LIVING ALONG AN ESTUARINE GRADIENT: JUVENILE PERFORMANCE, REPRODUCTIVE PATTERNS, AND HEAT-SHOCK PROTEIN EXPESSION IN THE BARNACLE *BALANUS GLANDULA*

by

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A DISSERTATION

Presented to the Department of Biology
and the Graduate School of the University of Oregon
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Title: LIVING ALONG AN ESTUARINE GRADIENT: JUVENILE PERFORMANCE, REPRODUCTIVE PATTERNS, AND HEAT-SHOCK PROTEIN EXPRESSION IN THE BARNACLE *BALANUS GLANDULA*

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The intertidal barnacle *Balanus glandula* is predominantly an open coast species in the Northeast Pacific. However, *B. glandula* commonly inhabits estuaries where environmental conditions such as salinity and temperature drastically differ from the open coast. The increased variability of environmental conditions within an estuary, and subsequent stress, can affect the survivorship and growth of recently settled juveniles and, in addition, adult reproductive dynamics. Along an environmental gradient, at the oceanic, mid-estuarine, and riverine end of the South Slough Estuary, Oregon, USA, I examined (1) seasonal variation in the reproductive cycle of adult *B. glandula* and (2) survivorship and growth rates of recently metamorphosed laboratory reared juvenile *B. glandula* outplanted to the field. High densities of adult *B. glandula* were observed at all sites. The oceanic site was characterized by a reproductive output that was higher than

the mid-estuarine site. However, higher survivorship and growth rates were observed at the mid-estuarine site than the oceanic site. Although a disparity was observed between the oceanic and mid-estuarine sites, both sites ranked closer to each other than to the riverine site. A low annual reproductive output coupled with high juvenile mortality and a low growth rate made the riverine site the least suitable habitat within the estuary.

In the intertidal, where organisms reside in an interface between an aquatic and terrestrial habitat, body temperatures of sessile marine organisms can reach 20-35 °C for extended periods of time during low tide. In response to an environmental stress, such as temperature, organisms will upregulate heat-shock protein synthesis in an effort to save existing protein pools from irreversible damage. I examined the physiological response of the intertidal barnacle, *Balanus glandula*, to thermal stress by measuring thermally induced heat-shock protein expression. Induction of hsp70 occurred as low as 23 °C regardless of field acclimatization or laboratory acclimation. Induced levels of hsp90 reached a maximum at 23-28 °C; levels decreased by 33 °C. Although a heat-shock response was observed, evidence of irreversible protein damage was not observed. The heat-shock response of *B. glandula* appears to be different than other intertidal organisms (e.g., molluscs). This dissertation includes my co-authored material.

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CHAPTER I

GENERAL INTRODUCTION

The complex life-cycles of marine invertebrates have intrigued researchers for a number of years. Each phase presents a different set of challenges and consequently a different set of adaptations that are necessary for success (Wilbur 1980). As an adult, enough surplus energy must be acquired via feeding to effectively produce offspring (Cody 1966, Sibly and Calow 1986). Once the offspring are produced and liberated into the plankton, mortality rates are extremely high (Morgan 1995; however see Johnson and Shanks 2003). Of the larvae that do survive the planktonic period and successfully settle, juvenile mortality rates are also high (Gosselin and Qian 1996, Gosselin and Qian 1997, Hunt and Scheibling 1997). The processes involved in successful reproduction and the recruitment of the resulting offspring into a population are dependent upon a complex interaction between biological and environmentally mediated physical factors.

In natural systems, environmental conditions vary across a wide range of spatial and temporal scales. In an intertidal marine habitat, a multitude of physical factors vary, such as wave exposure, temperature, desiccation, and salinity (Raffaelli and Hawakins 1996). These abiotic factors potentially can be stressful (Vernberg and Vernberg 1972) and as a result, decrease the growth, survivorship, or reproductive output of an individual (Barnes 1989, Graham et al. 1997, Chapman and Fletcher 2002).

An organism possesses a variety of adaptations to contend with stressful conditions. Mobile animals, such as gastropods, can behaviorally modify their exposure to stress to some degree by moving to an area with a lesser degree of stress, such as the cooler underside of a rock or a shaded crevice. However, sessile organisms, such as the barnacle *Balanus glandula*, do not have the option of being able to move when conditions become unfavorable. One specific physiological adaptation to stress is the upregulation of molecular chaperones (Hochachka and Somero 2002, Hofmann et al. 2002). Molecular chaperones (heat-shock proteins) rescue denatured proteins from irreversible damage, thus helping to conserve the existing pool of proteins (Parsell and Lindquist 1993, Buchner 1996, Fink 1999).

This dissertation contains a series of field and laboratory experiments aimed at understanding the response of a sessile intertidal barnacle (*Balanus glandula*) to environmental stress encountered in the field. A large component of this dissertation was devoted to examining juvenile survivorship, juvenile growth, and adult reproductive patterns along an estuarine gradient. An estuary presents a unique experimental system, such that over a spatial scale of kilometers, a gradient of environmental conditions occur well within the observed distribution of an adult population.

In Chapter II, I evaluated growth and survivorship as a comparative measure of juvenile performance within a population of *B. glandula* along an estuarine gradient. My results exemplify the temporal and spatial variability of survival and growth during the juvenile stage of a sessile intertidal invertebrate.

Chapter III describes the reproductive patterns of a natural population of *B. glandula* that resides along an estuarine gradient. By measuring reproductive indices such as percent of the population brooding and individual fecundity, I was able to quantify the reproductive effort of *B. glandula* along an estuarine gradient. My results indicate how the reproductive effort of an intertidal barnacle can rapidly decrease over a spatial scale of kilometers, as a transition occurs from a marine to a freshwater habitat along an estuarine gradient.

Chapter IV explores the physiological response of *B. glandula* to thermal stress by characterizing the heat-shock response. By measuring heat-shock protein expression levels, ecologically relevant temperatures that were physiological stressful to adult *B. glandula* were determined. Through a series of laboratory experiments, my results point out that *B. glandula* displays a heat-shock response during conditions regularly experienced in the intertidal.

Chapter V summarizes the research presented in this dissertation and suggests future work that would be complimentary to the research performed in this dissertation.

CHAPTER II

JUVENILE PERFORMANCE OF THE BARNACLE *BALANUS GLANDULA* ALONG AN ESTUARINE GRADIENT

Introduction

Understanding the processes involved in the successful recruitment into a population that is distributed along an environmental gradient, is an important aspect of ecology. Environmental gradients and resultant distributional patterns are commonly found in both terrestrial and marine habitats (Whittaker 1970, Lewis 1978, Newell 1979, Raffaelli and Hawkins 1996). Numerous studies have examined changes in community composition along an environmental gradient as one community is replaced with another (Whittaker 1956, Terborgh 1971, Harrold et al. 1988, Hoagland and Collins 1997, Vazquez and Givnish 1998, Attrill and Rundle 2002). Along an environmental gradient, the abundance of each species reaches a peak where conditions are presumed to be most favorable, whereas, at either end of the distributional range abundance will decrease due to physiological stress or biological interactions (Whittaker 1967, Vernberg and Vernberg 1972). For example, in terrestrial habitats, abiotic factors, such as desiccation and wind-induced abrasion, limit the upper vertical distribution of coniferous trees (Wardle 1968, Hadley and Smith 1986). In the interface between terrestrial and marine habitats, a combination of physiological stress and competitive interactions maintains the

distribution of vascular plants in salt marshes (Niering and Warren 1980, Bertness 1991). In a marine rocky intertidal habitat, the upper limit of a species distribution is typically driven by post-settlement mortality due to physiological stress (algae, Harley 2003; crabs, Jensen and Armstrong 1991; gastropods, Garrity 1984; bivalves, Peterson 1991; barnacles, Connell 1961, Foster 1971a, Wethey 1983, Raimondi 1988).

