Dehydrogenase Stabilization in Porcelain Crabs, Genus *Petrolisthes*, From Different Thermal Habitats

Abstract

Kinetic properties, such as substrate affinity (K_m), of orthologous homologs of proteins (orthologs - proteins from different organisms encoded by the same gene) from organisms distributed from polar to tropical habitats are conserved within a narrow range at normal body temperatures. However, whether structural properties of orthologs are correlated with body temperature is unclear. Here, the relationship of enzyme thermal stability to body temperature is examined in 22 congeneric species of porcelain crabs (Genus *Petrolisthes*). These crabs are distributed throughout the Eastern Pacific, and exhibit discrete patterns of intertidal vertical zonation, thus creating a diverse range of thermal microhabitat conditions. In these crabs, the thermal stability of lactate dehydrogenase (LDH) is higher than that previously reported for other crustacean and vertebrate LDHs. Temperatures required for a 50% loss of activity within 10 minutes ranged from 65°-75°C among species. Phylogenetic, comparative analyses do not indicate a general "adaptive" pattern of LDH thermal stability related to microhabitat temperature, although for two groups of sister species, there is a correlation between LDH thermal stability and microhabitat temperature. LDH stability was not affected by acclimation of two species to intertidal or subtidal conditions. Examination of the mechanistic causes of LDH thermal stability indicates that stability differences are due to

factors intrinsic to the LDH molecules and to stabilizing proteins extrinsic to the LDH molecules, the identity or mechanism of which are unresolved. Differences in apparent molecular mass found in SDS-PAGE analysis of purified LDHs suggest either interspecific variation in LDH monomer primary sequence, or in post-translational modification. Porcelain crab LDHs present a number of interesting questions for future research, such as identification of the selective factor which has produced variants with such a wide range of stabilities, and the elucidation of the mechanism of LDH stabilization in these species.

Introduction

Temperature is a dominant factor in limiting the distribution of ectothermic marine organisms (Fields *et al.*, 1993; Barry *et al.*, 1995). Whole organism thermal tolerance limits have been shown to reflect habitat temperature ranges and distribution patterns (Vernberg and Tashian, 1959; Edney, 1961; Chapter 4). The thermal tolerance limits of organisms are governed by a combination of tissue, cellular, and biochemical sensitivities to temperature (Hochachka and Somero, 1984; Cossins and Bowler, 1987; Somero, 1997).

Biochemical adaptation to temperature

Biochemical systems that are the most thermally sensitive include proteins and membranes. Proteins are structurally and functionally perturbed by temperature (Alexandrov, 1977; Somero, 1995). Enzymatic proteins must be marginally stable in order to possess enough conformational flexibility to catalyze reactions, and net stabilization energies are often on the order of a few weak bonds (Jaenicke, 1991).

Studies of orthologous homologs of proteins, defined as proteins from different organisms encoded by the same gene (abbreviated orthologs), have found that structural and functional properties are conserved within a narrow range at normal body temperatures (Graves and Somero, 1982; Dahlhoff and Somero, 1991, 1993b; Somero, 1995, 1997). Conservation of function across such a wide range of temperatures requires that the orthologs have evolved changes in amino acid sequence or tertiary structure enabling them to alter their temperature sensitivity (Fields and Somero, 1997, 1998; Holland *et al.*, 1997). Orthologs can have different thermal sensitivities; some orthologs can withstand a wide range of temperatures without alteration of kinetic properties, while others are very temperature sensitive (Somero, 1995).

Although generally consistent trends are seen in comparisons of kinetic properties in differently adapted orthologs, research on the correlations between body temperature and thermal stability of enzymatic activity has produced mixed results. Several studies have reported strong positive correlations between maximum body temperature and thermal stability of proteins (McFall-Ngai and Horowitz, 1990; Jaenicke, 1991; Somero, 1991; Dahlhoff and Somero, 1993b). However, other studies indicated that a positive correlation between enzyme thermal stability and body temperature is not always present (Place and Powers, 1984; Fields and Somero, 1997; Holland *et al.*, 1997). Comparative studies of enzyme thermal stability in closely related species have produced both types of results. In abalone congeners, the thermal stability of cytosolic malate dehydrogenase (cMDH) was positively correlated with habitat temperature (Dahlhoff and Somero, 1993b), but in barracuda congeners, thermal stability differences among LDH orthologs do not correlate with habitat temperature (Holland *et al.*, 1997). Alexandrov (1977) suggests that thermal stabilities of individual proteins are not necessarily correlated with whole organism thermal sensitivity because some proteins are more temperature sensitive

than are others. Whether enzyme thermal stabilities change adaptively with habitat temperature remains unclear.

LDH as a study system of biochemical adaptation to temperature

The choice of an appropriate group of enzymes to examine the relationship between molecular and whole organismal thermal properties requires careful consideration (Fields and Somero, 1997). Study species must be adapted to different thermal habitats, yet be closely related so that few accumulated mutations between orthologs are observed and those substitutions that have occurred can be interpreted within an adaptational framework. Additionally, the best choice of enzyme to study is one that has been well characterized structurally and mechanistically. These criteria have been the basis of a number of studies of orthologs of A_4-lactate dehydrogenase (A_4-LDH) from congeneric and confamilial fishes distributed over a large range of thermal habitats (polar to tropical) (Somero, 1995, for review; Fields and Somero, 1997, 1998; Holland *et al.*, 1997). Vertebrate A_4-LDH makes an excellent biochemical "study system" for such comparative-evolutionary studies because a great deal is known of its physical and chemical properties. The crystal structure of vertebrate A_4-LDH has been resolved (Abad-Zapatero *et al.*, 1987), and biochemical properties of the enzyme have been well characterized. Additionally, the expression of vertebrate LDH genes, which encode at least three classes of subunits (A, B, and C), has been described (e.g. Whitt *et al.*, 1975). The evolutionary relationships between these isozymes have been the subject of several studies, and while *Ldh*-A was initially suggested as the ancestral gene (Holmes, 1972), recent work indicates that the *Ldh*-c gene is the ancestral type (Li *et al.*, 1983; Baldwin *et al.*, 1988; Tsoi and Li, 1994). The A form, previously referred to as the M type, is principally found in skeletal muscle tissue and liver. A_4-LDH is poised for work in

anaerobic tissues because it favors the conversion of pyruvate to lactate, allowing recycling of the cofactor NAD^+ during anaerobic fermentation. In contrast, the B form, formerly referred to as the H form, is found in aerobic tissues such as heart, where it is poised to convert lactate back into pyruvate, which is then metabolized by the more efficient energy-producing pathways of the citric acid cycle and oxidative phosphorylation.

LDHs of crustaceans

In contrast to what is known about LDHs of vertebrates, comparatively little is known about crustacean LDHs. Early work on the LDHs of crustaceans revealed that while L-lactate was the preferred substrate stereo-isomer in most crustaceans (references below), as is the case in all vertebrates, D-lactate was preferred exclusively by crustaceans of subclass Cirripedia (barnacles) (Gleason *et al.*, 1971; Ellington and Long, 1978). Physical and chemical properties of crustacean LDHs were examined in a number of crustaceans, including the brine shrimp, *Artemia salina* (Ewing and Clegg, 1972), the shrimp, *Palaemon serratus* (Thébault and Le Gal, 1977; Thébault *et al.*, 1981) and the crayfish, *Orconectes limosus* (Scislowski *et al.*, 1982). By far the most thorough investigations of the physical, chemical and kinetic properties of crustacean LDHs were made on the LDH from the lobster, *Homarus americanus* (Kaloustian and Kaplan, 1969; Kaloustian *et al.*, 1969; Eichner and Kaplan, 1977a, 1977b). These studies have shown that while crustacean LDHs share many properties of vertebrate LDHs, there are some major differences. One of the most significant of these is that crustacean LDHs are catalytically active both as dimers and as tetramers, while vertebrate LDHs are only active as tetramers. Lobster LDH subunit association is salt dependent; under low salt conditions, the enzyme associates in a tetrameric state, and the presence of dimers

increases with increasing salt content until all LDH molecules are in the dimeric state at a salt content of 1.3 M ammonium sulfate (Eichner and Kaplan, 1977b). Given that the intracellular salt content of marine crustaceans is greater than zero, but less than 1.3 moles l^{-1} (Robertson, 1961), it is likely that under physiological conditions, the tetramer and dimer states both exist, and may be in an equilibrium (Taylor and Oxley, 1976). However, since the relationship between dimer to tetramer polymerization state is not linear with respect to ionic strength (Eichner and Kaplan, 1977b), it is possible that tetrameric forms predominate under cellular conditions. Factors other than ionic content, such as temperature and protein-protein interactions, may also influence monomer association, and thus alter the relative amounts of dimer and tetramer forms of LDH in the cell.

The substrate affinities of crustacean LDHs in the different association states are dissimilar: The K_m of pyruvate (K_m^{pyr}) was 0.6 mM in low salt (0.1 M Tris-Cl), as compared to 0.18 mM in high salt (0.1 M Tris-Cl plus 1.1 M ammonium sulfate) (Eichner and Kaplan, 1977a). Thus, in high salt, the dimeric lobster LDH has a K_m^{pyr} similar to that of the vertebrate A type, whereas in low salt the lobster tetrameric LDH is kinetically similar to the vertebrate B type. Lobster LDH functions very similarly to vertebrate LDH in the reverse reaction (conversion of pyruvate to lactate), but has different kinetic properties of the forward reaction (conversion of lactate to pyruvate) where sigmoidal kinetics are observed (Kaloustian and Kaplan, 1969). Sigmoidal kinetics were only observed in buffers of low ionic strength, suggesting cooperative interactions between binding sites of the tetrameric form (Thébault and Le Gal, 1977).

Tissue-specific expression patterns of crustacean LDHs have been observed; at least two isozymic variants occur and can combine to form five different electrophoretic species (Trausch and Schoffeniels, 1976). There are varying reports as to the prevalence

of heterotetramers *in vivo*. Studies have shown that heterotetramers do form and that expression patterns are tissue specific (Kaloustian *et al.*, 1969; Somero and Hochachka, 1969; Eichner and Kaplan, 1977b; Thébault and Le Gal, 1977; Scislowski *et al.*, 1982). However, in many crustaceans, LDHs are predominantly homotetramers, as only one electrophoretic species is observed (Ewing and Clegg, 1972; Trausch and Schoffeniels, 1976; Dendinger, 1980).

Determination of the evolutionary relationship of crustacean LDHs to vertebrate LDHs has been made by comparing total amino acid composition (Zietara *et al.*, 1996), and by examination of homologies in the active site and loop regions of the molecule (Taylor and Oxley, 1976). The results of these studies suggest that crustacean LDHs may be most homologous with either the vertebrate B or C LDH isoforms. Lobster LDH has also been shown to co-polymerize with pig B_4-LDH (Trausch and Schoffeniels, 1976), although this finding does not necessarily indicate homology with B isoforms. The complete amino acid sequence (or cDNA sequence) of a crustacean LDH has not yet been elucidated, and thus, the results of the above mentioned studies are not definitive in assessing the evolutionary origins and homologies of crustacean LDHs.

Examination of the effects of temperature on kinetic properties of crustacean LDHs has shown that K_m for pyruvate increases with increasing temperature in a similar fashion to vertebrate LDHs (Somero and Hochachka, 1969; Trausch, 1976; Thébault and Le Gal, 1978; Thébault *et al.*, 1980; Thébault, 1984). K_ms of both pyruvate and lactate of lobster LDHs were shown to be lowest at the acclimation temperature, and to rise sharply at higher temperatures (Trausch, 1976). There is no strong indication that LDH activity or kinetic properties change with thermal acclimation of shrimp (Thébault *et al.*, 1980; Thébault, 1984), although the rate at which K_m increases with increasing temperature was altered in one study (Thébault *et al.*, 1980). Multiple isoforms of crustacean LDHs do

not necessarily exhibit the same changes in kinetic parameters with respect to temperature (Somero and Hochachka, 1969; Thébault *et al.*, 1980; Thébault, 1984), although it is unclear as to whether this would have any effect at physiological temperatures.

A potentially confounding factor exists in previous studies of thermal dependence of kinetic properties of crustacean LDHs in that all of the studies were made in buffer systems that are now known to inadequately simulate intracellular conditions. In the above studies, buffers were used whose pHs changed with temperature in a manner that did not reflect the pH vs. temperature relationship of biological fluids. Buffers used in previous studies were primarily either phosphate buffers, which have a very low change in pH with temperature, or Tris buffers, whose pH changes nearly twice as much as that of intracellular fluids. To gain a more complete understanding of the effects of temperature on crustacean LDH kinetic properties, these studies should be repeated using a buffer system, such as imidazole-Cl, that has a change in pH with temperature that is the same as biological fluids (Yancey and Somero, 1978). Additionally, buffer chemical composition should be adjusted to match the intracellular osmolyte composition, as different osmolytes can profoundly affect kinetic properties (Bowlus and Somero, 1979).

Very little work has been done on the structural stability (defined here as the thermal stability of activity) of crustacean LDHs. In some studies, thermal denaturation curves are presented for one or two species (e.g. Thébault and Le Gal, 1978). Temperatures required for a 50% loss of activity after a 20 minute incubation range from 48 to 61°C among species (Gleason *et al.*, 1971; Thébault and Le Gal, 1978). Only one study of crustacean LDH structural stability has included data for more than a few species (Gleason *et al.*, 1971). In this study, the thermal stability of LDHs from a total of 9

species of shrimps, lobsters and crabs were examined. However, no comparative analysis of the correlation between LDH thermal stability and body temperature was made.

The thermal properties of crustacean LDHs have not been previously measured in an evolutionary, comparative context, akin to studies of vertebrate LDHs such as those in congeneric species of barracuda and gobies (Graves and Somero, 1982; Fields and Somero, 1997; Holland *et al.*, 1997). Here, we present a comparative analysis of the thermal stability of LDHs in a group of congeneric porcelain crabs, genus *Petrolisthes*. These crabs are distributed throughout the Pacific, and there are about 45 species in the Eastern Pacific distributed on both latitudinal and vertical, intertidal gradients (Figs. 1.2, 4.1). Maximum body temperatures of individual species range from 16-42°C (Chapter 4), thus presenting a much larger difference in maximum body temperatures than for previous studies of congeneric fishes (references above). Using porcelain crab LDHs, and employing modern comparative analyses, including phylogenetic methods, hypotheses regarding the adaptive significance of enzyme thermal stability are addressed. Mechanisms of LDH stabilization in these crabs are examined using studies of acclimation to intertidal and subtidal conditions, as well as an investigation into the macromolecular species involved with stabilization of porcelain crab LDHs.

Measurement of enzyme structural stability

Enzyme thermal stability is reported here as the loss of enzymatic activity during heat denaturation. While this measure of thermal stability is the most commonly used in comparative studies (e.g. Dahlhoff and Somero, 1993b; Fields and Somero, 1997, 1998; Holland *et al.*, 1997), it does not necessarily represent the enzyme's biophysical structural stability in terms of the net free energy of folding. This is because loss of activity can occur from only partial unfolding of the protein, leading to subunit disassociation or the

aggregation of multiple proteins. These processes do not quantitatively reflect the net free energy of stabilization of the proteins; they only indirectly reflect the stabilization energies of the regions that unfolded. Methods commonly used in biophysical studies to directly measure the net stabilization energies of proteins include circular dichroic spectroscopy (CD), Fourier transform-infra red spectroscopy, differential scanning calorimetry, and NMR-detected hydrogen-deuterium exchange (e.g. Kasimova *et al.*, 1998; Závodszky *et al.*, 1998). In two comparative studies of protein thermal stability, more direct physical measures have been employed. McFall-Ngai and Horowitz (1990) monitored changes in protein secondary structure during heat treatment using CD, and Donahue (1982) (in Somero (1991)) monitored protein unfolding by detection of tryptophyl residues as they were exposed to the solution during unfolding. These direct physical methods require large amounts of pure protein, which was not available from most species of *Petrolisthes*. Thus, following the common convention of comparative studies, enzyme thermal stability in this paper is indirectly measured as the heat denaturation of activity. Before final conclusions are made from these data, physical methods should be employed to directly measure enzyme structural stability in terms of the net free energy of stabilization for at least a few species that have LDH stabilities over the entire observed range of values.

Materials and Methods

Specimen collection and storage

For interspecific comparisons of the thermal stability of LDH with respect to microhabitat, crabs were collected from the below locations and held in aquaria for 24-48 hours at ambient temperatures. Following this brief acclimation, specimens were frozen

whole in liquid nitrogen, or solid carbon dioxide, or in a -70°C freezer. Collections were always made during the dates of the lowest spring tides of the month. Collection locations and dates of collection are as follows: *Petrolisthes manimaculis*, Monterey Bay, California (36°36'N, 121°53'W), collected bi-monthly, Nov., 1995-Dec., 1997. *P. cabrilloi*, La Jolla, California (32°51'N, 117°16'W), Jan., 1996. *P. eriomerus*, Cape Arago, Oregon (43° 21' N; 124° 19' W), collected bi-monthly, Aug., 1994-Aug., 1995, Aug., 1996, July, 1997. *P. cinctipes*, Cape Arago, Oregon collected bi-monthly, Aug., 1994-Aug., 1995, Aug., 1996, July, 1997 and Monterey Bay, California, collected bi-monthly, Nov., 1995-Dec., 1998. *P. armatus, P. gracilis, P. sanfelipensis, P. hirtipes, P. crenulatus*, Pelican Point, Puerto Peñasco, Sonora, Mexico (31°39'N, 113°15'W), Aug., 1997. *P. granulosus, P. laevigatus, P. violaceus, P. tuberculatus, P. tuberculosus*, Las Cruces, Chile (33°33'S, 71°36'W), Oct., 1997. *P. tridentatus, P. armatus, P. galathinus, P. edwardsii, P. agassizii, P. lewisi austrinsus, P. haigae*, Naos Island, Pacific Panama (8°50'N, 79°8'W), March, 1998.