Although numerous studies have focused on community changes along an environmental gradient, fewer studies have examined intraspecific responses along a gradient. Performance of individuals at specific locations along a gradient will determine their ability to successfully recruit into the population. For example, the growth of embryos of the macroalgae *Fucus serratus* decreases as light levels decrease along a light gradient (Chapman and Fletcher 2002). A similar pattern of decreased growth occurs for the algae *Macrocystis pyrifera* as wave exposure increased along a horizontal wave gradient (Graham et al. 1997). In a northern hardwood forest, the growth and mortality of saplings varies along a horizontal gradient in soil type (Kobe 1996, Bigelow and Canham 2002).

Many benthic marine invertebrates have a complex life cycle involving both a pelagic larval stage and a sessile adult stage. Pelagic larvae are typically weak swimmers and are likely to be transported into locations that vary environmentally. During the larval phase, pre-settlement processes such as supply and transport (Roughgarden et al. 1988, Devries et al. 1994), food availability (Fenaux et al. 1994), and predation (Morgan 1995) may influence the success of larval settlement. Upon attachment to a substrate, post-settlement processes such as juvenile mortality will determine the success of

individuals recruiting into a population (Gosselin and Qian 1997). Juvenile mortality can be caused by environmental stress (Foster 1971b, Wethey 1984), competitive interactions (Connell 1961), or predation (Osman and Whitlatch 1995, Barnes 1999). Whether pre-settlement processes or post-settlement mortality determines the abundance of an organism has been debated in the literature (Denley and Underwood 1979, Connell 1985, Jeffery 2003).

Growth of an intertidal barnacle is influenced by a variety of factors such as temperature, water flow, food availability, physiological stress, and competition (Crisp and Bourget 1985). Barnacles growing at a faster rate can undercut or overgrow adjacent barnacles (Connell 1961). Some benefits of a larger size are reaching a refuge from predation (Miller and Carefoot 1989) and an increased tolerance to physiological stress (Foster 1971b). From a life-history perspective, a faster growing individual could become reproductive sooner than an individual with a reduced growth rate (Arendt 1997). Therefore, the rate of growth can determine the success of an individual.

The mouth or "oceanic end" of a typical estuary in the Pacific Northwest, although seasonally variable, is characterized by water temperatures and salinity more similar to the open coast than the rest of the estuary. At the riverine end of an estuary, the water temperature is warmer and the salinity is lower due to the input of fresh water. Between the oceanic and riverine ends, mixing occurs, creating a natural temperature and salinity gradient along the estuary. Reduced salinity encountered by organisms at the riverine end of an estuary can be osmotically stressful (Kinne 1971, Vernberg and

Vernberg 1972), consequently reducing performance (e.g., survivorship and growth) and reproductive output (Chapter III).

When distinct habitats are present within the distributional range of a species, the potential for local adaptation and genetic differentiation is possible. Genetic differentiation between populations can be affected by larval dispersal mode (e.g., direct vs. planktonic development), larval retention, or environmentally mediated selection (Yamada 1989, Johannesson et al. 1990, Parsons 1996, Schmidt and Rand 1999). An estuary is a habitat where local adaptation can potentially occur due to larval retention (Bousfield 1955) or environmental spatial variability. Transplant experiments in estuaries suggest genetic differentiation can occur between populations. For example, variations in settlement preference and survivorship have been observed for populations of the barnacle *Semibalanus balanoides* with different genotypes residing in environmentally different habitats (Bergeron and Bourget 1986, Bertness and Gaines 1993, Brind'Amour et al. 2002). Therefore, based on the population source (e.g., genetic composition), measures of performance may vary spatially.

An estuary presents a unique opportunity for experimentation, where over a spatial scale of kilometers, a gradient of environmental conditions can occur well within the observed distribution of an adult population. I used field outplant experiments to measure performance of juvenile barnacles (*Balanus glandula*) along an environmental gradient within an estuary. Juveniles were outplanted to three sites along the environmental gradient during the spring and summer seasons, over a two year period. At the outplant sites, adult *B. glandula* were remarkably dense, with close to 100 percent

of the available substrate covered (M. Berger, pers. obvs.). Three possible alternative hypothesis are that juveniles would perform best at (1) the riverine end of the estuary, (2) a point midway along the environmental gradient in the estuary (i.e., mid-estuarine), or (3) the oceanic end of the estuary. As a complement to the field experiments, a controlled laboratory experiment was performed to examine how juvenile performance varied as a function of salinities and temperatures similar to those encountered at the outplant sites. I also examined within-site variation of survivorship and growth as a function of vertical height. Through a reciprocal transplant of juveniles reared from oceanic and riverine populations (i.e., both ends of the gradient), I measured juvenile performance to determine whether the populations were locally adapted to their respective habitat. If local adaptation along the environmental gradient occurred, juveniles would perform best within the habitats from which they originated.

Methods

Larval culturing

Adult *Balanus glandula* were collected on pilings in the intertidal at the oceanic end of the South Slough Estuary, Charleston, Oregon, USA (43° 20.4′ N, 124° 19.4′ W) and returned to the lab where egg lamellae were removed; the lamellae contained embryos in the latter stage of development and were ready to hatch. The egg lamellae from each adult were placed in separate beakers of 0.45 µl filtered sea water (FSW) and exposed to a fiber optic light source for up to four hours, to encourage nauplii hatching (Brown and Roughgarden 1985).

Equal numbers of larvae from six adults were pooled and reared in twelve 3.8 L jars at a density of 1500 nauplii in 1.5 L of FSW. Every two days, the culture water was changed days, the nauplii were fed the diatom *Skeletonema costatum* at a concentration of 1.0×10^5 cells/ml (Hentschel and Emlet 2000), and larval development stage was determined. The cultures were gently stirred with swinging paddles at 15 strokes minute^{-1} (Strathmann 1987). When greater than 80 percent of the larvae were cyprids, the cultures were divided in half and allowed to settle on three 6-cm^2 slate panels. Each panel contained 96 small pits and was coated with a settlement factor to enhance cyprid attachment (Rittschof et al. 1984). Following a four day period to allow for adequate settlement and metamorphosis to a juvenile stage, juveniles were thinned to one pit^{-1} (32 juveniles). I mapped the panels so each individual juvenile could be followed through the course of the experiment and not be mistaken for a juvenile that settled after outplanting the panels. As a proxy for size, the basal circumference of each juvenile was drawn with a camera lucida and the area within the circumference was calculated with Optimas image analysis software (version 6.0, Optimas Corp. 1996).

Experimental field outplants

Juveniles were outplanted at three sites within the South Slough Estuary, Charleston, Oregon, USA (Fig. 1). Site selection was based on location along the estuarine salinity gradient, with sites at the oceanic end, mid-estuarine, and riverine end of the gradient. All sites were within the distributional range of *Balanus glandula* in the estuary.