Laboratory acclimation to intertidal and subtidal conditions

Petrolisthes cinctipes and *P. manimaculis* were collected from Monterey Bay, California on April 9, 1997, and held in flow through aquaria for 5 days. Acclimation to intertidal and subtidal conditions was initiated on April 14, 1997 when crabs were moved to one of two shallow pools. The pools used were hard-sided children's swimming pools, and were roughly 2 m in diameter and 0.3 m deep. A drainage system was placed at one end of each pool, with water inflow at the other end. A 5 cm layer of washed gravel (1-2 cm in diameter) was placed on the bottom of each pool, and stones from the natural habitat were arranged on top of this gravel to provide under-rock habitat for the crabs. Water was allowed to flow through both pools for 2 days before the acclimation was

started. Water flow was regulated such that inflow was faster than outflow, in order to prevent drainage in the event of slight variation in inflow speed. Holes were made at the upper rim of each pool to prevent overflow from washing over the top of the pools, which may potentially have allowed crabs to escape. Crabs (n=182 *P. cinctipes* and n=94 *P. manimaculis*) were added to each pool. The allocation of crabs to the two treatments was made such that crabs of equal sizes were added to the two pools.

The simulated low tide condition in the "intertidal" pool was made by draining this pool every day for a period of 5 hours. The intertidal treatment was always made between 10 a.m. and 3 p.m., times that coincided with direct sunshine on the two pools. A trickle of water was allowed to flow in to the intertidal pool in order to assure that there was water in the interstices of the gravel bed, to prevent desiccation stress. Temperatures were measured by hand with a thermocouple thermometer in both pools immediately before and immediately after the low tide treatment was made. Ten measurements of under-rock temperatures were made from each pool at each time.

Specimens (n=6) of both species were collected weekly from both pools, except for an additional collection on the 4th day of acclimation. Collection was made in such a way as to minimize disturbing the rock arrangement in each pool - the first crabs encountered were collected as long as they did not have missing limbs, or had not recently moulted. Crabs were frozen on solid carbon dioxide at the time of collection and stored at -70°C.

Supernatant preparation

Whole claws (merus, carpus, and manus) were removed from frozen specimens and thawed on ice. The use of freshly molted specimens was avoided, although preliminary studies did not suggest any affect of moult cycle on enzyme thermal stability.

The number of claws used for one supernatant preparation depended on the body size of the species. While single claws could be used for many species, for some, such as *Petrolisthes tridentatus*, *P. gracilis*, and other small-sized species, as many as 20 claws were needed to obtain enough tissue for a single preparation. For large specimens, only a portion of one claw was used to conserve tissue.

Claw muscle tissue was dissected from exoskeleton and endoskeleton material, as well as any fatty tissue, and weighed to the nearest 0.0001g. Muscle tissue was homogenized in Kontes-Duall ground glass tissue homogenizers in 6 volumes (w/v) of 50 mM potassium phosphate buffer, pH 6.8 (i.e. 6 ml buffer g^{-1} tissue) (buffer referred to hereafter as homogenization buffer). Each homogenate was removed from the tissue homogenizer with a fresh Pasteur pipette and placed into a 1.7 ml microcentrifuge tube. The homogenates were then centrifuged at 16,000***g*** for 35 minutes in a microcentrifuge at 4°C. Supernatants were transferred to fresh microcentrifuge tubes and stored on ice until use. Care was taken to avoid any of the lipid rich material that floated at the surface of the supernatant and any of the flocculent material on the top layer of the pellet. If such material was removed along with the supernatant, the sample was re-centrifuged for 10 minutes at 16,000***g*** and the supernatant was transferred to a third fresh microcentrifuge tube for storage. All procedures were performed on ice, unless otherwise noted.

Stabilizer identification experiment

For identification of the molecular factors responsible for protein stabilization, the supernatant (generated as above) was aliquotted into four fractions. One fraction was unaltered, representing the supernatant condition (code: supernatant); a second fraction was dialyzed to remove small molecules (code: -small molecules); a third was subjected to ammonium sulfate precipitation to remove non-protein macromolecules (code: protein

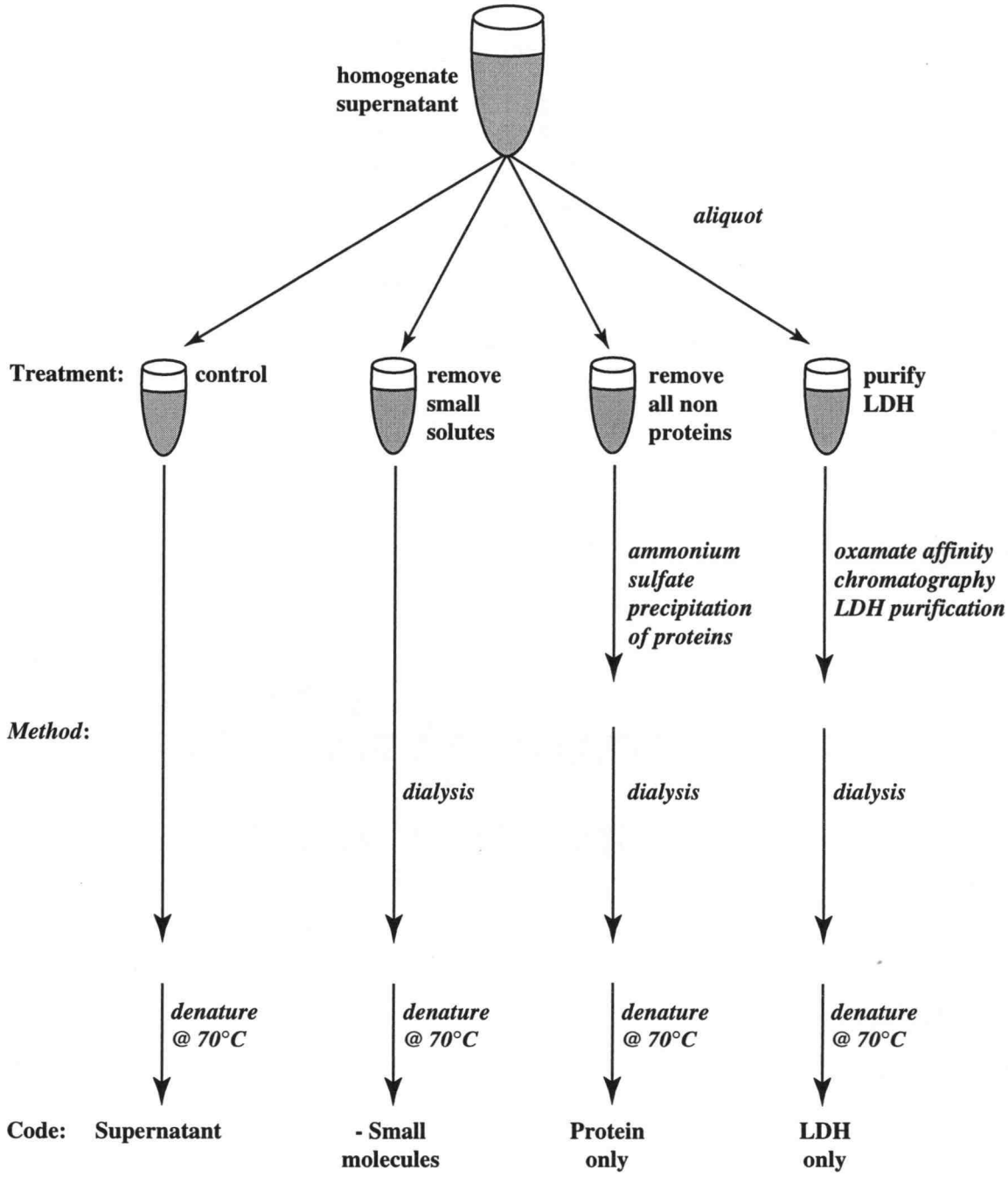

Figure 5.1. Diagram of the experimental procedure used in the stabilizer identification experiment. Sample identification is in normal text, and methods are in italicized text. See text for details.

only); and the final fraction was used in purification of LDH, thereby removing all macromolecules except LDH from the sample (code: LDH only) (Fig. 5.1).

Description of the preparation of each of these groups is as follows: Dialysis was simultaneously performed on all of the experimentally altered fractions following other preparation. Dialysis was accomplished using Slide-a-lyzer dialysis cassettes with a molecular weight cutoff of 10,000 Daltons (Pierce Chemical, #66406T and 66407T). Samples were added to the cassettes directly, without any pre-treatment of the cassettes. For the "- small molecules" and "protein only" fractions, sample volume was from 1-1.5 ml, and cassettes of a volume range of 0.5-3.0 ml were used. For dialysis of column fractions during LDH purification, cassettes of a volume range of 3-15 ml were used, and sample volume was generally between 10-12 ml. Dialysis at 4°C was performed against 4 L of homogenization buffer for 4-6 hours followed by 4 L of fresh buffer for 10-12 hours. Following dialysis, samples were removed from the cassettes and placed into fresh microcentrifuge tubes (for "- small molecules" and "protein only" fractions) or, in the "LDH only" fractions, samples were first concentrated by Centricon 30 concentrators (Amicon, #4209) in a Sorvall RC-5C centrifuge with a SS34 rotor at 4°C.

Proteins were separated from the mix of macromolecules in the supernatant by ammonium sulfate precipitation. Supernatant aliquots were made to 100% ammonium sulfate by slowly adding crystals to the supernatant sample while slowly stirring with a magnetic stirrer. Precipitates were collected by centrifugation at 16,000***g*** for 30 min, and the resultant pellet was resuspended in a volume of homogenization buffer that was 25% less than the original volume of supernatant used. The solution was then dialyzed as described above. Less buffer was used during resuspension to obtain a similar sample volume following dialysis as that of the original supernatant used. During resuspension and dialysis, sample volume increased due to two factors; firstly, the pellet itself

occupied a volume, and secondly, sample volume increased during dialysis. The volume increase occurs because the water concentration in the sample before dialysis is lower than that of the dialysis buffer, as the sample has a greater number of dissolved particles, including macromolecules. As some of these particles do not diffuse through the dialysis membrane, volume of the sample increases when the water concentration in the sample reaches an equilibrium with the water concentration of the dialysis buffer. Following dialysis, the sample was briefly centrifuged, and the supernatant was stored in a fresh microcentrifuge tube. All procedures were performed at 4°C or on ice.

LDH was purified following the procedure of Yancey and Somero (1978) using oxamate affinity chromatography. Oxamate-Sepharose beads were gravity packed into a glass column and pre-equilibrated with 100 ml (10 bed volumes) of 50 mM potassium phosphate, 500 mM potassium chloride, 0.2 mM NADH, pH 6.0, (column buffer). Supernatant aliquots (usually 4 ml) were made to 500 mM potassium chloride and 0.2 mM NADH by addition of appropriate amount of dry chemicals; pH was not adjusted. This solution was gravity fed onto the column, and was followed by 250 ml (25 bed volumes) of column buffer to wash non-LDH proteins off of the column. LDH was eluted by changing the wash buffer to 50 mM potassium phosphate, with 500 mM potassium chloride, and 10 mM pyruvate, pH 6.0 (elution buffer). Fractions were collected immediately upon the change to the elution buffer, and LDH elution was monitored by activity assay (see below). Fractions containing significant levels of LDH activity were pooled, dialyzed, and concentrated (see above). Silver stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) indicates that LDH prepared in this manner is nearly, if not completely purified from other proteins (Figs. 5.2, 5.3). Non-denaturing native PAGE indicated that only one isoform of LDH was present when prepared in this manner (Fig. 5.4).

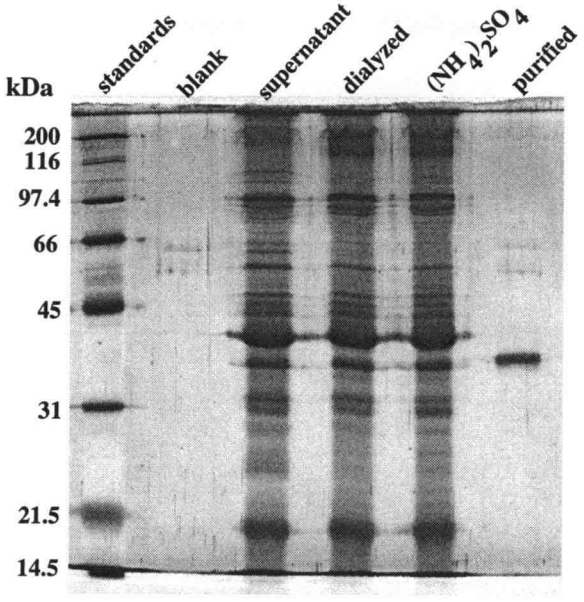

Figure 5.2. Representative SDS-PAGE of protein banding patterns of *Petrolisthes cinctipes* samples. Sample preparations described in the text and Figure 5.1.

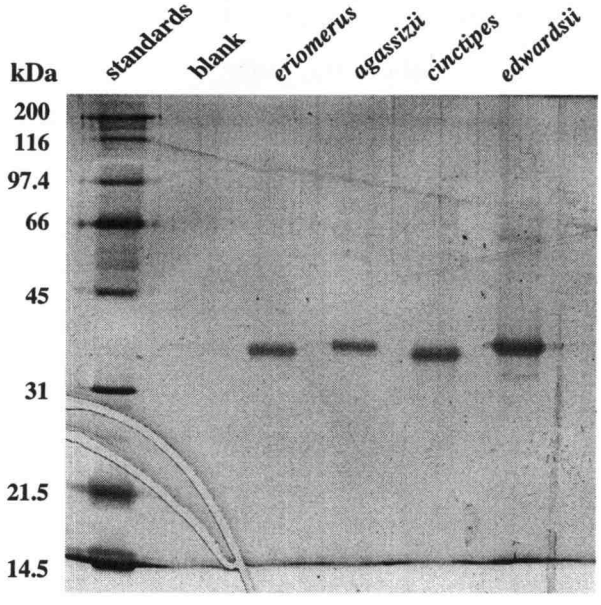

Figure 5.3. SDS-PAGE of purified LDH from four species of *Petrolisthes*.

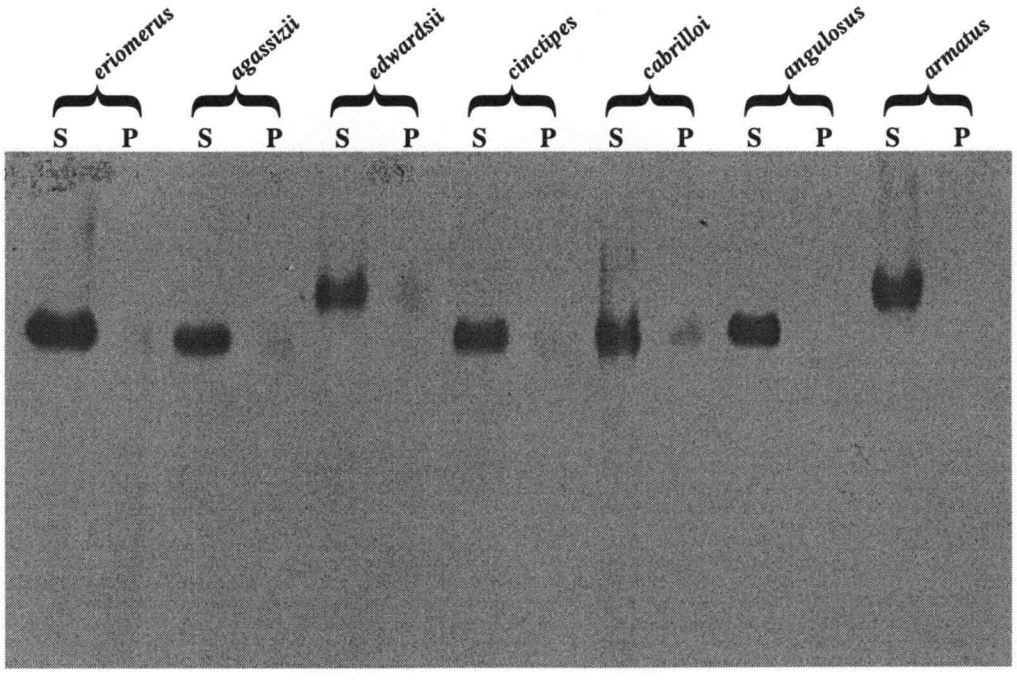

Figure 5.4. Western-stained native PAGE of LDHs from seven species of *Petrolisthes*. For each species, a sample of supernatant (S) and pure LDH (P) have been loaded in adjacent lanes. The two lanes were loaded for equal LDH activity, measured as described in the text. Western blot staining with enhanced chemiluminescence was used. The primary antibody was commercially prepared against *P. cinctipes* native LDH.

Lactate dehydrogenase assay procedure

Enzyme activity was determined using a Perkin Elmer Lambda 3B spectrophotometer equipped with a temperature controlled cuvette cell connected to a Lauda RM6 recirculating water bath. Activity assays were always performed at 20°C. LDH activity was measured by following the oxidation of NADH to NAD^+ as pyruvate was converted to lactate. Reaction conditions were: 80 mM imidazole-Cl, 150 μM NADH, 5 mM pyruvate, pH 6.9, in a volume of 2 ml. Examination of the pH dependence of activity indicated that pH 6.9 was near or at the optimal pH for the conversion of pyruvate to lactate (Fig. 5.5). Thermally equilibrated cuvettes were placed into the spectrophotometer, allowed to thermally re-equilibrate for about 30 seconds, and then the reaction was initiated by addition of 10 μl of enzyme containing solution (supernatant or other). The solution in the cuvette was rapidly stirred for less than 3 seconds, and activity was monitored for about 1 minute. Two replicate assays were made for each sample. If the slopes on the strip-chart records from the two assays looked different (judged by eye), then, a third assay was performed. The two most similar assays were used in subsequent analyses.

Thermal denaturation of LDH activity

To determine the thermal stability of LDH activity in each sample, the following thermal denaturation procedure was used. Aliquots of each sample were placed into 200μl thin walled thermal-cycler tubes (Robbins Scientific # 1045-21-9). Aliquot volumes ranged from 25 to 100 μl, depending on sample volume available, but were usually 60 μl. No effect of aliquot size on thermal stability was seen in preliminary studies. For supernatant, dialyzed supernatant, and ammonium sulfate precipitated samples, aliquots were added without any additional modification. Pure LDH was

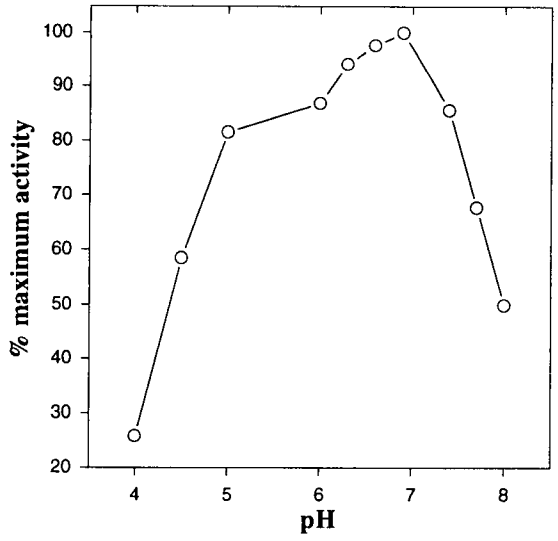

Figure 5.5. The pH dependence of LDH activity in the catalysis of the reverse reaction (pyruvate → lactate). Measurements were made on purified *Petrolisthes cinctipes* LDH. Activity is expressed as a percentage of maximal activity, which was measured at pH 6.9.

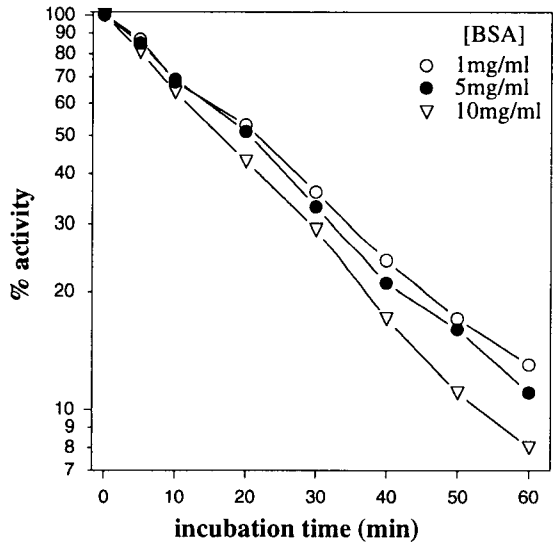

Figure 5.6. The protein concentration dependence of LDH thermal stability in purified LDH. Thermal denaturations were made on purified *Petrolisthes cinctipes* LDH with 1, 5, or 10 mg ml^{-1} bovine serum albumin (BSA). Activity is expressed as a percentage of that from the unheated sample. There was no increase of activity with increasing BSA concentration.

diluted to activities of 0.2-0.3 change in absorbance per minute, and made to 1 mg ml^{-1} bovine serum albumin (BSA) to standardize protein concentration among purified samples. Protein concentrations of supernatant samples were about 10 times higher, but increasing the amount of BSA to 5 or 10 mg ml^{-1} did not increase the LDH stability (Fig. 5.6). Thus 1 mg ml^{-1} BSA was used throughout. Tubes were held on ice, or in an ice-water bath during all times, except during thermal denaturation.

Thermal denaturation was performed in a MJ-Research thermal cycler with a heated lid (model # PTC-100). The heated lid prevented evaporation, and the thermal cycler was set to hold a single temperature during denaturations. The first method of thermal denaturation was to place 6-8 tubes of each sample into the thermal cycler and remove them over time. This method was used to determine the half-life of a sample (see below), and 70°C was the most commonly used temperature, although in some cases, 68°C was used. The second method was to place a single tube of each sample into the thermal cycler and incubate the tube at that temperature for 10 minutes, followed by a rapid cool down of the thermal cycler. This was repeated, at 1°C intervals, over a range of temperatures that resulted in from no loss of activity to total loss of activity during the 10 minute incubation. This method was used to determine the T_{50} for each sample (see below). In both methods, after incubation, tubes were immediately placed on ice. When cool, each tube was centrifuged at 16,600***g*** in a microcentrifuge for 2 min to pellet any precipitate that formed during the thermal incubation. Supernatants were then used directly in activity assays.

Data analysis

For determination of the time at one temperature required for a loss of 50% of activity (the half-life), the following procedure was used. Replicate activity

measurements at each time point were averaged. Percent residual activity was calculated based on the activity of the unheated sample, which never varied during the time that it took to measure the activity of all of the samples. These percentages were log transformed to make them linear with respect to time, and regression analysis was employed to calculate the slope of the correlation line between activity and time (Fig. 5.7). The slope was then used to calculate the half life.

For determination of the temperature required for 50% loss of activity after a 10 minute incubation (the T_{50}), the following procedure was used. Replicate activity measurements at each temperature were averaged. Average activity at each time point was expressed as a percentage of the residual activity of the sample incubated at a temperature just below the level required for loss of activity to be measurable. This sample was used as the 100% rather than unheated sample, because activity increased slightly at elevated, but non-denaturing temperatures. Percentages were transformed by taking the arcsine of the square root of the percent residual activity, which made the activities linear with respect to incubation temperature over most of the denaturation temperature range (Fig. 5.8). Regression analysis was performed over this linear range, and the slope of the correlation line was used to calculate the T_{50}.

Half-life and T_{50} are log-linear when plotted against one another (Fig. 5.9) and the relationship between these two measures of thermal stability was used to generate half-life values from T_{50} values to compare species whose half-lives at 70°C were too short to accurately measure. Half-lives were calculated for each T_{50} value using the equation given in the legend of Figure 5.9.

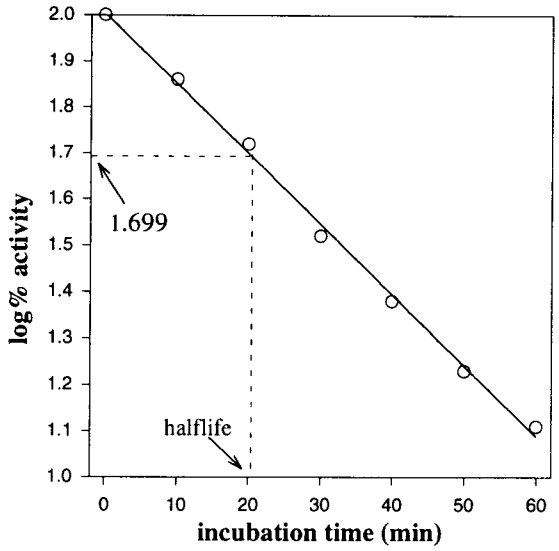

Figure 5.7. Determination of the half-life measure of protein thermal stability. Data are from the denaturation of a *Petrolisthes cinctipes* supernatant sample.

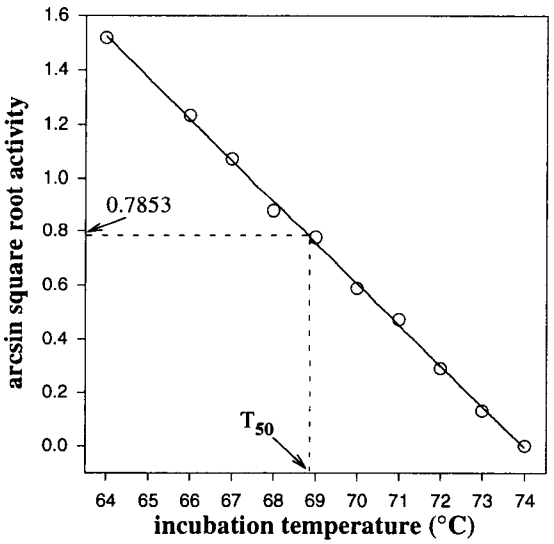

Figure 5.8. Determination of the T_{50} measure of protein thermal stability. Data are from the denaturation of a *Petrolisthes eriomerus* supernatant sample.

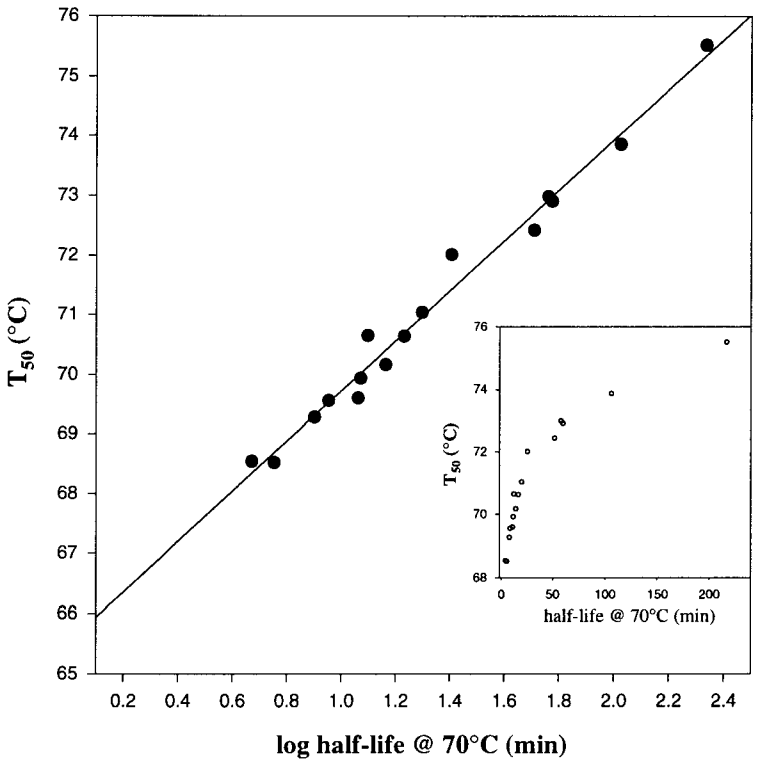

Figure 5.9. The relationship between half-life and T_{50} measurements of protein thermal stability. The insert figure represents the same data, but with half-lives that have not been log-transformed. The equation of the regression line is $T_{50}=65.5135+4.2075x$, $r^2=0.98$, where x is the $\log_{10}$ of half-life at 70°C in minutes.

Independent contrasts analyses

The phylogenetic tree used for independent contrast analyses was generated as described in Chapter 3, but using species for which thermal stability data were collected. Briefly, the phylogenetic tree was generated from a distance matrix based on a maximum likelihood model, with neighbor-joining used to construct the tree. Phylogenetic analyses were performed using several programs from the suite of phylogenetic software known as PHYLIP, version 3.5c (Felsenstein, 1989).

Phylogenetic independent contrasts (Felsenstein, 1985) of T_{50}, vertical position, and maximum habitat temperatures were generated using the CAIC software package (Purvis and Rambaut, 1995), and used in regression analyses.

Results

Patterns of LDH thermal stability

The thermal stability of LDH, as indexed by the half-life measurement, varies by over two orders of magnitude among species of Eastern Pacific *Petrolisthes* (Fig. 5.10). Half-lives at 70°C range from 0.8 ± 0.08 minutes for *P. lewisi austrinsus* (n=3) and *P. crenulatus* (n=3), to 240 ± 13 minutes for *P. edwardsii* (n=5) (Fig. 5.10). Corresponding measures of T_{50} range from 65 to 75.5°C for the above mentioned species. Although there is a large range of LDH thermal stabilities among species, there is no clear overall correlation of LDH stability with maximal habitat temperature (Fig. 5.10). In some cases of sister taxa (Fig. 5.11) living in different vertical zones (Fig. 4.1), LDH thermal stability appears to be correlated with maximal habitat temperatures. For example, in a group of four North Temperate sister species, the two intertidal species, *P. cinctipes* and *P. cabrilloi*, have LDHs that are significantly more stable than the LDHs of the two

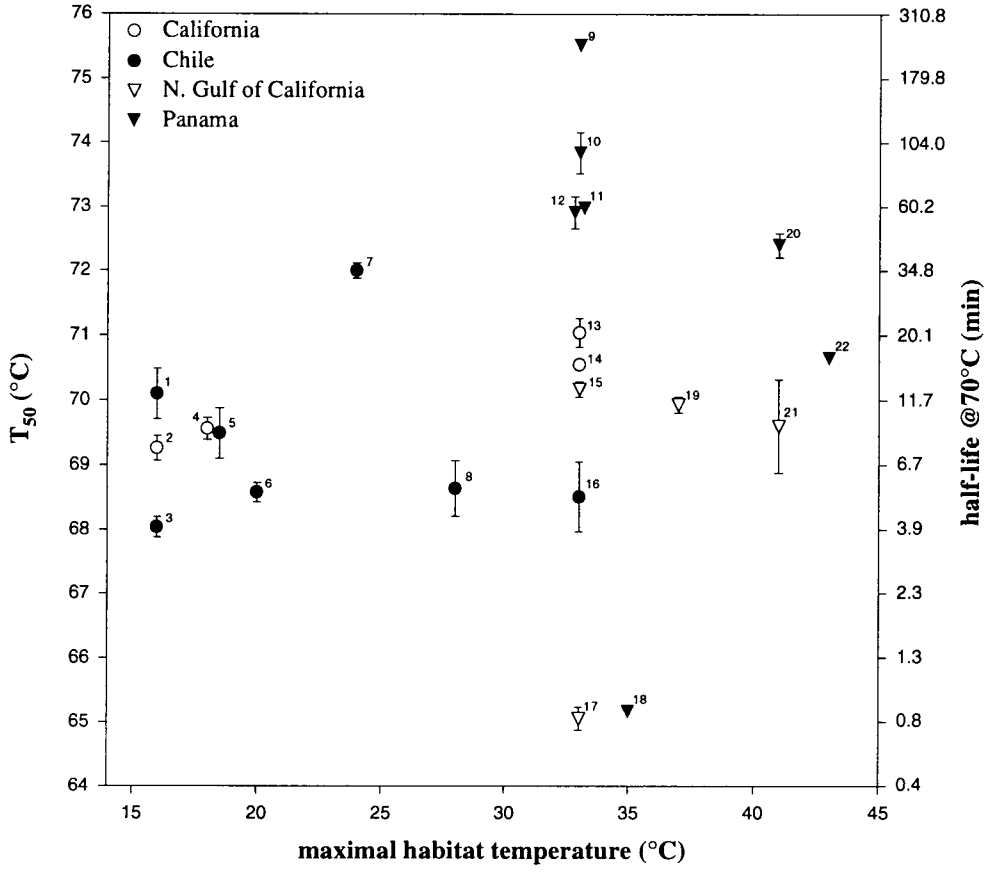

Figure 5.10. Thermal stability of LDHs in muscle homogenate supernatants from 22 species of Eastern Pacific porcelain crabs, genera *Petrolisthes* and *Allopetrolisthes*. Data plotted are T_{50} values; half-lives have been added to the right-hand y-axis for reference. Symbols denote geographical distribution. Each point represents the mean ± 1 S.D. for a species. Species are numbered as follows: 1. *P. tuberculosus*, 2. *P. eriomerus*, 3. *A. punctatus*, 4. *P. manimaculis*, 5. *P. tuberculatus*, 6. *P. violaceus*, 7. *A. angulosus*, 8. *P. laevigatus*, 9. *P. edwardsii*, 10. *P. agassizii*, 11. *P. haigae*, 12. *P. galathinus*, 13. *P. cinctipes*, 14. *P. cabrilloi*, 15. *P. sanfelipensis*, 16. *P. granulosus*, 17. *P. crenulatus*, 18. *P. lewisi austrinsus*, 19. *P. hirtipes*, 20. *P. armatus*, 21. *P. gracilis*, 22. *P. tridentatus*.

subtidal species, *P. eriomerus* and *P. manimaculis*. The measurements of LDH thermal stability in *P. cinctipes* yielded a half-life of 21.16±0.78 minutes (n=5) and T_{50} of 71.04°±0.05°C (n=5), significantly greater values than those of *P. eriomerus* LDH which has a half-life of 7.89±0.81 minutes (n=5) and T_{50} of 69.28°±0.04°C (n=5) (t-test, $p<0.0005$). In other cases of sister species occupying different thermal microhabitats, similar patterns were seen. *Allopetrolisthes punctatus*, which inhabits the low intertidal and subtidal zones, had a half-life of 4.1 ± 1.2 minutes (T_{50} 68.04°±0.49°C) (n=5) while its sister species, *A. angulosus*, which lives in the mid intertidal zone, had a significantly greater half-life of 35.2 ± 6.21 minutes (T_{50} 72.00°±0.33°C) (n=5) (t-test, $p<0.01$). For most species, there was little variation in the T_{50} values among individuals, or among preparations consisting of multiple individuals (Fig. 5.10).