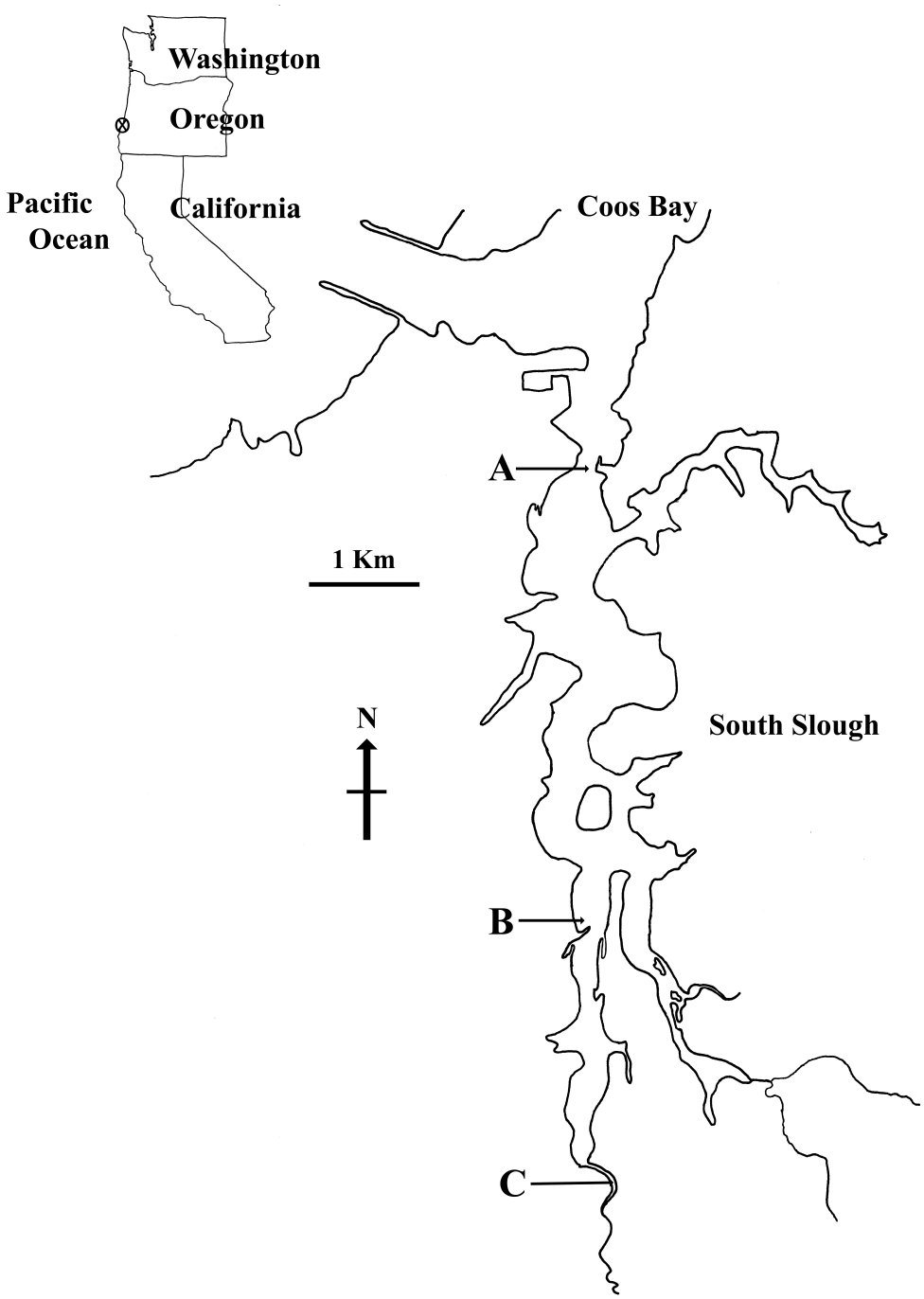


Figure 1. Location of study sites within the South Slough Estuary, Charleston, Oregon, USA. The three study sites are labeled as A) oceanic, B) mid-estuarine, and C) riverine. See inset for an approximate location along the west coast of the United States.

A total of seven outplant experiments were performed during 2002 - 2003. The first and second set of the experiments (outplants I and II) occurred over a 20 day period between 2 – 22 May 2002 and 18 June – 8 July 2002, respectively. At all sites, experimental panels with juveniles were bolted to a PVC frame attached to a wooden piling and oriented so the panels faced east. Panels were outplanted at one intertidal height with six replicate panels for all experiments. The panels were randomly assigned from each culture jar, so that each jar was represented at all sites in all experiments.

As a tidal wave moves up an estuary it is dampened, decreasing the tidal amplitude (Juza 1995, Dyer 1997). Therefore, equivalent elevation would not result in equivalent submergence times between the study sites. An equivalent submergence time was determined by observing the change in water level at all three sites during the same high tide; this procedure was repeated on three separate dates. Panels were outplanted at equivalent submergence times at all sites. The panels at the oceanic site were outplanted at 1.28 m above mean lower low water (MLLW). Any mention to height above MLLW refers only to the oceanic site and should be interpreted as an approximation at all other study sites.

The third experiment (outplant III) to examine juvenile performance during mid-summer occurred over a 12-day period between 11 August – 23 August 2002. This particular experiment was terminated 8 days earlier than the previous experiments due to high juvenile mortality. In outplant III, a second intertidal height was added into the design so that panels were outplanted at 1.28 and 1.53 m above MLLW in reference to the oceanic site.

A fourth experiment (outplant IV) occurred over a 20 day period between 19 September – 9 October 2002. In this experiment, the effect of vertical height on juvenile performance at the oceanic site was investigated with six replicate panels outplanted at five intertidal heights (1.08, 1.20, 1.28, 1.41, and 1.53 m above MLLW).

The fifth and sixth experiments (outplants V and VI) occurred over a 20-day period between 6 – 26 May 2003 and 23 June – 13 July 2003, respectively. The experimental design was similar between outplant V and VI except three vertical heights (1.28, 1.41, and 1.53 m above MLLW) were used in outplant V and only two vertical heights (1.28 and 1.53 m above MLLW) were used in outplant VI.

Outplant VII tested for differences between juveniles reared from oceanic and riverine populations. Larval rearing and settlement procedures were similar to those outlined previously with the following exceptions: (1) in addition to collecting adult *B. glandula* from the oceanic site, *B. glandula* were collected on submerged dead tree branches from the riverine site and (2) cyprids were allowed to settle on 7.5-cm^2 slate panels containing 84 pits. Outplant VII occurred over a 20 day period between 18 July – 7 August 2003. Six replicate panels with juveniles reared from both the oceanic and riverine populations were outplanted at one height at both the oceanic and riverine sites in a reciprocal design. Outplant VII data were contributed by Abby Darrah.

For the duration of the outplant experiments, the panels were retrieved from the field during low tide every four days and returned to the lab where survivorship was determined by inspecting mapped juveniles. A juvenile was determined to be dead if (1) the juvenile was missing, (2) the opercular plates were missing, or (3) the juvenile did not

respond to gentle stimuli on the opercular plates. The basal circumference for each surviving juvenile was drawn using a camera lucida. Processing all of the panels was a time consuming procedure that required, on average, nine hours. During the processing period, all the panels were maintained in a flowing sea water table at a salinity of approximately 34. The panels were returned to the field as soon as possible, usually during the second low tide of the day. On occasion, the panels were kept overnight in the laboratory if the tides did not permit returning the panels on the same day.