Evolutionary analysis

As stated above, the "adaptive" pattern of increased LDH thermal stability with increasing microhabitat temperature is only apparent in a few groups of sister taxa. Phylogenetically independent contrasts, based on the phylogenetic tree shown in Figure 5.11, do not offer any greater support for the hypothesis that LDH thermal stability is positively correlated with microhabitat temperature within the Eastern Pacific *Petrolisthes* (Fig. 5.12). There is no correlation between contrasts of T_{50} and maximum habitat temperature, or T_{50} and vertical position (Fig. 5.12 - regression lines not shown).

Acclimation experiment

Acclimation of *Petrolisthes cinctipes* and *P. manimaculis* to intertidal or subtidal conditions did not affect LDH thermal stability (Fig. 5.13). In both species, there were no significant differences between half-lives of samples from subtidal or intertidal pools.

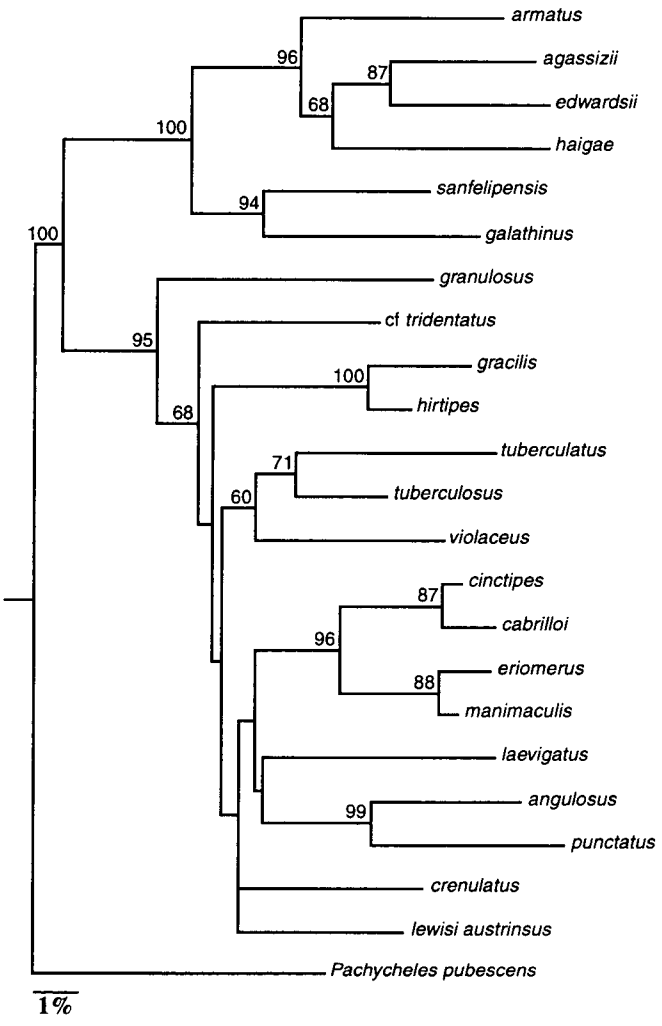

Figure 5.11. Phylogenetic tree of porcelain crabs used in this study. Tree construction was based on the 16sRNA gene. Generation of phylogenetically independent contrasts was performed using this phylogenetic tree. The tree was generated using distance analysis, and numbers next to nodes are bootstrap values out of 100 trees. Bootstrap values less than 60 have not been added to the tree. This tree was rooted with *Pachycheles pubescens*, although this terminal node was not used to generate an independent contrast.

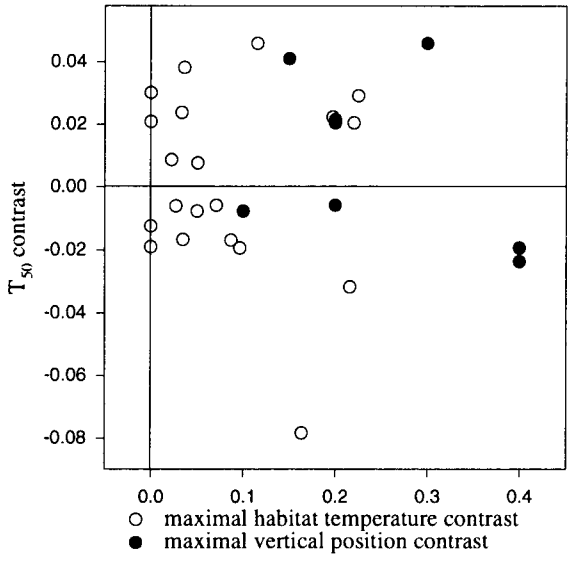

Figure 5.12. T_{50} phylogenetically independent contrasts plotted against contrasts of maximal habitat temperature and maximal intertidal zone vertical position.

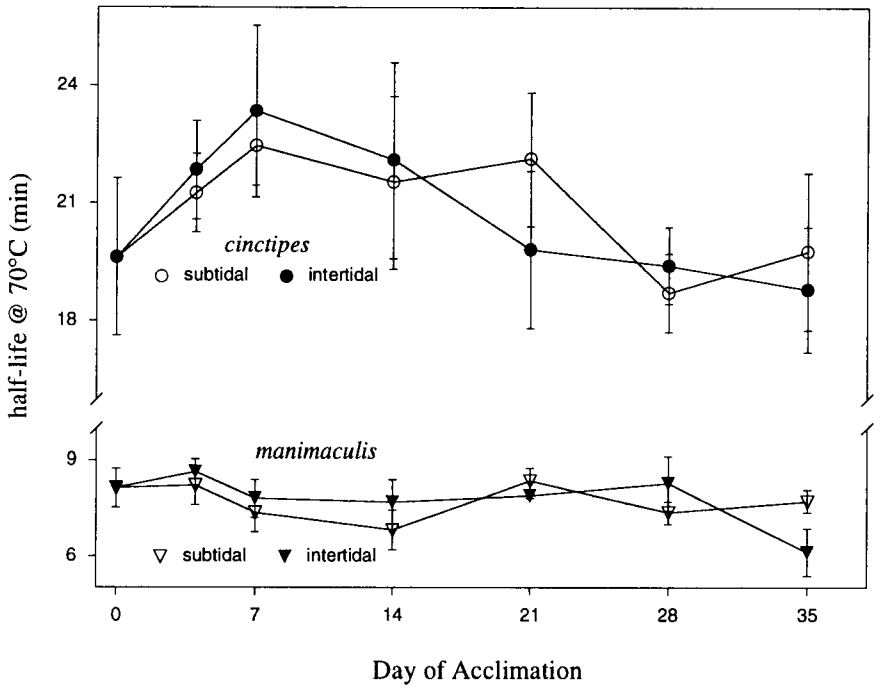

Figure 5.13. Acclimation of LDH thermal stability by intertidal or subtidal treatment in *Petrolisthes cinctipes* and *P. manimaculis*. Each point is the mean ± 1 S.D. of 5 individuals.

Additionally, the half-lives of LDH for each species remained constant during the 5 week acclimation. Half-lives of *P. cinctipes* LDHs were consistently from 19 to 23 minutes, similar values to field-collected specimens. Likewise, half-lives of *P. manimaculis* LDHs matched those of field-collected specimens and were consistently 7-8 minutes throughout the acclimation period (Fig. 5.13). Under-rock temperatures in the intertidal treatment increased during each simulated low tide period, with temperature ranges from 8-15°C above the ambient sea water temperature of 11-13°C (i.e. maximal temperatures of 19-28°C). Under-rock temperatures in the subtidal pool did not vary from the ambient sea water temperature throughout the acclimation period.

Factors affecting the stability of LDH

To investigate the mechanisms of stabilization in porcelain crab LDHs, we selected five species (*Petrolisthes eriomerus*, *P. cinctipes*, *P. armatus*, *P. agassizii*, and *P. edwardsii*) whose LDHs had stabilities over a wide range of the observed variation within the genus. LDH half-lives in muscle homogenate supernatants of these species ranged from 8 to 240 minutes at 70°C (Fig. 5.10). Interspecific differences in thermal stability were consistent regardless of what classes of intracellular macromolecules were present (Fig. 5.14 - note log scale). That is, the ranking of LDH thermal stability was always the same among species in every treatment (Fig. 5.14). Thus, at least part of the LDH stabilization is due to factors intrinsic to the LDH molecules themselves. The half-lives of LDH in the "- small molecules" and "protein only" fractions were consistently slightly higher than the supernatant samples (Fig. 5.14). In "LDH only" fractions, the difference in half-life relative to the "supernatant" fractions varied among species. Half-lives of LDH in "LDH only" fractions from *P. eriomerus* and *P. cinctipes* (7.58±0.47 and 20.88±0.71 minutes respectively) (mean±S.E.M.) were not statistically different from

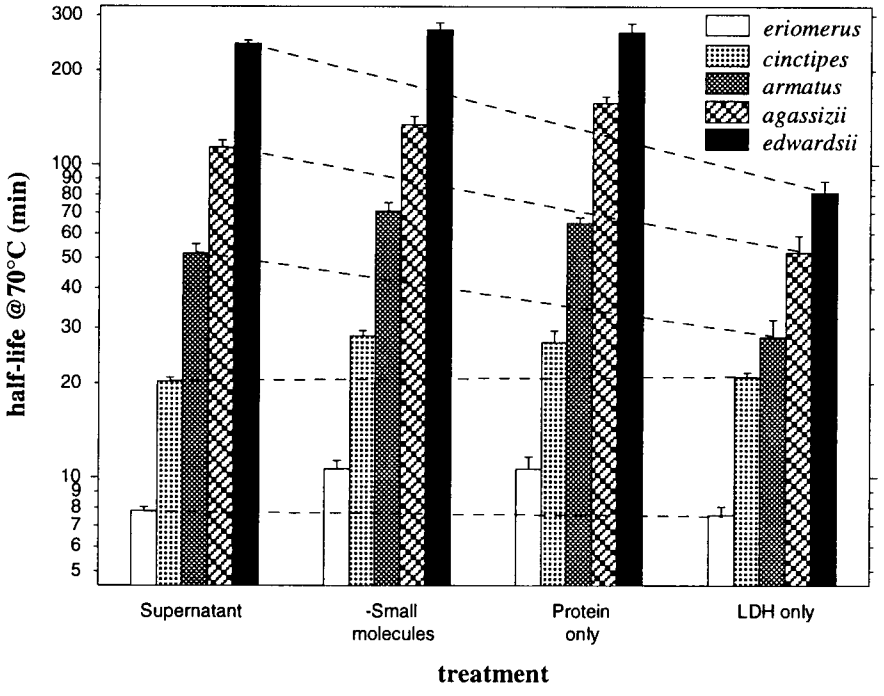

Figure 5.14. Analysis of the molecular classes responsible for stabilization of *Petrolisthes* LDHs. Each bar represents the mean ± S.E.M. half-life @ 70°C for each sample. Sample sizes are as follows: for *P. eriomerus* and *P. edwardsii* n=2; for *P. cinctipes*, *P. armatus* and *P. agassizii* n=3. Dashed lines connect the "Supernatant" and "LDH only" samples for each species.

those in "supernatant" fractions (7.80±0.22 and 20.27±0.54 minutes respectively) (t-test, p=0.69 for *P. eriomerus* (n=2) and p=0.53 for *P. cinctipes* (n=3)) (Fig. 5.14, note dashed near horizontal lines). However, in *P. armatus*, *P. agassizii* and *P. edwardsii*, half-lives of LDH in "LDH only" fractions (28.01±3.94, 52.18±6.95, and 81.63±6.92 minutes respectively) were significantly lower than those in the supernatant samples (51.65±3.98, 113.47±5.98, and 243.44±5.89 minutes respectively) (t-test, p=0.013 for *P. armatus* (n=3), p=0.003 for *P. agassizii* (n=3) and p=0.003 for *P. edwardsii* (n=2)) (Fig. 5.14, note dashed lines). This result suggests that there are differences among the LDHs of these species that are intrinsic to the LDH molecules (as noted by the consistent ranking of half-lives among species), and that in *P. armatus*, *P. agassizii* and *P. edwardsii*, extrinsic protein stabilizers also influence the LDH stability. The presence of extrinsic protein stabilizers in these three species can be inferred from the observation that LDH stability only decreases when the LDH is removed from the pool of supernatant proteins (i.e. the difference occurs from the "Protein only" to the "LDH only" samples).

The protein pools in the "supernatant", "- small molecules", and "protein only" fractions were similar (Fig. 5.2), and purified samples were commonly represented by a single band on silver stained SDS-PAGE (Figs. 5.2, 5.3).

The intrinsic differences between LDH molecules are apparent from SDS-PAGE of samples with widely varying thermal stabilities (Fig. 5.3). LDHs of *Petrolisthes eriomerus* and *P. cinctipes* are about 1kD smaller than LDHs from *P. agassizii* and *P. edwardsii* (Fig. 5.3). Whether the difference in size among these LDHs is due to differences in primary structure, or in post-translational modification, such as phosphorylation or glycosylation, is unknown.

Potential evidence for the interaction of LDH with other protein molecules in the supernatant is provided by Western blot analysis of native-PAGE of LDH from

supernatant and purified samples of conspecifics (Fig. 5.4). In adjacent lanes of the native gel, supernatant and purified samples from seven species of porcelain crabs were loaded with equal amounts of enzymatic activity, but Western staining indicates that there are many more molecules of LDH in the supernatant sample. Enzymatic staining (native stain) produces a similar result, indicating that the LDH molecules in the supernatant sample are enzymatically active (gel not shown). These results suggests that the activity of some molecules of LDH in the supernatant may be masked, potentially by interaction with other groups of macromolecules. This effect is seen in LDHs of species with and without extrinsic protein stabilization. Thus, LDHs of all species may interact with extrinsic proteins, but only in some species are those extrinsic proteins stabilizing the LDHs.

Discussion

Examination of the patterns and mechanisms of LDH thermal stability among congeneric species of porcelain crabs has revealed that while most porcelain crab LDHs are extremely thermally stable, there is no overall significant evolutionary correlation of thermal stability with maximal microhabitat temperature. The thermal stability of LDHs from two North Temperate species did not change following acclimation to intertidal and subtidal conditions, indicating that the bases for LDH stability are genetic or ontogenetic, and LDH stability is not phenotypically plastic within the adult organism. Mechanistic studies have revealed that interspecific variation in LDH thermal stability is caused both by characteristics intrinsic to the LDH molecule, and by extrinsic stabilizing proteins, whose identity and mechanism remain unidentified. Further elaboration on some of these points is given below.

Thermal stabilities of porcelain crab LDHs

Porcelain crab LDHs are unusually thermally stable in comparison to previously studied vertebrate and crustacean LDHs. The temperature required to fully denature the most stable porcelain crab LDH (from *Petrolisthes edwardsii*) within a 10 minute period was 79°C. In previous studies of crustacean LDH thermal stabilities, T_{50}s (after 20 minutes incubation as opposed to 10 minutes as presented here) were as high as 61°C, but were commonly lower (Gleason *et al.*, 1971). In fish A_4-LDHs, temperatures required for total loss of enzymatic activity following a 20 minute incubation are generally well under 60°C (Fields and Somero, 1997, 1998). Incubation of LDHs from all species of *Petrolisthes*, except for the two less stable LDHs from *P. crenulatus* and *P. lewisi austrinsus* (Fig. 5.10), for 10 minutes at temperatures below 65°C resulted in almost no measurable loss in activity. Measurable loss of activity in *P. edwardsii* did not occur at incubation temperatures below 71°C. A direct comparison of LDH thermal stabilities between the data reported here and data from the literature is possible in species, such as *P. cinctipes* and *P. cabrilloi*, that have half-lives of approximately 20 minutes at 70°C. Those species would have T_{50} values of approximately 70°C if thermal incubations lasted for 20 minutes, much higher values than previously found in any eucaryotic organism. Comparisons of thermal stabilities of crustacean LDHs to vertebrate A_4-LDHs must be tempered with the understanding that the crustacean LDHs may not be homologous to vertebrate A_4-LDHs (Zietara *et al.*, 1996).