Physical site characteristics

Salinity, water temperature, and air temperature were continuously monitored during all outplant experiments. Salinity was recorded with a YSI model 6600 data logger at both the oceanic and riverine sites, and a YSI model 6000 data logger at the mid-estuarine site. Air and water temperature were recorded with a data logger (Optic StowAway, Onset Inc.) that was bolted to a 6-cm^2 slate panel and covered with PVC piping to avoid direct solar radiant heating at the mid intertidal height for all sites. In addition, point measurements of chlorophyll-*a* concentrations were determined by collecting three replicate surface water samples during high tide at each site at least once during outplant experiments I, II, V, and VI. The water samples were filtered onto glass fiber filters and analyzed for chlorophyll-*a* concentration following the method of Parsons et al. (1984).

Experimental laboratory study

A laboratory experiment examined how temperatures and salinities similar to those at the field sites affected survivorship and growth. Larvae were reared, settled on slate panels, and mapped following the methods previously described. Each panel was attached to a swinging paddle and immersed in an individual beaker with 1 L of FSW. The beakers were maintained at three experimental temperatures of 10 °C (9.5– 10.5), 15 °C (14.0 – 15.5) or 20 °C (18.5 – 20.5) in a temperature controlled room with a 16L : 8D light cycle. At each temperature, three salinities were tested: 14, 24, and 34. Every two days: (1) the water in each beaker was changed with FSW at the appropriate temperature and salinity and (2) the juveniles were fed the diatom *Skeletonema costatum* at a concentration of 1.0×10^5 cells/ml. *Skeletonema costatum* was cultured at 14, 24, and 34 in an incubator at 20 °C throughout the experiment. Every 4 days, over a 20 day period, survivorship was determined and the basal circumference for each juvenile was drawn.

Analysis

All data examined in this study were analyzed with both Systat (version 9.0, SPSS Inc. 1999) and Statistica (version 6.0, Statsoft 2002). The assumptions of normality and homoscedasticity were assessed with a Komogorov-Smirov test with a Lilliefors option and Cochran's C test, respectively. The assumption of normality was violated in a few analyses. However, ANOVAS are robust to departures from normality (Sokal and Rohlf 1995, Underwood 1997). All repeated measures ANOVAS were subjected to Mauchly's

test of sphericity. Violations of sphericity were interpreted by using the Greenhouse-Geisser epsilon determining the appropriate degrees of freedom.

To test the effect of estuarine location and intertidal height on survivorship, I used a repeated measures ANOVA with location and height as fixed factors. All data were arcsine transformed and analyzed for days 4, 8, 12, 16, and 20 from outplanting for all experiments except outplant III, when only days 4 and 8 were analyzed. To test for significant differences between sites, univariate contrasts were performed on the last day of the outplant (von Ende 1993). A repeated measures ANOVA was used to test the effect of population source and estuarine location on survivorship. Both source and location were treated as fixed factors. In addition, to test the effect of temperature and salinity on survivorship in the laboratory experiment, a similar repeated measures ANOVA was performed; both temperature and salinity were treated as fixed factors.

Growth rates were calculated for each juvenile by using a natural log transformation of basal area to obtain a linear relationship over time. The slope of each line was then estimated via linear regression to calculate a growth rate.

To test the effect of estuarine location and intertidal height on growth rate, I used a nested ANOVA design with panel nested in location and height. Panel was treated as a random factor, while location and height were fixed factors. In outplants I and II, panel was nested only in location. To further explore differences between treatments, a Tukey post hoc test was performed. To test the effect of population source and estuarine location on growth rate, I used a nested ANOVA design with panel (random factor) nested in population source and estuarine location (both fixed factors).

To test for an effect of intertidal height on growth rate (outplant IV), I used a nested ANOVA design with panel (random factor) nested in intertidal height (fixed factor). The estimation of growth rate was identical to methods previously outlined. A linear contrast was performed to test the specific hypothesis that growth rate decreased as intertidal height increased. The analysis for the laboratory experiment examining the effect of temperature and salinity on growth rate was similar to the outplant experiments. Panel (random factor) was nested in temperature and salinity (both fixed factors). A Tukey post hoc test was performed to explore significant differences.

Results

Physical site characteristics

The South Slough Estuary showed seasonal patterns for salinity, water temperature, air temperature, and chlorophyll-*a* over the duration of the study period. Salinity decreased away from the oceanic end of the estuary, with all sites experiencing lower salinity levels during the spring and higher levels during the summer (Fig. 2). The salinity was below 10 over a longer duration during spring 2003 (outplant V) than during spring 2002 (outplant I). Water temperature was warmer at both the mid-estuarine and riverine sites than the oceanic site (Fig. 3). Air temperatures were highly variable and depended on atmospheric conditions for a given day. Typically air temperature was a few degrees higher at mid-estuarine and riverine sites, especially during summer (Fig. 4). Though the data are not continuous, chlorophyll-*a* concentrations were lowest at the mid-

estuarine site on four of six sample dates distributed over outplants I, II, V, and VI (Fig. 5).

Juvenile performance along an environmental gradient

Location within the estuary significantly affected the survivorship of juvenile *Balanus glandula* (Table 1). However, survivorship of juveniles varied between both seasons and year. At the termination of the spring 2002 experiment (outplant I), juvenile survivorship was highest (> 90 %) at the mid-estuarine site; with significantly lower survivorship (< 50 %) at both the oceanic and riverine sites (Fig. 6A). Survivorship at the oceanic site and riverine sites were not significantly different at the termination of the experiment on day-20 (Univariate ANOVA contrast, $F_{1,15} = 3.06$, $p = 0.101$). However, when the field outplant was repeated during summer 2002 (outplant II), survivorship was consistently high and did not vary significantly between estuarine sites (Table 1, Fig. 6B).

As in spring 2002, juvenile survivorship was highest at the mid-estuarine site in spring 2003 (outplant V) (Fig. 6C). In contrast to spring 2002, when survivorship was similar between the oceanic and riverine sites, survivorship during spring 2003 was significantly lower at the riverine site than the oceanic site at the termination of the experiment (Univariate ANOVA contrast, $F_{1,45} = 90.63$, $p = 0.000$). Only one intertidal height was examined in spring 2002, but when three intertidal heights were examined in spring 2003 no significant effects of height over time on survivorship were observed at any of the study sites (Table 1).

Survivorship in summer 2003 (outplant VI) and summer 2002 (outplant II) were dramatically different. Compared to the high and non-significant difference in survivorship between locations in 2002, site significantly affected juvenile survivorship in summer 2003 (outplant VI) (Table I). Two intertidal heights were examined in outplant VI. Survivorship at the mid-estuarine site was significantly higher than at the riverine site on the final day of the experiment (Univariate ANOVA contrast, $F_{1,30} = 27.06$, $p = 0.000$) (Fig. 6D). At all sites (outplant VI), survivorship was significantly lower for juveniles outplanted to the higher intertidal height. Survivorship at the oceanic site was more complex than at the other two sites. A significant three-way interaction of location by height over time supports the observation that survivorship of oceanic juveniles at the lower intertidal height was similar to the mid-estuarine site, while survivorship of juveniles at the high intertidal height was similar to the riverine site (Fig. 6D).