Evolutionary patterns of LDH thermal stability

Comparative analyses of LDH thermal stability with respect to maximal microhabitat temperatures or vertical intertidal zone position did not indicate that the LDHs of *Petrolisthes* congeners have evolved different thermal stabilities in response to

environmental factors (Fig. 5.12). However, in two cases, closely related species living in different thermal microhabitats had LDH thermal stabilities that were correlated with maximal microhabitat temperatures (Fig. 5.10). Specifically, this was seen in two groups of sister species, the Californian species *P. cinctipes*, *P. cabrilloi*, *P. eriomerus* and *P. manimaculis*, and the Chilean species, *Allopetrolisthes punctatus* and *A. angulosus* (Fig. 5.11). Comparison of closely related species that have different LDH stabilities, but do not live in dramatically different thermal microhabitat conditions (e.g. *P. edwardsii* and *P. agassizii*, and *P. tuberculosus* and *P. tuberculatus*), indicates that LDH thermal stability is not necessarily correlated with microhabitat temperature in sister species. Thus, explanations relating LDH thermal stabilities and thermal microhabitats are not presently possible.

If the thermal stabilities of LDH among species of *Petrolisthes* did not evolve in response to temperature, then what other factors may have resulted in the large observed diversity in LDH thermal stabilities? Locomotor activity varies considerably throughout the genus, from the extremely active *P. tuberculatus* and *P. tuberculosus*, to the slow moving, but warm-living species *P. crenulatus* and *P. lewisi austrinsus*. The latter two species possess the least stable LDHs of any species of *Petrolisthes*, and perhaps this is correlated with their locomotor activity levels. Decreased locomotor activity might prevent any sort of exercise acidosis from occurring in muscle cells, and thus free the LDHs from the need to be acid-stable. Decreased stability to low pH could result in a decreased thermal stability. In a related fashion, perhaps thermal stability is also related to a combinatorial factor of body size, metabolic rate, and respiratory strategy. Further analysis of a variety of physiological parameters of porcelain crabs may shed light onto the adaptive significance of LDH thermal stabilities.

The evolutionary history of LDH thermal stability in porcelain crabs indicates that overall levels of thermal stability can be divided between the two main clades of the genus (Fig. 5.11). Most of the species that are in the "spiny" *Petrolisthes* clade (including *P. armatus*, *P. agassizii*, *P. edwardsii*, *P. haigae*, and *P. galathinus*) have LDHs that are generally more stable than those of any of the species in the other clade, with the exception of *P. sanfelipensis* (Fig. 5.10). Porcelain crabs in the genus *Pachycheles* have LDHs with T_{50}s of 66°-68°C (pers. obs.), which are less thermally stable than those of most species of *Petrolisthes*. The thermal stability of LDHs of Galatheid crabs (genus *Munida*) vary, with *M. hispida* and *M. quadrispinosa* having T_{50}s of 65° and 73°C, respectively (pers. obs. - these two species co-occur in cold-temperate zone marine habitats to depths of 1500 m). Thus, it would seem that while the LDHs from some species of *Petrolisthes* may be more thermally stable than LDHs from other crabs, it is unclear as to when this increased stability first arose, and how widespread it is throughout the superfamily Galatheidae. The evolutionary relationships among anomuran genera are not well resolved, preventing any inferences about the ancestral LDH thermal stabilities or their thermal microhabitats.

Mechanisms of LDH thermal stability

Fractionation of the muscle homogenate supernatant into classes of molecules suggests that porcelain crab LDHs are stabilized both by characteristics intrinsic to the LDH molecules themselves, and by other extrinsic intracellular stabilizing proteins (Fig. 5.14). The slight increase in stability observed in the "- small molecules" and "protein only" samples was probably due to the removal of destabilizing solutes (e.g. Cl^- ions) and the relative increase of stabilizing PO_4^{2-} ions from the dialysis buffer (Timasheff, 1992). The consistent interspecific differences in LDH stability seen in all 4 fractions (Fig. 5.14)

indicates that intrinsic properties of the LDH molecules differ among species. Stabilization by extrinsic proteins is suggested in *Petrolisthes armatus*, *P. agassizii* and *P. edwardsii* by the reduction of LDH stability in "LDH only" fractions as compared to the stability of LDH in "supernatant" fractions (Fig. 5.14). As no decrease in LDH stability occurred when small solutes or large- non-protein molecules were removed in the "- small molecules" and "protein only" treatments (Fig. 5.14), the logical deduction is that the extrinsic stabilizer is a protein or multiple proteins.

Various methods, such as immunoprecipitation, can be used to isolate and eventually identify unknown proteins that interact with a known protein. While we have not been successful in our attempts to isolate or identify an extrinsic stabilizing protein, we present several logical candidates of types of proteins that would likely interact with LDH, and could potentially increase thermal stability. Proteins known to have a role in the rescue and recovery of thermally damaged proteins include the family of chaperone proteins known as heat-shock proteins, or stress proteins (Parsell and Lindquist, 1993). There are many classes of stress proteins with known functional targets and requirements (review in Parsell and Lindquist, 1993). Many of the stress proteins (e.g. hsp-70) are ATP dependent in their action, and because no ATP was present in samples that had been dialyzed, we can rule out these classes of stress proteins as candidates. Potential families of heat-shock proteins that can function in the absence of ATP, and that are known to bind folded or unfolded proteins, include the hsp27 family, the TF55 family, and the cyclophilins (Parsell and Lindquist, 1993).

Non-chaperone proteins may also interact with LDH and confer increased stabilization. Glycolytic enzymes, including LDH, have been shown to be associated with ultrastructural components of muscle contractile apparati (Amberson *et al.*, 1965), such as the intracellular microtrabecular lattice (Clegg, 1984; Masters, 1984; Pagliaro,

1993). The physical arrangement of enzymes that catalyze reactions in a common metabolic pathway into a single metabolic unit (known as the metabolon) may be advantageous for maximally efficient substrate-product trafficking (Weber and Bernhard, 1982). The microtrabecular lattice may provide a framework for such physical arrangements to be made. Additionally, in some cases, glycolytic enzymes require binding to actin for proper function (Bronstein and Knull, 1981), suggesting that these enzymes are readily poised to bind to the cellular microtrabecular lattice or contractile apparatus (Clegg, 1984). The above organization of glycolytic enzymes (and for that matter, all cytosolic enzymes) is probably a closer approximation to actual intracellular conditions than the image of cytosolic enzymes floating randomly within the cell (Clegg, 1984; Pagliaro, 1993). Further investigation of the extrinsic protein stabilization of *Petrolisthes* LDHs may reveal new types, or new modes of protein-protein interaction and stabilization.

Native-PAGE western blot analysis suggests that in the supernatant there may be greatly more LDH present than is indicated by measurement of enzymatic activity (Fig. 5.4). Potentially, this observation is due to "cloaking" of LDH molecules by other proteins during activity assays. This "cloaking" may represent an important intracellular regulatory control. During periods of energy demand that exceed oxygen availability, glycolytic activity increases, and high LDH activities are required to restore the pool of NAD^+, allowing glycolysis to continue (Stryer, 1988). During non-energetically demanding periods of time, however, having high LDH activities in muscle cells is not advantageous, because pyruvate, instead of being reduced to lactate, is shuttled into the citric acid cycle during aerobic cellular conditions (Stryer, 1988). The maximal activity and kinetic properties of vertebrate LDHs are sensitive to small changes in pH (Yancey and Somero, 1978; Walsh and Somero, 1982; Coppes *et al.*, 1992), such that slight

increases in intracellular pH could effectively disable the pyruvate-reductase activity of the enzyme. In some crustacean LDHs, the forward and reverse reactions have been shown to be catalyzed at maximal (although different) rates at a common pH (Scislowski *et al.*, 1982). Thus, potentially in crustaceans, a mechanism to provide the necessary LDH boost when needed, but prevent excess activity when not needed would be to catalytically "cloak" the LDH during periods when activity is not required.

Differences intrinsic to the LDH molecules may represent differences in primary structure, or may represent post-translational modification through addition of phosphate, sugar, or other groups to the LDH molecules. Size analysis by SDS-PAGE suggests that there is an approximately 1 kDa difference in apparent molecular mass between, for example, *P. cinctipes* and *P. edwardsii* LDHs (Fig. 5.2). This mass difference could represent the presence of approximately 10 additional amino acid residues in the primary sequence of *P. edwardsii* (and *P. agassizii*). Certainly, whatever modification has resulted in the 1 kDa increase is implicated in the concomitant increase in thermal stability.

Examination of the intrinsic differences in LDH stability between species is hindered by our deficit of knowledge of the primary, secondary, tertiary and quaternary structure of crustacean LDHs. To begin to deduce the primary structure of crab LDH, we attempted to sequence cDNA of *Petrolisthes cinctipes* LDH. All of our attempts were unsuccessful. A wide variety of primers based on conserved regions among fish (*Sphyraena* - barracuda) and fly (*Drosophila*) LDHs were tested for their ability to amplify parts of the crab LDH cDNA, but none were successful in generating fragments that matched any known LDH sequence. Further studies of intrinsic interspecific differences in LDH thermal stability of Porcelain crabs will be greatly improved if the primary sequence of the LDHs can be obtained. The dramatic interspecific differences in

thermal stability may be determined by only a few mutations in primary structure, as has been seen in fish LDHs (Holland *et al.*, 1997; Fields and Somero, 1998). Knowledge of the structure of crab LDHs, and the locations of specific differences in primary structure may reveal new mechanisms that enzymes use to adjust thermodynamic properties. For example, given the large difference in thermal stability between crustacean LDHs and fish LDHs, it would be interesting to see whether site specific mutations shared any common characteristics, such as has been shown in genetically engineered protein variants with hugely increased thermal stabilities (Van Den Burg *et al.*, 1998).

In summary, we have shown that the thermal stability of an enzymatic protein is not necessarily related to body temperature in ectothermic organisms. Examination of the diversity and evolutionary history of the thermal stability of LDH in a group of marine crustaceans reveals that there is no general adaptational response of enzyme thermal stability to body temperature. LDHs in these species are stabilized by factors that are intrinsic to the LDH molecule and by extrinsic proteins, whose identity and mechanism remains unidentified. The diversity of LDH thermal stability among congeneric species suggests that there is some selective force at work. Further research may resolve both ultimate and proximate causes of LDH stability in these crabs.

Chapter 6

Summary

In the preceding chapters, I have presented studies of the environmental and evolutionary physiology of intertidal and subtidal porcelain crabs. Porcelain crabs in the genus *Petrolisthes* occur over a wide range of microhabitat conditions, in terms of maximal temperatures, temperature ranges, and frequency and duration of emersion. These studies have shown that among species of *Petrolisthes*, there is a great diversity of phenotypes for traits on morphological, physiological and biochemical levels. In question were the proximate and ultimate causes of the observed phenotypic diversity. Proximate causes, those involving the mechanistic bases for observed diversity, were investigated through experimentation. Ultimate causes, those involving evolutionary change through the processes of natural selection, were addressed using phylogenetic analyses. Phylogenetic trees were constructed and used in two ways. 1) Biological traits and microhabitat characteristics were mapped onto the phylogenetic trees and the adaptive significance of the trait was inferred by the correlation of the first appearance of the trait and the microhabitat characteristic (or other biological trait) being investigated. 2) Phylogenetically independent contrasts were generated to ameliorate statistical problems of non-independence of biological data. Standard statistical analyses of the independent contrasts were used to determine the correlations between biological traits and microhabitat or other characteristics.

Morphological and physiological differences among species vary in correlation with microhabitat characteristics. Whole animal thermal tolerance limits and the thermal tolerance limits of heart rate are positively correlated with maximal microhabitat

temperatures in *Petrolisthes* from throughout the Eastern Pacific. Phylogenetic analyses support an adaptive hypothesis of thermal tolerance limits evolving in response to maximal habitat temperatures. In two groups of temperate zone species, whole organism thermal tolerance limits were similar with respect to vertical position in the intertidal zone. This was also the case for groups of tropical and seasonally tropical species. The range of observed thermal tolerance limits, from subtidal to intertidal zone species, was greater in temperate zone species than in tropical or seasonally tropical species. Acclimation studies suggest that temperate zone intertidal species may have adjusted their upper thermal tolerance limits to nearly the maximum extent possible. Further studies of the proximate cause(s) of thermal tolerance in *Petrolisthes* may provide additional insight into the cellular bases of heat death.

Aerial respiration of large-bodied intertidal zone *Petrolisthes* is facilitated by accessory respiratory structures, thin membranous regions on the ventral merus of each walking leg (leg membranes). While no direct correlation between leg membrane size and vertical distribution was observed, there was a positive correlation between leg membrane size and body size. Examination of the distribution of species with large body sizes indicates that leg membrane size is large on all large-bodied intertidal zone species. Phylogenetic analyses indicate that leg membranes are the ancestral condition, but that they probably were not adaptations for aerial respiration. However, the presence of leg membranes can be implicated as exaptations in the evolution of larger body sizes and increased metabolic rate in some species.

Interspecific differences in the thermal stability of LDH were not correlated with microhabitat conditions in phylogenetic and non-phylogenetic analyses, although in several cases of sister species, there was a positive correlation between thermal stability of LDH and maximal microhabitat temperature. LDHs of *Petrolisthes* are the most

thermally stable of any LDHs ever studied for crustaceans, other invertebrates, and vertebrates. However, due to uncertainties in the homology of crustacean LDHs to the LDHs of other organisms, direct comparisons may be inappropriate. LDH thermal stability in several species did not change with acclimation to intertidal or subtidal conditions. The interspecific diversity of LDH thermal stabilities is produced by differences intrinsic to the LDH molecules and by differential effects of extrinsic protein stabilizers. The identities of the specific intrinsic and extrinsic stabilizing factors have not yet been identified. The elucidation of the mechanistic bases for LDH stabilization may provide novel insight to the field of protein stabilization.

Taken together, the results of all of these studies suggest that some organismal traits may have a larger fitness component than other traits, and thus show a clearer adaptive pattern. Thermal tolerance limits arguably have the greatest fitness consequences because having a thermal tolerance limit below the maximal microhabitat temperature would result in death. Without leg membranes, large bodied *Petrolisthes* may not be able to efficiently respire while emersed, but they could definitely endure the low tide period producing energy *via* anaerobic fermentative pathways. Lower metabolic efficiency does have a fitness consequence, but it is likely not as great as that of having an inadequate thermal tolerance. Thus, it is not surprising that in analyses of the evolutionary adaptation of organismal thermal tolerance limits, there is a tighter correlation between thermal tolerance and maximal microhabitat temperatures than there is between leg membrane size and body size. The lack of adaptive significance indicated by phylogenetic analyses of LDH thermal stability suggests that there is not an appreciable fitness consequence of having a more or less stable LDH. Since in all LDHs denaturation would not occur within days at normal body temperature, the non-correlation of LDH stability with environmental characteristics is not surprising.

However, the analysis of LDH structural stability requires further study using biophysical methods before any final conclusions are drawn.

Consideration of additional aspects of the above-mentioned traits, or additional traits of porcelain crabs, as they relate to environmental stresses, may strengthen our understanding of how evolutionary adaptation to abiotic stresses occurs. For example, a comparative analysis of lower thermal tolerance limits, such as was reported for two species in Chapter 2, may refine the comparison of organismal thermal tolerance and thermal microhabitat characteristics. Addition of analyses of salinity tolerance to the above analyses might add another facet upon which a comparative investigation of adaptation to environmental stress could be analyzed. Examination of the proximate causes of these additional traits may also provide new models for the general understanding of the mechanistic bases of cold tolerance, or of osmotic and cell volume regulation.

Evolutionary inferences made using comparative analyses are only as good as the approximation to the true phylogeny of the phylogenetic tree used in those analyses. Inclusion of data from additional species and additional genes may produce a more accurate phylogenetic tree, and thereby may improve comparative analyses. Reasons for continuing on the phylogenetics of this group of crabs include the potential for comparative analyses, but a complete world-wide phylogeny of porcelain crabs will also provide one of most speciose groups available for analyses of factors involved in the establishment and maintenance of global marine biogeographic patterns.

The studies of the biology of porcelain crabs presented in this thesis highlight the great potential of these species as study organisms. I hope that the biological diversity provided by these crabs is the subject of many more fruitful research efforts in the future.

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Appendices

Appendix 1. Descriptions of terms used in studies of comparative biology and evolutionary adaptation.