Location within the estuary also had a strong significant effect upon growth rates (Table 2). The growth rate of juvenile *B. glandula* during spring 2002 (outplant I) was significantly higher at the mid-estuarine site than at the oceanic site (Tukey HSD, d.f. = 15, $p = 0.000$) (Fig. 7A). The lowest growth rate was observed at the riverine site, where the growth rate was significantly lower than the oceanic site (Tukey HSD, d.f. = 15, $p = 0.001$). Although significant, the effect of location on growth rate was not as strong when the experiment was repeated in summer 2002 (Table 2). The growth rate of *B. glandula* (outplant II) was significantly lower at the oceanic site than both the mid-estuarine site (Tukey HSD, d.f. = 15, $p = 0.034$) and the riverine site (Tukey HSD, d.f. =

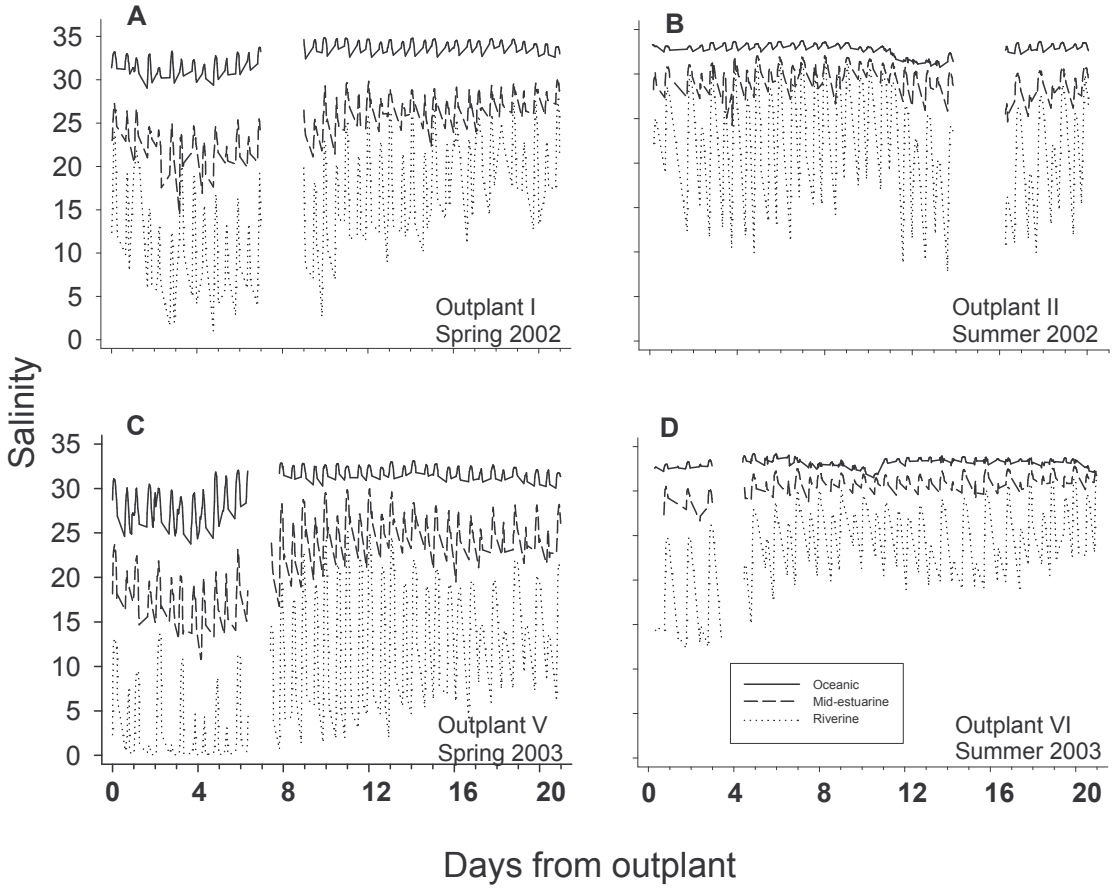


Figure 2. Seasonal salinity profiles at the three experimental study sites along an estuarine gradient for A) spring 2002, B) summer 2002, C) spring 2003, and D) summer 2003. Data points represent salinity measured during submergence of the outplanted juveniles only.

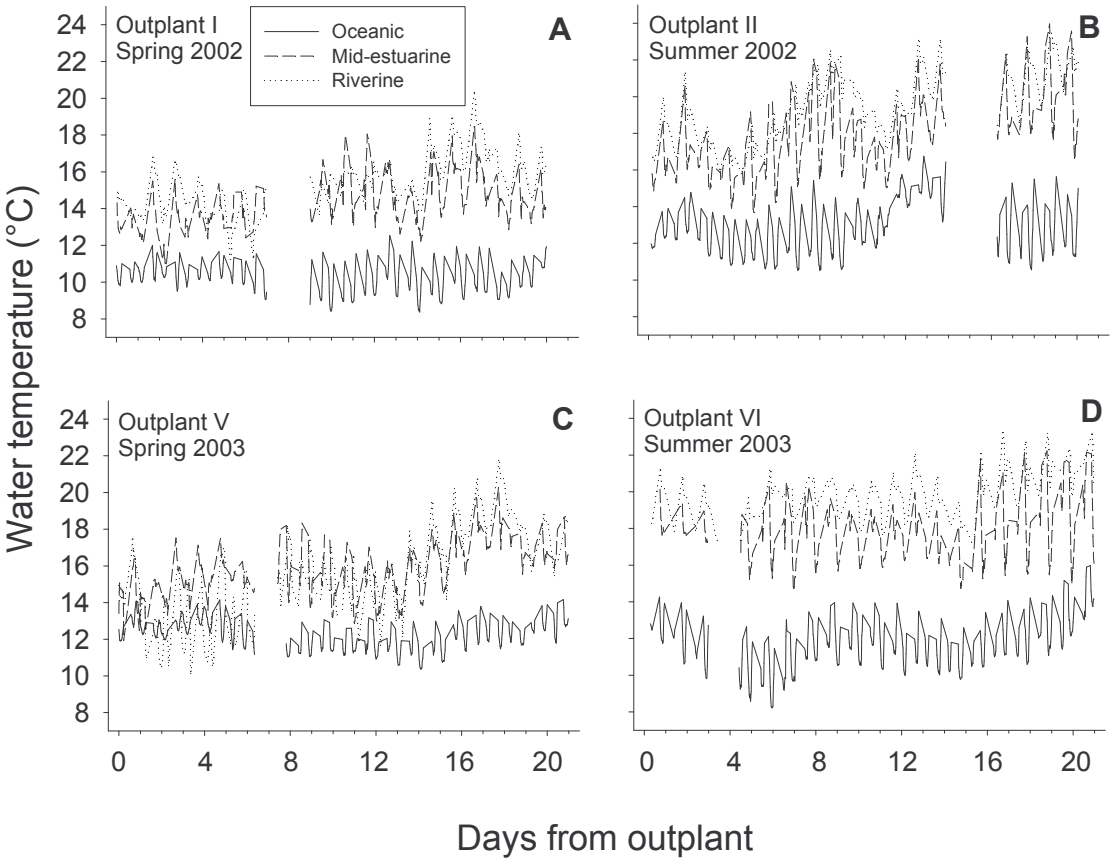


Figure 3. Seasonal water temperature profiles at the three experimental study sites along an estuarine gradient for A) spring 2002, B) summer 2002, C) spring 2003, and D) summer 2003.

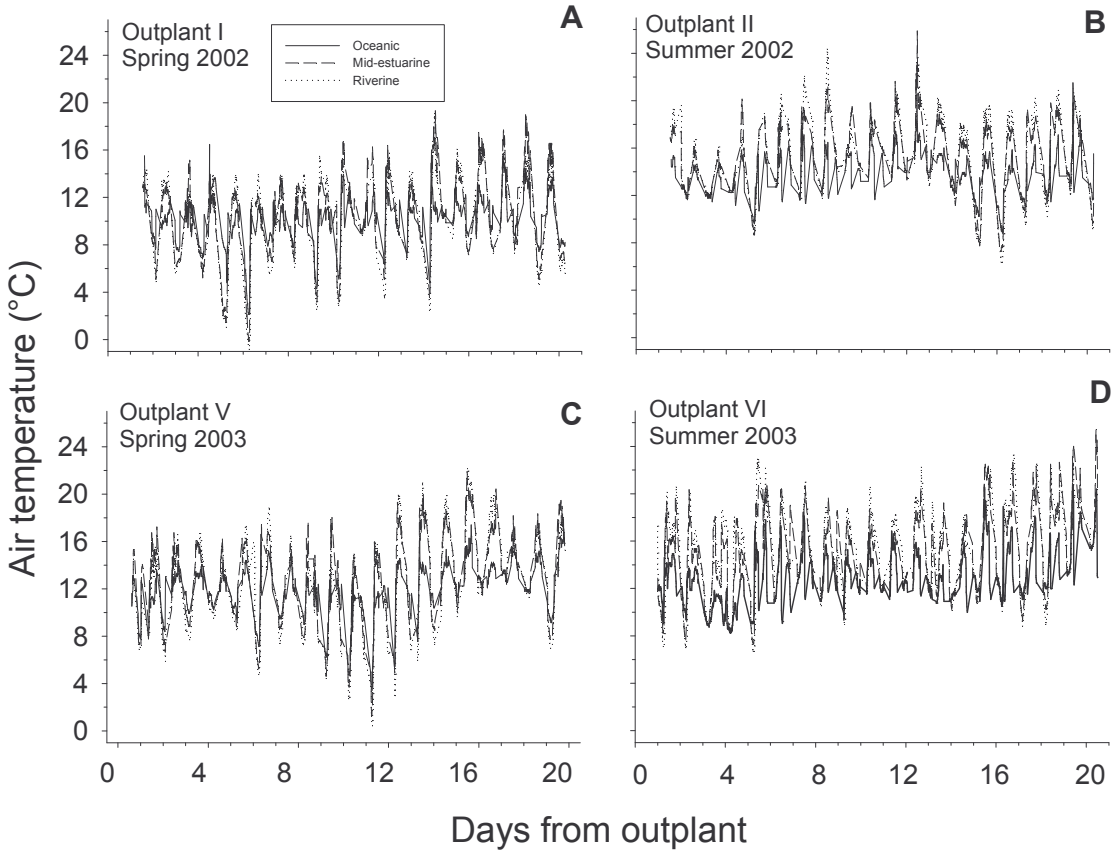


Figure 4. Seasonal air temperature profiles at the three experimental study sites along an estuarine gradient for A) spring 2002, B) summer 2002, C) spring 2003, and D) summer 2003.

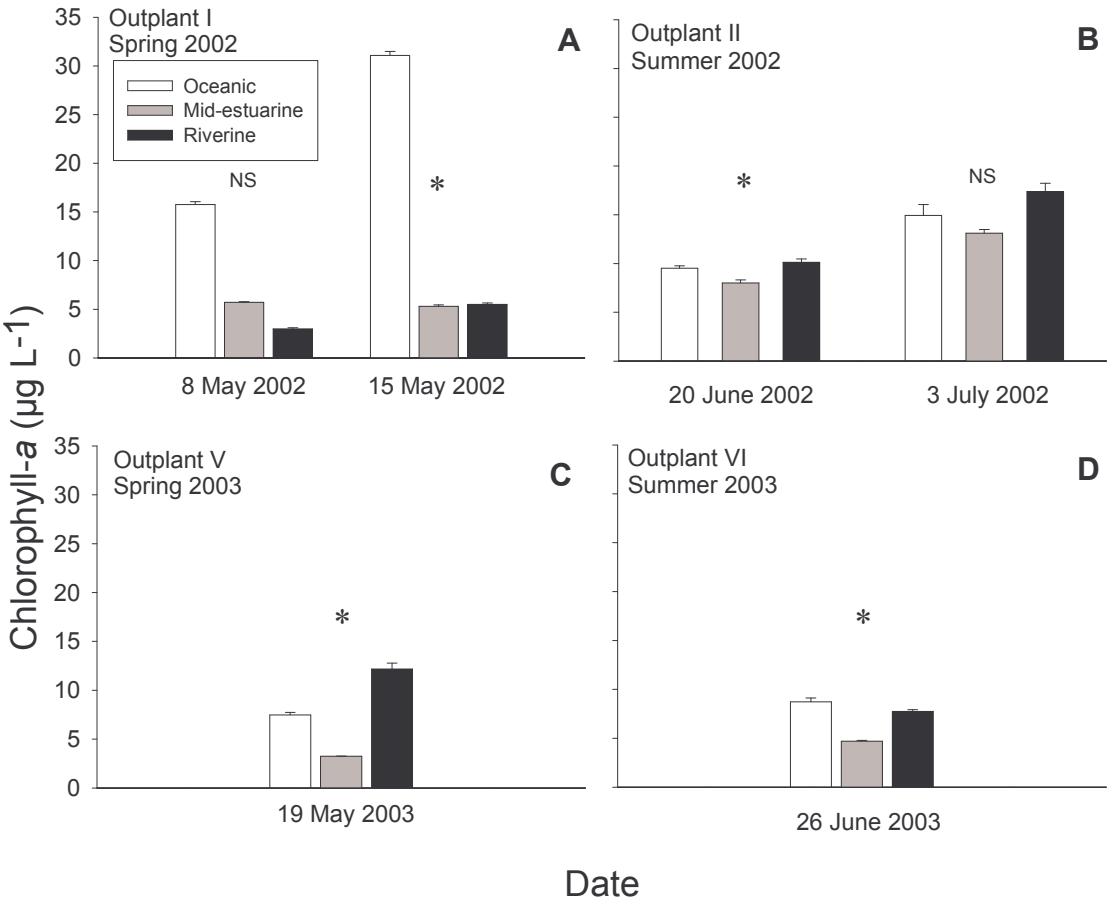


Figure 5. Surface chlorophyll-*a* concentrations at three study sites along an estuarine gradient for A) spring 2002, B) summer 2002, C) spring 2003, and D) summer 2003. Each bar represents the mean of three replicate samples; error bars are 1 standard error. Asterisks indicate chlorophyll-*a* concentrations were significantly lower at the mid-estuarine site based on a one-way ANOVA analysis ($p < 0.05$); NS indicates chlorophyll-*a* levels were not significantly lower at the mid-estuarine site.