Biologists comparing the responses of organisms to biotic or abiotic stresses commonly measure phenotypic characteristics that have two fundamentally distinct types of genetic bases, and two-time courses of response. On the one hand, genetic change that occurs during the response to stress over many generations is the accumulation of mutations in gene sequence resulting in the production of a new variant of a specific protein. The new protein may possess structural or kinetic properties that fit more closely to the specific stress. Or, the new protein may interact with other proteins in a different manner (perhaps by altering the form of a structural element, or the actions of other proteins in a biochemical pathway), which increases the overall fitness of the organism. On the other hand, evolutionary modification of gene regulatory systems, notably in species from highly variable environments, may facilitate the expression of the needed types of proteins, in the needed quantities, during environmental stress. The terminology used to discuss these varied responses to environmental stress thus needs to reflect whether differences observed between organisms are a consequence of differences in routinely expressed gene products or in differential expression of genes in response to environmental variation.

If the organisms can adjust a character during their lifetime, then the adjustment is appropriately termed an "acclimation" (if the adjustment is in laboratory settings, where only a single factor is varied) or an "acclimatization" (if the adjustment is in the natural habitat). Adjustments occurring within the lifetime of an organism are commonly referred to as "adaptations" in the older comparative physiology literature, but current

uses are to reserve this term only for cases where processes of natural selection are occurring throughout multiple generations. What distinguishes "adaptation" from "acclimation/acclimatization" is that, in the former, organisms can acquire new genetic information over multiple generations, while in the latter, they cannot. Precisely what is meant by evolutionary biologists when using the term "adaptation" is a complicated issue (Gould and Lewontin, 1979), and as a result, new terminology has been invented for purposes of most accurately communicating the evolutionary nature of a trait (Gould and Vrba, 1982). Definitions of some of these terms are listed below:

Adaptation: A beneficial trait that arose by processes of natural selection for the current function served by the trait. This is an implicitly historical term, and requires knowledge of the evolutionary history of the trait, the need for the trait, and the fitness advantage at the time that the trait arose.

Aptation: A trait that currently has beneficial value but is discussed without regard to the historical framework in which it arose. Most of the description of characters as "adaptations" in the comparative physiology literature would probably be considered "aptations" by those who strictly adhere to the importance of historical reference.

Exaptation: A trait that currently has a beneficial value for which it was not selected. An "exaptation" can be thought of an "aptation" that was a "nonadaptation", or a "preadaptation" or "adaptation" that was "exapted". One example of an exaptation is the use of the second ceratobranchial bone to expand the dewlap of anole lizards, facilitating communication (Larson and Losos, 1996). This bone is a component of the hyoid apparatus, which is homologous to the gill arches of the ancestral aquatic

vertebrates (Larson and Losos, 1996). The gill arches presumably arose by natural selection for the purpose of respiration, and thus are adaptations for respiration, not communication (Larson and Losos, 1996). Another example is the white coat color of Arctic rabbits. Currently, white coloration may be advantageous both for its thermal properties and for its cryptic coloration against the whiteness of the snowy Arctic landscape. If white color was an adaptation for thermal properties, then rabbits living in cold but forested areas might also be white, and the cryptic coloration would be considered an exaptation. If the only white rabbits are those found in Arctic habitats, then the cryptic coloration would be termed an adaptation, and the thermal advantages of whiteness an exaptation (Gould and Vrba, 1982).

Nonadaptation: A trait that arose but has no (known) beneficial function. This term is useful in the context of discussion of "exaptation". For example, the functionless human chin, which forms as the by-product of the formation of the lower jaw, would be considered a nonadaptation (Gould and Vrba, 1982).

Preadaptation: A state where a trait that was already present and had a particular beneficial function could potentially be used by a descendent species for a different beneficial function. More correctly, "preadaptation" should be termed "preaptation" since the state of "preadaptation" is valid without regard to the historical context.

For excellent summaries of the historical and non-historical concepts used in the study of evolutionary adaptation, (and the historical construction of such concepts), I recommend Amundson (1996) and Larson and Losos (1996).

Appendix 2. An overview of the logical framework involved in phylogenetic (tree construction) and comparative (independent contrasts) analyses.

In this appendix, I provide a brief description of methods used in phylogenetic and comparative analyses. I will focus on logical, and not mathematical, points of each method. For a detailed treatment see Swofford *et al.* (1996) and the documentation of the Phylogeny Inference Package (PHYLIP) by Felsenstein (1989). I stress that phylogenetic trees are hypotheses about the evolutionary history of taxa, and do not necessarily represent the true evolutionary history of those taxa. Thus, inferences made from phylogenetic trees need to be qualified appropriately.

Phylogenetic tree construction

Three commonly employed methods in constructing phylogenetic trees are distance, parsimony, and maximum likelihood analyses. The methods can use the same original data set and work with any kind of data (e.g. morphological, allozyme variation, or molecular sequences), but analyze the data differently. Distance and maximum likelihood analyses (described below) require a specific mechanistic basis for how a homologous character evolves from one form to another. Molecular sequence data (DNA) satisfy that requirement because the processes involved when a nucleotide changes from one base to another are understood. My presentation of the methods is in the context of analysis of molecular sequence data.

Preceding comparison of multiple sequences, the sequences must be properly aligned so that bases occupying the same site, or position in the sequence, are homologous. This alignment is probably the most difficult, and most overlooked, aspect

of molecular phylogenetics. Alignment can be performed using computer programs, and when knowledge of the secondary structure of the particular gene is available, that information can also help in the alignment process. For example, sequences of ribosomal genes can be aligned using the stem and loop regions of the ribosome secondary structure. Sometimes sequences cannot be unambiguously aligned. This is especially true when comparing sequences that have different size ribosome loop regions. When regions cannot be unambiguously aligned, a conservative (and correct) approach is to remove those data during phylogenetic analyses.

Distance analysis is a phenetic, or numerical, method that generates a matrix of pair-wise comparisons of the amount of sequence divergence between two taxa. The method compares the number of differences between two sequences as a function of the total length of the sequences. The calculation of divergence is based on a model of evolution selected by the researcher. The models used each have a set of assumptions about the data, such as base composition, equal rates of mutation among sites, and the ratio of transition to transversion mutations. Using the correct model with appropriate parameters is important in precise estimation of the amount of sequence divergence.

With the matrix of pair-wise distances generated, the tree can be drawn by one of two methods: unweighted pair group method with arithmetic means (UPGMA), or by neighbor-joining. UPGMA assumes that the rate of evolution is constant among taxa. UPGMA constructs trees as follows: First, the two taxa with the smallest distance between them are placed together and treated as a single taxon. Next, additional taxa are added in a step-wise fashion based on their distance to the taxa already in the tree (considered a single "composite" taxon by UPGMA), from most similar to least similar, until only one species remains. Lastly, the remaining taxa is assigned to the outgroup, the taxa that is the least related to all of the other taxa.

Unlike UPGMA, Neighbor-joining does not assume equal rates of evolution among taxa, and also allows the researcher to assign an outgroup species. This flexibility of neighbor-joining makes it a better analysis for problems of biological relevance, such as issues pertaining to the molecular clock. Neighbor-joining constructs trees by minimizing the total branch length of the tree. The analysis starts by assuming a "star-phylogeny" where all species are equally related at a common ancestral node. The analysis searches all possible tree groupings until it finds the tree with the shortest total branch length.

Parsimony analysis is a cladistic method that constructs trees based on the minimum number of sequence mutations necessary to explain the evolutionary relationships between taxa. This method assumes that DNA mutations occur over time, and that the closest relatives will have the least number of mutations. Thus, the number of sequence mutations required (by the analysis) to change the sequence of one species to that of another should be at a minimum for the most closely related species. Parsimony analysis, unlike distance analysis, only considers phylogenetically informative sites, that is, sites of nucleotide differences that affect tree topology. Thus, parsimony analysis makes fewer assumptions about the model of sequence evolution than do distance methods. Parsimony analysis is superior to distance analysis in finding the correct branching order of a tree, but distance analysis more accurately portrays branch lengths.

In both distance and parsimony analyses, statistical confidence is commonly placed on nodes using bootstrap or jackknife methods. These methods involve the construction of multiple data sets by re-sampling the original sequence data. Bootstrapping generates sequences that are the same length as the original data set by randomly re-sampling sites from the original data set. In bootstrap generated data sets, the same site of the original data set may be sampled more than once. Jackknifing creates

re-sampled data sets by randomly deleting sites from the original data set. Bootstrapping is the more frequently used method, although jackknifing has been found to perform equally well, and has an advantage in that data sets are smaller and computations will be faster. Regardless of the method used, generally 100-1000 re-sampled data sets are created and trees from each set are generated. A consensus tree is generated based on the topologies that are most frequently represented in the set of trees. The frequency of occurrence of each node is referred to as the bootstrap (or jackknife) value, and is placed on the node of the consensus tree. Generally, bootstrap values of 60% or greater are accepted as statistical support for a node.

Maximum likelihood, a third method for the construction of phylogenetic trees, does so based on the probability that by a given model of evolution and a given evolutionary history, the observed data set would have been produced. The method begins by generating one form of the tree based on a distance model (see above). Branch lengths of the tree are then adjusted until the probability (the likelihood) of the data set is maximized. The likelihood of this tree is compared to the likelihoods of other trees similarly constructed. The tree with the topology that has the highest (maximum) likelihood is the final result. This method offers a basis for statistical inference, and branch lengths are accompanied by confidence intervals and are tested against the null hypothesis that they are equal to zero. Maximum-likelihood is the most robust method against sampling error, and generally gives estimates of branch length with the least variance. The only drawback to maximum-likelihood analysis is that it is extremely computationally intensive, and analyses can occupy computers for very long periods of time.

Comparative analysis by independent contrasts

Comparative analyses ask about the relationship between two biological variables, or between biological and environmental variables. They use biological species or populations as their comparison points. Generally, regression analysis is used to determine the relationships outlined above, and therein lies a problem. A fundamental assumption of regression analysis is that all of the data points be independent. Biological data rarely satisfy this assumption, and to do so, the true evolutionary history of a group of taxa would have to be a star-phylogeny (see above and Fig. 1.1). To overcome the lack of independence, a new set of independent data, known as independent contrasts, are generated from the original data set and from the topology and branch lengths of a phylogenetic tree. The method for generation of independent contrasts was first presented by Felsenstein (1985), and several software packages are available to carry out the analyses.

Independent contrasts are generated as the average value for a trait of two taxa corrected for the distance from the common ancestor of those two taxa. The contrast is a standardized linear contrast between the two taxa with a variance proportional to the branch lengths of those taxa. Contrasts of distant taxa will have greater variances so linear contrasts are standardized by dividing each contrast by its expected standard deviation, thereby satisfying the equal variances assumption of regression analysis. Contrasts are generated for each node of a phylogenetic tree, moving from the tips to the base of the tree, so that for n species, n-1 contrasts are generated. Each pair of tip species gives rise to one contrast, which becomes the tip for the next round of calculations. Contrasts are generated for each variable in this way, and the contrasts of those variables are then used in regression analyses.

Regression analyses of one contrast on another must be forced through the origin. Logically, this makes some sense if one considers that the origin represents the overall ancestral type for the group of taxa from which contrasts were generated. This ancestor is the same for all of the taxa, and there is no other species to which it is compared, thus it cannot possibly have a value for its contrast other than zero. Forcing the regression through the origin is sometimes dissatisfying, especially in the cases where the origin really has a lot of statistical leverage (such as in Fig. 3.7, solid symbols), yet is required when analyzing the relationship between independent contrasts.

Independent contrasts analysis takes into account aspects of the phylogenetic tree including branch length and branch order, but does not take into account the statistical significance of each node. One way to get around this limitation is to change the point in the analytical process when the bootstrapped data sets are condensed into one. Instead of using one consensus tree from bootstrapped analyses, independent contrasts can be generated for each of the bootstrapped trees, (i.e. generate 100-1000 sets of independent contrasts). For each of these sets of independent contrasts, one could then perform regression analyses and formulate a consensus of the results of the regression analyses, thereby incorporating the statistical rigor of the bootstrap into the independent contrasts analysis (Dave Swofford, pers. comm.).

There are additional methods besides independent contrasts that remove or control for the effects of phylogeny and which could be employed for the analysis of comparative data, including: hierarchical ANOVA, phylogenetic autocorrelation, and phylogenetic regression (summarized in Miles and Dunham, 1993). Each method has its own advantages and disadvantages, and may provide a different interpretation of the data. Thus, a more conservative approach may be to employ several methods to test the same hypotheses of the relationship of biological and environmental variables.

Appendix 3. Species morphologically examined in this study.

Species examined	D[1]	S[2]
Petrolisthes agassizii	E	1
amoenus	A	9
annuleipes	W	?
armatus	E,A	1
artifrons	E	1
asiaticus	W	2
australiensis	W	2
bispinosus	W	2
borradailei	W	2
boscii	W	2
brachycarpus	E	1
cabrilloi	E	1
caribensis	A	10
carinipes	W	14
celebesensis	W	5
cessacii	A	8
cinctipes	E	1
coccineus	W	16
cocoensis	E	1
crenulatus	E	1
decanthinus	W	16
dentatus	W	2
desmarestii	E	1
dissimulatus	A	20
edwardsii	E	1
eldredgei	W	4
elegans	W	5
elongatus	W	2
eriomerus	E	1
extremus	W	3
fimbriatus	W	2
galathinus	E,A	1
galapagensis	E	1
gertrudae	A	7
glasselli	E	1
gracilis	E	1
granulosus	E	1
haigae	E	1
hastatus	W	2
haswelli	W	2
heterochrous	W	11
hians	E	1
hirtipes	E	1
hirtispinosus	E	1
holotrichus	E	1
indicus	W	2
japonicus	W	2
jugosus	A	8
kranjiensis	W	1
lachnaeus	W	?
laevigatus	E	1
lamarckii	W	16
leptocheles	W	2
lewisi lewisi	E	1
lewisi austrinsus	E	1
Petrolisthes lindae	E	13
magdalenensis	A	19
manimaculis	E	1
marginatus	A	10
masakii	W	2
mesodactylon	W	2
militarus	W	16
miyakei	W	2
moluccensis	W	16
nigrunguiculatus	E	1
nobilii	E	1
novaezelandiae	W	2
ornatus	W	2
ortmanni	E	1
penincillatus	W	16
perdecorus	W	5
platymerus	E	1
politus	A	8
polymitus	E	1
pubescens	W	2
quadratus	A	8
rathbunae	E	1
robsonae	E	1
rosariensis	A	8
rufescens	W	16
sanfelipensis	E	1
scabriculus	W	2
schmitti	E	1
speciosus	W	2
teres	W	2
tiburonensis	E	1
tomentosus	W	11
tonsorius	E	1
tridentatus	E,A	1
tuberculatus	E	1
tuberculosus	E	1
unilobatus	W	2
violaceus	E	1
virgatus	W	2
zacae	E	18
Allopetrolisthes angulosus	E	1
punctatus	E	1
spinifrons	E	1
Liopetrolisthes mitra	E	1
Neopetrolisthes maculatus	W	2
Parapetrolisthes tortugensis	A	?

Species not examined[3]		D	S
Petrolisthes	*alobatus*	W	2
	columbiensis	A	12
	donanensis	W	6
	haplodactylus	W	15
	limicola	W	15
Neopetrolisthes	*ohshimai*	W	2
Liopetrolisthes	*patagonicus*	E	17

Appendix 1. Continued

Notes:

1. D (Distribution) is Eastern Pacific (E), Western Pacific (W) or Atlantic (A).
2. S (Sources) are as follows: 1. (Haig, 1960), 2. (Hsieh, 1993), 3. (Kropp, 1994), 4. (Haig and Kropp, 1987), 5. (Haig, 1981), 6. (Osawa, 1997), 7. (Werding, 1996), 8. (Werding, 1982), 9. (Haig, 1966), 10. (Scelzo, 1980; Scelzo and Varela, 1988), 11. (Kropp, 1986), 12. (Werding, 1983), 13. (Gore, 1982), 14. (Haig, 1987), 15. (Haig, 1988), 16. (Haig, 1983), 17. Weber Urbina (1991), 18. Haig (1968), 19. (Werding, 1978), 20. (Gore, 1983).
   ? indicates specimens were present in museum collections, but literature mention was not found.
3. Species not examined include those for which literature mention was found, but no museum specimens were encountered.

Appendix 4. Sequences of 16sRNA genes from porcelain crabs (Anomura: Porcellanidae). Shaded regions indicate regions that were not unambiguously aligned, and were omitted from analyses. Species are all in the genus *Petrolisthes*, unless specific name is preceded by an A (*Allopetrolisthes*), L (*Liopetrolisthes*) or P (*Pachycheles*). Sequence data represented by "." indicate synonomy with the sequence of *Petrolisthes cinctipes*. For *Petrolisthes armatus* collections were made at three locations: m = Northern Gulf of California, p = Pacific Panama, and a = Caribbean Panama. For *Petrolisthes galathinus*, 7 different "types" were sequenced: 1=Pacific Panama, 2-7 are from the Caribbean Panama and have different colorations where 2=yellow colored from San Blas Islands (SB), 3=white colored (SB), 4=red colored from Galeta (G), 5=red leg color (SB), 6=yellow colored (G), 7=red-bodied but white-legged (SB).

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                1                                                          60
cinctipes       GTCTA-TTCT GCCCATTGAG GAA-AGTTCT T-AAAAGGCC GCGGTATTCT AAC-TGTG-C
cabrilloi       .......... .......... T......... .......... .......... ..........
eriomerus       .......... .......... T......... .......... .......... ..........
manimaculis     .......... .......... T..M...... .......... .......... ...A......
tiburonensis    .......... .......... T...-..... .......... .......... ..........
laevigatus      .......... .......... T.T..AA... .......... .......... ..........
A. angulosus    .......... .......... A..A-A.... .T-..C.... .......... ..........
A. punctatus    ....G..... .......... A..G-A.... .......... .......... ..........
A. spinifrons   A......... .......... A..A.A.... .......... .......A.. ..........
tuberculatus    ...A...... .......... AG.AG-.... ..G....... .......A.. ..........
tuberculosus    .......... .......... A...-A.... .......... .......A.. ..........
crenulatus      ...Y..A... .........A A.TATT..T. .......... ..A....A.. ..........
holotrichus     ....T..... .......... ..-A...... .......... .......A.. ..........
lewisi austrinsus .......... .......... -.-AGA.... .......... ..A....A.. ..........
platymerus      ....T..... .......... ..-A.A.... .......... C......A.. ..........
violaceus       .......... .......... .....-.... .......... .......A.. ..........
L. mitra        .......... .......... A....A.... .......... ......AA.. ..........
gracilis        ....G.AG.C .........A A.-..A.... .......... .......ATA ........G.
hirtipes        ....G..... .......... A.-..A.... .......... .......AT. ..........
schmitti        ....G..... .......... A.-..A.... .......... .......A.. ..........
cf tridentatus  ....T..... .......... A....A.... .......... .......A.. ..........
tridentatus     ....C..... .......... A....A.... .......... .......A.. ..........
granulosus      .......... .......... A.TA.--... .......... .......A.. ..........
elongatus       ....G.MC.. .....C...A T.TT--.... .TT..C.... ......C... G.........
novaezelandiae  .......C.. .....C...- T--.--..T. ...G.G.... ........T. G.........
armatus (m)     ....G..... ....G....A T.TT.TAGT. ..G....... ..A....CT. G.........
armatus (p)     ...AG..... ....G....A T.TT.TAGT. ..G....... ..A....CT. G.........
armatus (a)     ....G..... ....G....A T.TT.TAGT. ..G....... ..A....CT. G.........
zacae           ...CG..... ....G..... T-TT.TAA.. ..G....... ..A.....T. G.........
agassizii       ....G..... ....G....A TG-..AAAT. ..G....... ..A....CT. G.........
edwardsii       ....G..... ....G....A TG-..AAAT. ..G....... ..A....CT. G.........
haigae          ....G..... ....G....A TG-T.AAAT. ..G....... ..A....CT. G.........
sanfelipensis   .......... .........A T...TAAAT. .......... ..A....... G.........
galathinus (1)  .......... .........A T..T.-AGT. .......... ..A....... G.........
galathinus (2)  .......... .........A T...-AAAT. .......... ..A....... G.........
galathinus (3)  .......... .........A A..T-AAAT. .......... ..A....... G.........
galathinus (4)  .......... .........A T...-AAAT. .......... ..A....... G.........
galathinus (5)  .......... .........A T..T-AAAT. .......... ..A....... G.........
galathinus (6)  .......... .........A T...-AAAT. .......... ..A....... G.........
galathinus (7)  .......... .........A T..T-AAGT. .......... ..A....... G.........
P. chilensis    .......... .........A --TA.A.AT. .......... ..A....C.. ..........
P. crinimanus   .......... .........A --TA.A.AT. .......... ..A....C.. ..........
P. grossimanus  .......... .........A --TA.A.AT. .......... ..A....C.. ..........
P. calculosus   .......... .........A --TA.A..T. .......... ..A.....T. ..........
P. rudis        .......... .........A --TA.A.AT. .......... ..A....C.. ..........
P. pubescens    .....A.... .........A --TA.A.AT. .......... C.A....C.. ..........
P. setimanus    .......... .........A T.TATA.AT. .......... ..A....C.. ..........
P. trichotus    ...M...... .........A TGTATA.AT. .......... ..A....C.. ..........
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Appendix 4. Continued

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                61                                                                                                  120
cinctipes       AAAGGTAGCA TAATCATTAG TTTCTTAATT GGAGGCTTGT ATG-AATGAT TGGATAAAAA
cabrilloi       .......... .......... .......... .......... ......G... ..........
eriomerus       .......... .......... .......... .......... ...G...... ..........
manimaculis     .......... .......... .......... .......... .......... ..........
tiburonensis    .......... .......... .......... .......... .......... ..........
laevigatus      .......... ......G... .......... ......C... ......C... .......R..
A. angulosus    .......... ......G... C......... .......... ......C... .A.....G..
A. punctatus    .......... ......G... C......... .......C.. .......... .A.....G..
A. spinifrons   .......... ......G... .......... .......... .......... .......G..
tuberculatus    .......... ......G... .......... .......... .......... .......G..
tuberculosus    .......... ......G... .......... .......... .......... .......G..
crenulatus      .......... ......G... .......... .......... .......... ..........
holotrichus     .......... ......G... .......... .......... .......... .......G..
lewisi austrinsus .......... ......G... ...T...... .......... .......... .......G..
platymerus      .......... ......G... .......... .......... .......... .......G..
violaceus       .......... ......G... .......... .......... .......... .......G..
L. mitra        .......... ......G... C......... A......... .........G .......G..
gracilis        .......... ......C..K ...T...... .A........ .......... ..........
hirtipes        .......... ......G... ...T...... .A........ .......... ..........
schmitti        .......... ......G... ...T...... .A........ .......... ..........
cf tridentatus  .......... ......G... ...T...... .......... .......... .......G..
tridentatus     .......... ......G... ...T...... .A........ .......... .......G..
granulosus      .......... ......G... .......... .......... .......... ....C..G..
elongatus       .......... .......... C..T...... .AGA...G.A ......A.G. C.A.C..G..
novaezelandiae  .......... ......C... ...T...... .A........ .........GG ..A.C..G..
armatus (m)     .......... .......... ...T...... .A.......A .......... C.A.C..G..
armatus (p)     .......... .......... ...T...... .A.......A .......... C.A.C..G..
armatus (a)     .......... .......... ...T...... .A.......A .......... C.A.C..G..
zacae           .......... .......... ...T...... .........A .......... C.A.C..G..
agassizii       .......... ......C... ...T...... .A.......A .......... CT..C..G..
edwardsii       .......... ......C... ...T...... .A.......A .......... CT..C..G..
haigae          .......... ......C... ...T...... .A.......A .......... C...C..G..
sanfelipensis   .......... .......... ...T...... .A........ .......... ....C..G..
galathinus (1)  .......... .......... ...T...... .A........ .......... ....C..G..
galathinus (2)  .......... .......... ...T...... .A........ .......... ....C..G..
galathinus (3)  .......... .......... ...T...... .A........ .......... ....C..G..
galathinus (4)  .......... .......... ...T...... .A........ .......... ....C..G..
galathinus (5)  .......... .......... ...T...... .A........ .......... ....C..G..
galathinus (6)  .......... .......... ...T...... .A........ .......... ....C..G..
galathinus (7)  .......... .......... ...T...... .A........ .......... ....C..G..
P. chilensis    .......... .........K ...T...... .......... ......CR.. ....C..GGG
P. crinimanus   .......C.. .........T ...T...... .......... ......CA.. ....C..GGG
P. grossimanus  .......... .......... ...T...... .......... ......CR.. ....C..GGG
P. calculosus   .......... .......... ...T...... .A........ ......C... ....C..G.G
P. rudis        .......... .......... ...T...... .......... .......... ....C..G.G
P. pubescens    .......... .......... ...T...... .......... ......C... ....C..GGG
P. setimanus    .......... .......... ...T...... .......... ......C... ....C..R..
P. trichotus    .......... .......... ...T...... .......... ......C... ....C..G..
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Appendix 4. Continued

	121					180
cinctipes	TTG-AACTGT	CTTTTTTT-A	ATAAATTGAA	TTTTATTTTT	GAGTGAAAAA	G-CTTAAATA
cabrilloi	....	........A.	T-........	....	....	....
eriomerus	.C.G......	.....C....	....	....	....	....C....G
manimaculis	....	.....C....	....	....	....	....C.....
tiburonensis	....	....	...G......	....	....	....C....G
laevigatus	....	....	.A........	....	A.........	.........G
A. angulosus	..A.......	.....C..A.	T.G.G.....	....	A.........	....
A. punctatus	..A.......	....CC..A.	T...C.....	....	A.........	.........G
A. spinifrons	..A.......	.....C....	.A........	....	A.........	.........G
tuberculatus	..A.......	..C.CC..T.	.A.G.C....	....	A.........	....
tuberculosus	..A.......	.....C....	.A........	....	A.........	....
crenulatus	..A.......	.....C....	.A........	....	A.........	.........G
holotrichus	..A.......	.....C....	.A........	....	A.........	.........G
lewisi austrinsus	..A.......	.....C....	GA........	....	....	....C....G
platymerus	..A.......	.....C....	GA.G......	....	A.........	.........G
violaceus	..A.......	.....C....	.A...C.A..	....	A.........	....
L. mitra	..A.......	....AC....	RA.T..AA..	....	A.........	....
gracilis	..A.......	.....C....	.A..G.....	....	A.........	.C........
hirtipes	..A.......	.....C....	.A........	....	A.........	....
schmitti	..A.......	....	.A..G.....	....	A.........	....
cf tridentatus	..A.......	.....C....	.A........	....	A.........	....
tridentatus	..A.......	.....C....	.A........	....	A.........	....
granulosus	..A.......	......AAA-	.AR.......	....	A.........	....
elongatus	..A.G...A.	..A.A.G..T	TA.....T..	G..A......	.T........	...A......
novaezelandiae	..A..T....	..C.......	....	.........A	A.........	.....T....
armatus (m)	C.C..G....	.....C.GAT	TA........	....	A.........	....
armatus (p)	C.C..G....	.....C.GAT	TA........	....	A.........	....
armatus (a)	C.C..G....	.....C.GAT	TA........	....	A.........	....
zacae	C.T.......	......AAAC	....	....	AG........	...C......
agassizii	CCA.......	..C....GG.	GA.-......	....	A.........	....
edwardsii	C.A.......	..C....GAT	TA.-..C...	G.........	A.........	.........G
haigae	C.A.......	....C.CGAT	TA.G......	....	A.........	....
sanfelipensis	..A..G....	..C....GAT	TA..T.....	....	A.........	.........G
galathinus (1)	..A..G....	..C...CAAT	.AC.C.....	....	A.........	....
galathinus (2)	..A..G....	..C...CAAT	.AT.T.....	....	A.........	.........T
galathinus (3)	..A..G....	..C...CAAT	.A..T.....	....	A.........	....
galathinus (4)	..A..G....	..C...CAAT	.GTGT.....	....	A.........	....
galathinus (5)	..A..G....	..C...CAAT	.A..T.....	....	A.........	....
galathinus (6)	..A..G....	..C...CAAT	.AT.T.....	....	A.........	.........G
galathinus (7)	..A..G....	..C...CAAT	.AG.T.....	....	....	....C.....
P. chilensis	C.A.......	..C.....T-	.A........	.......A..	A.........	.........G
P. crinimanus	C.A.......	..C.....T-	.A........	.......A..	A.........	.........G
P. grossimanus	C.A.......	..C.....T-	.A........	.......A..	A.........	.........G
P. calculosus	C.A.......	..C.....T.	..T.......	.......A..	....	.........G
P. rudis	C.A.......	..C.....T.	.A-.......	.......A..	A.........	.........G
P. pubescens	C.A.......	..C.....T.	.A-.......	.......A..	A....G....	....
P. setimanus	C.A.......	..C....CT.	..T.G.....	........C.	A.........	......G...
P. trichotus	C.A.......	..C.....T.	..T.......	........C.	A.........	......G...

Appendix 4. Continued

	181					240
cinctipes	AACC-AGAGG	GACGATAAGA	CCCTATAAAT	CTTTATGAAG	GTG-TTTTTG	TTTAATGAAA
cabrilloi	....	....	....	....	....	....
eriomerus	..T.......	.....C....	....	....	A.T...C...	....
manimaculis	..T....G..	.....C....	....	....	A.T...C...	....
tiburonensis	..TT......	.....C....	....	....	A.C......A	..C.G.A...
laevigatus	..T.......	....	....	.......T.A	T..TYC....	..A.G.A...
A. angulosus	TTT....G..	....	....	...C...T.A	T..G..C...	AA.GG.A.T.
A. punctatus	CGT.......	....	....	.......T..	...A......	.G..G.A.T.
A. spinifrons	..TT......	....	....	.......TTA	T.TA......	......A...
tuberculatus	.GTT......	....	....	.......TGA	A.-A......	.A.GGC..T.
tuberculosus	..TT......	....	....	.......TGA	A.........	......A.T.
crenulatus	..TT......	....	....	...A...T.A	T.-G.....A	....GCT.T.
holotrichus	..AT......	....	....	...C...T.K	T.-G..C...	.....CA.T.
lewisi austrinsus	..T.......	....	....	.......T.A	T.-A..C..A	......T.T.
platymerus	..AT......	....	....	.......T..	T.-G.CC...	......A.T.
violaceus	..TT......	....	....	....	T.AT......	..CG.CA...
L. mitra	...T......	.....C....	....	.......TGA	T.TT....CA	......A.T.
gracilis	..T.......	....	....	.......T..	T.-T......	CC.T..T...
hirtipes	..T.......	....	....	.......T..	T.-T......	C..T..T.T.
schmitti	..T.......	....	....	.......T..	T.-T......	C..T..T.T.
cf tridentatus	..T.......	....	....	.......T.T	A.-G.....A	...-..T.T.
tridentatus	..T.......	....	....	.......TGT	..-G.....A	...-..T.T.
granulosus	..TG......	....	....	.......T.A	TATG.C...C	.AA.G.A.T.
elongatus	T....G..AA	....	....	......A.TA	.C.AAA...T	G...TAT...
novaezelandiae	..TT......	....	....	......ATCA	T.TT.C..AA	AAA...A..G
armatus (m)	..TA....AA	....	....	.........A	T.TAG....T	AAA.T.A...
armatus (p)	..TA....AA	....	....	.........A	T.TAG....T	AAA.T.A...
armatus (a)	..TA....AA	....	....	.........A	T.TAG....T	AAA.T.A.T.
zacae	..TA.....A	....	....	.........A	T.TAAC.C.T	GAA.T.A...
agassizii	CTTA..A.AA	....	....	.......T.A	A.TAA.C..T	AA.TC.A.-.
edwardsii	.TTA..A.AA	....	....	.......T.A	A.TAG....T	AA.TC.A.-.
haigae	..TA....AA	....	....	...G...T.A	T.TAG.AC.T	AGGTT.A.T.
sanfelipensis	.TTA.....A	....	....	.......T.A	A.CAAC...T	.AAGT.T...
galathinus (1)	.TAA....AA	....	....	.......T.A	A.TAG-...T	.AAGT.A...
galathinus (2)	.TTA....AA	.....T....	....	.......T.A	A.TAA-...T	.AAGT.A...
galathinus (3)	.TTA....AA	....	....	.......T.A	A.TGA-...T	.AAGT.T...
galathinus (4)	.TTA....AA	....	....	.......T.A	A.TAA-...T	.AA.T.A...
galathinus (5)	.TTA....AA	....	....	.......T.A	A.TGA-...T	.AAGT.T...
galathinus (6)	.TTA....AA	....	....	.......T.A	A.TAA-...T	.AAGT.A...
galathinus (7)	.TTA..A.AA	....	....	...A...TGA	..AAG-...T	.AAGT.T...
P. chilensis	..AA....A.	....	....	......A..A	A.TAG-...C	.GA.CAA.R.
P. crinimanus	..AA....A.	....	....	......A..A	A.TAG-...C	.GA.CAA.G.
P. grossimanus	.GAA....A.	....	....	......A..A	A.TAG-...T	.AA.CAA.G.
P. calculosus	..TT......	....	....	......A..A	A.TAA-...T	AAAG.AA...
P. rudis	..AA....A.	....	....	......A..A	T.TAA-...T	.GA..AAG..
P. pubescens	..TA....A.	....	....	......A..A	A.TAG-...C	.AA.TMA...
P. setimanus	.GTA......	....	....	......A..A	A.CTG-...C	.A...CA.T.
P. trichotus	.GTAC.....	....	....	......A..A	AGTTA-...C	.A....A.C.