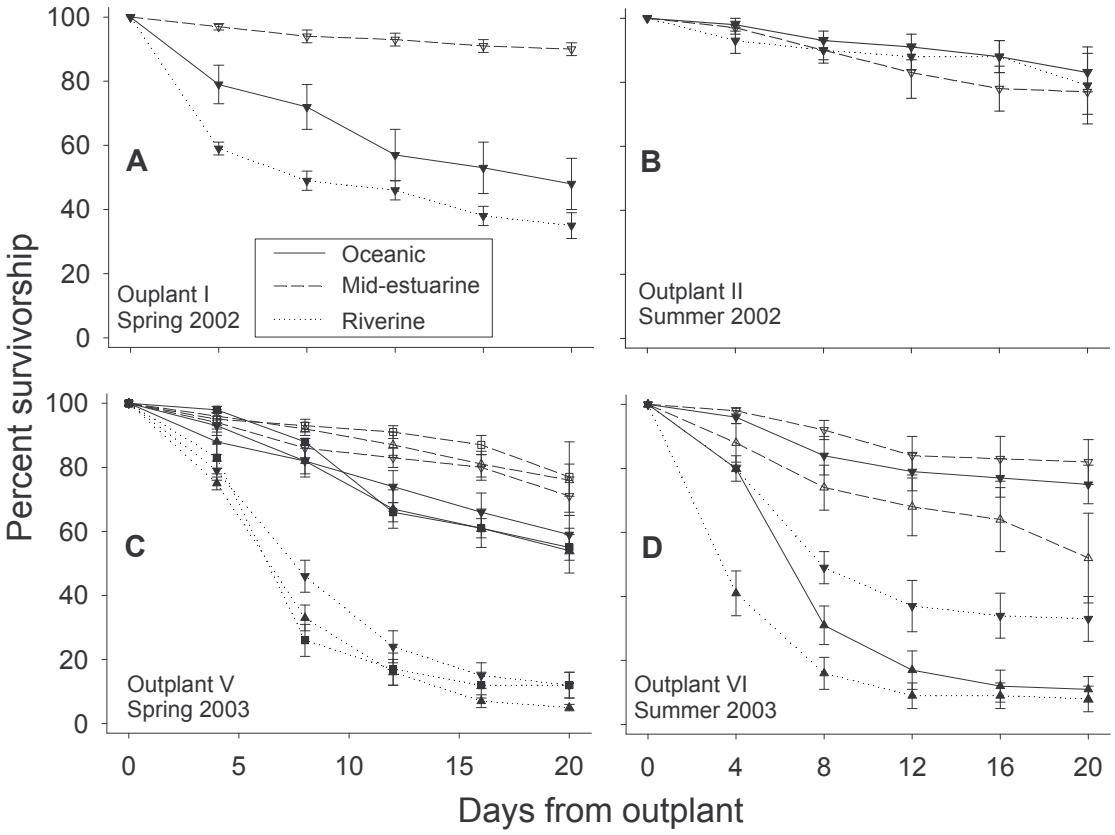


Figure 6. Percent survivorship of *Balanus glandula* juveniles outplanted along an estuarine gradient for A) spring 2002, B) summer 2002, C) spring 2003, and D) summer 2003. Symbols represent relative intertidal height; triangle up = high, square = mid, and triangle down = low. Each data point represents the mean of six panels; error bars are ± 1 standard error. See Table 1 for statistics.

Table 1. Repeated measures ANOVA on the effect of location and height on survivorship for juvenile *Balanus glandula* for 4, 8, 12, 16, and 20 days from outplant (see Fig. 6).

Source*	ss	df	Mean-square	*F*-ratio	*P*
Outplant I – Spring 2002					
Between subjects					
Location	5.53	2	2.767	34.79	0.000
Error	1.19	15	0.080		
Within subjects[I]					
Time	0.76	4	0.191	47.97	0.000
Time X location	0.89	8	0.011	2.78	0.032
Error	0.24	60	0.004		
Outplant II – Summer 2002					
Between subjects					
Location	0.08	2	0.040	0.22	0.806
Error	2.72	15	0.181		
Within subjects[II]					
Time	1.00	4	0.250	20.59	0.000
Time X location	0.08	8	0.010	0.82	0.529
Error	0.73	60	0.012		
Outplant V – Spring 2003					
Between subjects					
Location	22.04	2	11.018	1.34	0.000
Height	0.09	2	0.044	0.54	0.586
Location X height	0.28	4	0.071	0.86	0.492
Error	3.69	45	0.082		
Within subjects[V]					
Time	10.41	4	2.602	444.00	0.000
Time X location	2.00	8	0.250	42.64	0.000
Time X height	0.07	8	0.009	1.56	0.163
Time X location X height	0.28	16	0.017	2.98	0.002
Error	1.06	180	0.006		
Outplant VI – Summer 2003					
Between subjects					
Location	10.46	2	5.233	28.74	0.000
Height	8.50	1	8.502	46.68	0.000
Location X height	1.05	2	0.524	2.88	0.072
Error	5.46	30	0.182		
Within subjects[VI]					
Time	5.64	4	1.411	127.00	0.000
Time X location	0.30	8	0.037	3.34	0.019
Time X height	0.24	4	0.059	5.35	0.009
Time X location X height	0.40	8	0.049	4.46	0.004
Error	1.33	120	0.011		

*The assumption of sphericity (Mauchly's test) was violated in all four repeated measures ANOVAs. The degrees of freedom were multiplied by the Greenhouse-Geisser Epsilon and an adjusted *P*-value was calculated. The adjusted *P*-values are reported. Greenhouse-Geisser Epsilon values for within subjects: [I]0.6122, [II]0.5413, [V]0.6634, and [VI]0.4578.

15, p = 0.039) (Fig. 7B). Growth rates during summer 2002 were similar between both the mid-estuarine and riverine sites (Tukey HSD, d.f. = 15, p = 0.999).

In comparison to spring 2002 (outplant I) where growth rates were highest at the mid-estuarine site, growth rates during spring 2003 (outplant V) were similar between the oceanic and mid-estuarine sites; with significantly lower rates observed at the riverine site across all heights (Tukey HSD, d.f. = 45, $p < 0.05$) (Fig. 7C). In addition to a significant effect of location, an interaction between site and height supported the observation that vertical height had an effect on growth rate at both the oceanic and mid-estuarine sites, but not at the riverine site (Table 2, Fig. 7C). As intertidal height decreased, growth rates increased at the oceanic and mid-estuarine sites (see Fig. 7C for Tukey HSD results).

The effect of estuarine location on growth rate was significant during summer 2003 (outplant VI) (Table 2), although different patterns were observed when compared to summer 2002 (outplant II). A significantly higher growth rate was observed at the mid-estuarine site, compared to the oceanic site (Tukey HSD, d.f. = 30, p = 0.000) and riverine site (Tukey HSD, d.f. = 30, p = 0.000) across both heights (Fig. 7D). However, similar growth rates occurred between the oceanic and riverine sites (Tukey HSD, d.f. = 30, p = 0.451). In addition, intertidal height had a significant effect such that growth rates increased as intertidal height decreased across all sites (Table 2, Fig. 7D).