Appendix 4. Continued

	241					300
cinctipes	TATAAGT---	TGT-TAAA-T	TGTTTACAAT	AAAAAAATTC	ATTATGCTGG	GGCGGTAGAG
cabrilloi	.....A....	C	....	....	....	....
eriomerus	....	.......G.-	....A.T..G	...C.G....	.....A....	....
manimaculis	.....A....	.........-	....A.T..G	...C......	.....A....	....
tiburonensis	....GT....	.........-	.AC.G.T..A	.GGTT.....	...G.A....	....
laevigatus	A..G.AATTG	.T-.-..GA.	....A.T..A	GG-.......	...G......	.......A.A
A. angulosus	...TTT.GAG	.......T..	.A..GTT..G	..-.......	...G......	.........A
A. punctatus	...T.A.TT.	.....GGT..	.A..AG...G	..-G......	...G......	.........A
A. spinifrons	...T.AAT..	.......-A.	...-AG...G	..-T......	...G......	.....C.A..
tuberculatus	.G.G.TRA..	......GTG.	....A....A	.G-T..G...	...G......	.....C...A
tuberculosus	.....A....	.......G.A	.........G	..C.G.....	...G......	.....C...A
crenulatus	A..GTAAA..	.......-GA	..C.A.T..G	..-T......	...G......	....
holotrichus	.....A-...	.......-G.	.......T.G	G.-T..G...	...G......	.........A
lewisi austrinsus	.....TATT.	.......-G.	.A..A.T..G	..-T..G...	....	.........A
platymerus	....GA-...	.......-AG	CA....TT.G	G.-T..G...	...G......	.........A
violaceus	...T.A....	-.......G.	...C..T..A	..-.G.....	...G......	.....C...A
L. mitra	...C.AG...	-.........	......TG.C	..-.R.....	...G......	.....C.A.A
gracilis	.....T.A..	.......-G.	...A.G...A	..-...G...	...G......	.....C...A
hirtipes	.......G..	.......-G.	.....G...A	..-G..G...	...G......	.....C...A
schmitti	.....K.G..	.......-G.	...A.G...A	..-...G...	...G......	.....C...A
cf tridentatus	...C.T.C..	........A.	AA-AA.T..A	C.-C.G....	.....A....	.........A
tridentatus	...T...T..	........AG	AAGAA.T..G	GG-C.G....	.....A....	.........A
granulosus	.....A.CAG	.T.G....AC	G.C...AG.G	.G-...T...	.....A....	........T.
elongatus	.T.TTAG...	---..C..AA	AAA.A....G	G.GTTG-..T	......T...	....AC.TTA
novaezelandiae	...TTAGT..	---..T..AA	...G.TA.T.	..--.....-	...G.A....	...A..GA.A
armatus (m)	.T.T.AG...	---..T..AC	AAA..TT..A	..TT.T-A..	...G......	..T....A..
armatus (p)	.T.T.AG...	---..T..A.	AAA..TT..A	..TT.T-A..	...G......	..T....A..
armatus (a)	.T.T.AG...	---..T..GC	AAA..TT..A	.GTT.T-A..	...G......	..T....A..
zacae	.T.T.CG...	---..T..GG	AAA..TT...	.GTTT.-A..	...G.A....	..T....A..
agassizii	.C...AA...	---..T..AA	AAA..G-..G	..TT.G-...	...G......	.....C....
edwardsii	.T....G...	---..T..AA	AAG...-..A	.GTT..-...	...G......	.....C..G.
haigae	......G...	---..T..G.	AAA.CCT..G	TGCTG.-G..	...G......	....
sanfelipensis	......G...	---..T..G.	.AAC.TG..G	.GTC..-...	.....A....	....
galathinus (1)	......G...	---..T..C.	A.A..TA..G	..TT..G...	...G......	........T.
galathinus (2)	......G...	---.CT..T.	A.GC.TA..G	..TT..-...	...G......	........T.
galathinus (3)	......G...	---..T..T.	AAAC.TA..G	..TT..-...	...G......	........T.
galathinus (4)	......G...	---..T..C.	A.A..TA..G	..TT..-...	...G......	........T.
galathinus (5)	......G...	---T.T..T.	AAAC.TA..G	..TT......	...G......	........T.
galathinus (6)	......G...	---..T..T.	A.GC.TA..G	..TT.G-...	...G......	........T.
galathinus (7)	......G...	---T.T..T.	AAA..TA..G	..CT......	...G......	....
P. chilensis	...TTA....	---..T..AA	.....TGG.R	.TTT..T..-	....	......GTTA
P. crinimanus	...TTA....	---..T..AA	.....TGG.G	.TTT..T..-	....	......GTTA
P. grossimanus	...CTA....	---..T..AA	.....TAG.G	.TTT..T..-	....	......GTTA
P. calculosus	...TTA....	---..T..A.	A....TT..A	.TG..GT..T	...G......	..T....ATA
P. rudis	....TA....	---..T..GA	.A...TAG.A	.TTT.....-	...G......	..T...GTTA
P. pubescens	...CTA....	---..T..AA	.A...TAG.A	.TTT..T..-	....	......GTTA
P. setimanus	...TTA....	---..T..AG	.....TGG.A	.T.T..T..T	...G.A....	.......TTA
P. trichotus	...TTA....	---..T..A.	.....TAG..	GT.T..T..T	...G......	......GTTA

Appendix 4. Continued

	301					360
cinctipes	-ATATAAAA-	--AAACTATT	TTAAAAAATT	AA--C--AAA	TGTGTTTGTA	AA--GT-AAA
cabrilloi	....	....	.......G..	....	C.........	G.........
eriomerus	....	....	.A.......A	.....CA...	.A.A......	......T...
manimaculis	....	....	.A....--.A	..A..A....	.A.A......	....
tiburonensis	......C...	....	.A....TTCA	......A...	.A.A.....G	CG..AGT...
laevigatus	....	....	..T....GA.	......A...	.A.A....C-	....AGT...
A. angulosus	......GTG.	.......G..	.GT.....A.	....	.A.A......	.......T..
A. punctatus	.......T..	.......G..	.GT.TG.GGA	T.........	.A.A.....T	.......TGG
A. spinifrons	.G....G...	....	.-T.....A.	....	.A.A.....G	T.........
tuberculatus	....A.G...	....C.....	.CT.G...A.	........G.	.A.A......	-...A.....
tuberculosus	....	....C.....	.CT.G..GAC	....	CA.A......	G.........
crenulatus	......G...	....	.-...T..AA	....	A..A......	........G.
holotrichus	.......T..	....	.....TT.A.	....	.A.A....-.	....
lewisi austrinsus	....	....	.A..T...A.	....	.A.A....-T	........G.
platymerus	.......T..	....	.....TT.A.	....	.A.A....-.	....
violaceus	....	....	......T.AA	T.........	.A.A.....T	.....C..G.
L. mitra	.......T..	....	.A....T.A.	....	.A........	....
gracilis	.......T..	....	......T.A.	....	AA.A.....T	T.........
hirtipes	......GT..	....	......T...	....	AA.A.....T	T.........
schmitti	......GT..	....	.A....T.A.	....	AA.......T	T.......G.
cf tridentatus	....	....	........G.	.........T	GT.AG...-.	....
tridentatus	....	....	........G.	.........T	GT..G...-.	........G.
granulosus	....	....	A.T....-A.	.TA.......	.A.A....AG	....AAA...
elongatus	.....TT...	.T....CG.-	--T...TTA.	..AA......	.A.AA...AG	..AAAGT..T
novaezelandiae	....	.T.....G..	...TT---G.	..AA......	.A.AA...AG	TTT.AAT...
armatus (m)	.........A	AT.....G.-	CAG..-...A	..A.......	AA.A.....T	T-........
armatus (p)	.........A	AT.....G.-	CAG..-...A	..A.......	AA.A.....T	T-........
armatus (a)	.........A	AT.....G.-	CAG..-...A	..A.......	AA.A.....T	T-........
zacae	.........A	AT.....G.-	.GT..-....	..A.......	AA.A.....T	T-........
agassizii	.......T.A	AT.....G.-	C.CT..TGAA	..A.......	...T.....G	TG.....G..
edwardsii	.........A	AT.....G.-	C.TT.-T.AA	..A.......	AA.TC.....	TT..A..T.T
haigae	.......T.A	GT.....G.-	CATTT.T..A	..A.......	.A.A.....G	T-........
sanfelipensis	........CA	AT.....G.-	CAT..-..A.	..A.....T.	AA.C....A.	....
galathinus (1)	....A....A	ATT....G.-	CATT.-T.AG	..A.......	.A.T......	....
galathinus (2)	........GA	AAG....G.-	CATT.-T.AA	..A......G	.A.A......	....
galathinus (3)	....A...GA	AT.....G.-	CATT.-T.AA	..A.......	.A.T.....G	....
galathinus (4)	....A...GA	AT.....G.-	CATTT-..AG	..A.......	.A.T.....G	.G..A.....
galathinus (5)	....A...GA	AT.....G.-	CAT.T-..AA	..A.......	.A.T.....G	.......T..
galathinus (6)	........GA	AAG....G.-	CATT.-T.AA	..A......G	.A.A......	....
galathinus (7)	....A....A	AT.....G.-	CATT.-T.AA	..A......G	.A.T......	T.........
P. chilensis	....	.T.....G.-	.A.T.-...A	..A.......	.A.A....A.	.-..T.....
P. crinimanus	....	.T.....G.-	.A.T.-...A	..A.......	.A.A....A.	.-..T.....
P. grossimanus	....	.T.....G.-	.A.T.-...A	..A.......	...A....A.	.-..T.....
P. calculosus	....	.T.....G.-	.A...-....	..A.......	.A.A....AG	.-..T.....
P. rudis	....	.T.....G.-	.A.T.-...A	..AA......	.A.A....A.	.-..A.....
P. pubescens	....	.T.....G.-	.A...-...A	..A.......	.A.A....A.	.-..T.....
P. setimanus	....	.T.....G.-	.A.T.-.G..	G.A.......	.A.A....A.	.-..T..T..
P. trichotus	T.....TC.D	.T.....G.A	.A.T.T...A	..A.......	.A.A....A.	.-..T..T..

Appendix 4. Continued

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                361                                                                                                  420
cinctipes       AGATCTTA-G TTATAGATTT AAAGAATAAG TTACT-TTAG GGATAACAGC ATAATTTTTT
cabrilloi       .........A .......... .......... .......... .......... ..........
eriomerus       .........A .......... .......... .......... .......... .........C
manimaculis     .......... .......... .......... .......... .......... .........C
tiburonensis    .........A .......... .......... .......... .......... .........C
laevigatus      .......... .......... G..A...... .......... .......... .......C..
A. angulosus    .......... .......... .......... .......... .......... ..G....C..
A. punctatus    .......... .......... ......C... .......... .......... .......C..
A. spinifrons   .........A .......... T......... .......... .......... .......C..
tuberculatus    .........A .......... .......... .......... .......... .......C.C
tuberculosus    .........T .......... .......... .......... .......... .......C..
crenulatus      .........A .....A.... .......... .......... .......... .......C..
holotrichus     .......... .......... .......... .......... .......... .......C..
lewisi austrinsus .......... ....G..A.. ...A...... .......... .......... .......C..
platymerus      .......... ....G..... .......... .......... .......... .......C..
violaceus       .....C...A ....G..... .......... .......... .......... ......CC.C
L. mitra        .........A A........A .......... .......... .......... .......C..
gracilis        .....C...T ....G..... T......... .......... .......... .......C..
hirtipes        .....C...T ....G..... T..A...... .......... .......... .......C..
schmitti        .....C...A ....G..... T..A...... .......... .......... .......C..
cf tridentatus  .....C...A ...AG....A .......... .......... .......... .......C..
tridentatus     .....C...A ...AG....A .......... .......... .......... .......C..
granulosus      .....C.G.T ...CG....A .......... .......... .......... .......C..
elongatus       .......... ...A.A.... ...AT..... .......... .......... .......C..
novaezelandiae  ...C.C.T.T C..AG....A T......... .......... .......... .........C
armatus (m)     .....C...T A...G....A .....C.... .......... .......... .......C..
armatus (p)     .....C...C A...G....A .....C.... .......... .......... .......C..
armatus (a)     .....C...T A...G....A .......... .......... .......... .......C..
zacae           .....C...T ....G....A .....T.... .......... .......... .......C..
agassizii       .....C...A ....G....A .....G.... .......... .......... .......C..
edwardsii       .....C...A ....G..... .......... .......... .......... .......C..
haigae          .....C.G.A C..CG....A .....GA... .......... .......... .......C..
sanfelipensis   .....C.G.T ..TCG....A .....GA... .......... .......... .......C..
galathinus (1)  .....C.GAA A.T.G..... ....TGA... .......... .......... .......C..
galathinus (2)  .....C.GAT A.T.G..... ....TGA... .......... .......... .......A..
galathinus (3)  .....C.GAT A.T.G..... ....TGA... .......... .......... .......C..
galathinus (4)  .....CC.AA A.T.G..... ....TGA... .......... .......... .......C..
galathinus (5)  .....C.GAA A.T.G..... ....TGA... .......... .......... .......C..
galathinus (6)  .....C.GAT A.T.G..... ....TGA... .......... .......... .......C..
galathinus (7)  .....C.GAA A.T.G....A .....GA... .......... .......... .......C..
P. chilensis    .....C.T.A ....G..... ......A... .......... .......... .......C..
P. crinimanus   .....C.T.A ....G..... ......A... .......... .......... .......C..
P. grossimanus  .....C.T.T ....G..... ......A... .......... .......... .......C..
P. calculosus   .....C.T.A ...G.....A .....GA... .......... .......... .......C..
P. rudis        .....C.T.. ....G....A ......A... .......... .......... .......C..
P. pubescens    .....C...A ....G..... ......A... .......... .......... .......C..
P. setimanus    T....C.T.A .........A G.....A... .......... .......... ..........
P. trichotus    T....C.T.. ...A.....A ......A... .....G.C.. .......... .....C....
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Appendix 4. Continued

	421					479
cinctipes	TTGAGAGTTC	GGATTGAAAA	AAAAGTT-TG	TGACCTCGAT	GTTGAATTAA	AAATT-CCT
cabrilloi	....	....	....	....	....	....
eriomerus	..A.....C.	.A..C....G	....	....	....	....
manimaculis	..A.....C.	AA..C....G	....	....	....	....
tiburonensis	....	.A..C....G	.G........	....	....	..T..T...
laevigatus	....	AT..C....G	.G........	....	....	G......TC
A. angulosus	....	AA..C.....	.G........	....	....	..T......
A. punctatus	....	AA..C....G	.G........	....	....	..G......
A. spinifrons	....	AA..C.....	.G........	....	....	..T......
tuberculatus	....	TA..C....G	.G...C....	....	....	..T......
tuberculosus	....	AA..C....G	.G........	....	....	..T.....G
crenulatus	....	A...C.....	.G........	....	....	....
holotrichus	....	A...C.....	.G........	....	....	..-A.T.-.
lewisi austrinsus	........C.	AA..C.....	.G........	....	....	G........
platymerus	....	A...C.....	.G........	....	....	..T......
violaceus	........C.	AA..CT...G	.GG..C....	....	....	C
L. mitra	........C.	AA..C.....	.G........	....	....	....
gracilis	....	AA..C.....	.G.....C..	....	....	...C...-.
hirtipes	....	AA..C.....	.G........	....	....	.......T.
schmitti	....	AA..C.....	.G........	....	....	.......T.
cf tridentatus	........C.	AA..C.....	.G........	....	....	..T......
tridentatus	........C.	AA..C.....	.G........	....	....	..T......
granulosus	.AA.......	.T..CA....	.G........	....	....	..G......
elongatus	.AA.....C.	TA..C..C..	.G........	....	....	.......-.
novaezelandiae	....	TA..C....G	..G......A	....	....	.TT....-.
armatus (m)	........C.	TA..C.....	.G...C....	....	....	GG.A.....
armatus (p)	........C.	TA..C.....	.G...C....	....	....	GG.A.....
armatus (a)	........C.	TA..C.....	.G...C....	....	....	GG.A.....
zacae	....	TA..C.....	.G...C....	....	....	GGTA.....
agassizii	....	AA..C.....	.G...C....	....	....	GG.A.....
edwardsii	....	AA..C.....	.G...C....	....	....	GG.A.....
haigae	....	TA..C.....	.G...C....	....	....	GG.-.....
sanfelipensis	..A.....C.	AA..C.....	.G...C....	....	....	G.TA.....
galathinus (1)	........C.	AA..C.....	.G...C....	....	....	G.GA.....
galathinus (2)	..C.......	AA..C.....	.G...C....	....	....	G.GA.....
galathinus (3)	........C.	.A..C.....	.G...C....	....	....	G.GA.....
galathinus (4)	........C.	AA..C.....	.G...C....	....	....	G.GA.....
galathinus (5)	........C.	.A..C.....	.G...C....	....	....	G.GA.....
galathinus (6)	........C.	AA..C.....	.G...C....	....	....	G.GA.....
galathinus (7)	....	AA..C.....	.G...C....	....	....	G.GA.....
P. chilensis	....	AA..C.....	.G........	....	....	G........
P. crinimanus	....	AA..C.....	.G........	....	....	G........
P. grossimanus	....	AA..C.....	.G........	....	....	G.G......
P. calculosus	....	AA..C.....	....	....	....	G..G.....
P. rudis	....	AA..C.....	.G........	....	....	G........
P. pubescens	....	AA..C.....	.G......C.	....	....	G..Y.....
P. setimanus	....	AA..C.....	....	....	....	G..A.....
P. trichotus	....	AA..C.....	.....C....	....	.K........	GC.A.....