In August 2002, an outplant experiment (III) was conducted to examine juvenile performance when the estuary was influenced by oceanic conditions and warmer summer weather. An acute thermal event occurred during the first four days of this outplant, in

which recorded air temperatures reached levels 10 °C higher than the highs experienced on any other day during the study (Fig. 8A). Juveniles survivorship was significantly greater at the mid-estuarine site than the oceanic and riverine sites (repeated measures ANOVA time by site interaction, $F_{2,30} = 25.41$, $p = 0.000$) (Fig. 8B). Observed growth rates varied significantly as a function of estuarine location (ANOVA $F_{2,30} = 53.37$, $p = 0.000$), with a post hoc analysis confirming that juvenile growth rate was higher at the mid-estuarine site compared to the oceanic (Tukey HSD, d.f. = 30, $p = 0.000$) and riverine sites (Tukey HSD, d.f. = 30, $p = 0.000$) (Fig. 8C). No significant differences in growth rates were observed between the oceanic and riverine sites (Tukey HSD, d.f. = 30, $p = 0.787$). A significant intertidal height effect was also observed; juvenile growth rate was higher at a lower intertidal height (ANOVA $F_{1,30} = 4.62$, $p = 0.040$).

To examine the relationship between intertidal height and growth rates, a single outplant with five intertidal heights at the oceanic site (outplant IV) was designed. The results of a linear contrast analysis supported the prediction that as intertidal height decreased, growth rates significantly increased ($F_{1, 688} = 102.66$, $p = 0.000$) (Fig. 9).

In a reciprocal transplant experiment during summer 2003 (outplant VII), the source of the juvenile population (e.g., oceanic or riverine) had no effect on survivorship (Table 3, Fig. 10A) or growth of juveniles (Table 4, Fig. 10B). However, outplant location significantly affected survivorship over time (Table 3). Survivorship was higher at the oceanic site than riverine site for juveniles reared from both the oceanic and riverine populations (Fig. 10A). Growth rates were similar between the oceanic and

riverine populations, and did not significantly differ as a function of location within the estuary (Table 4, Fig. 10B).

Juvenile growth and survivorship in the laboratory

Since survivorship and growth rates were observed to be highest at the mid-estuarine site in many of the outplant experiments, a laboratory experiment was designed to test the effects of temperatures and salinities that juvenile *B. glandula* encountered in the field. Neither temperature (repeated measures ANOVA time x temperature interaction, $F_{4,8} = 1.0$, $p = 0.415$) nor salinity (repeated measures ANOVA time x salinity interaction, $F_{4,8} = 1.81$, $p = 0.134$) over the time course of the experiment significantly affected survivorship of juvenile *B. glandula*. In fact, survivorship was greater than 95 percent at all experimental temperatures and salinities.

The effect of temperature and salinity on the growth rate of juvenile *B. glandula* was surprisingly small. A significant interaction between temperature and salinity best explains the observed pattern that within a temperature range of 10 – 20 °C, growth was temperature dependent only at a salinity of 14 (Table 5, Fig. 11). At salinities of 24 and 34, the growth rate of *B. glandula* was not temperature dependent at 10, 15, or 20 °C (Tukey HSD, d.f. = 36, $p > 0.05$) (Fig. 11). Salinities of 24 and 34 typically occurred at the mid-estuarine and oceanic site during spring, respectively (Figs. 2A, C).

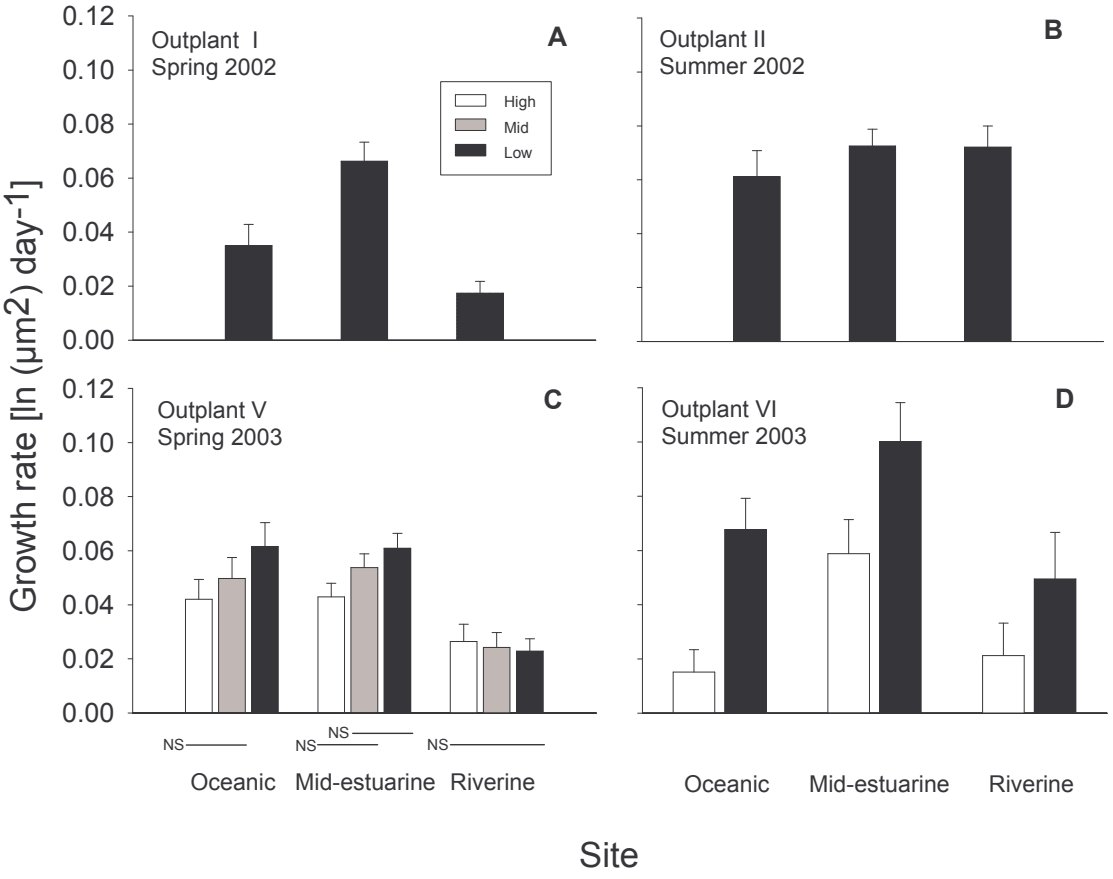
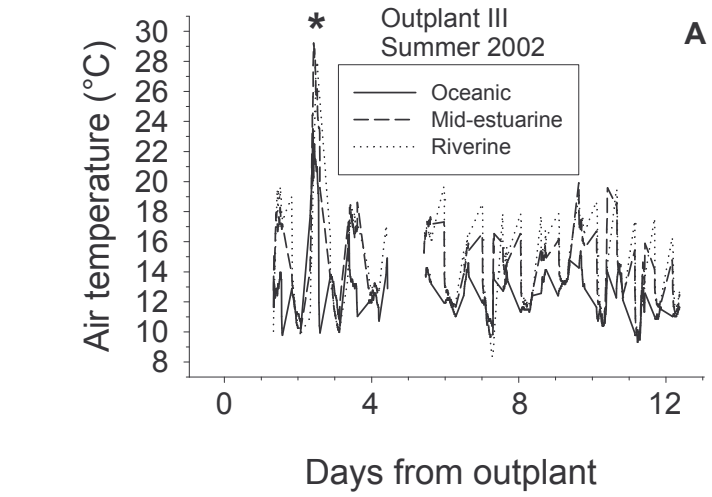
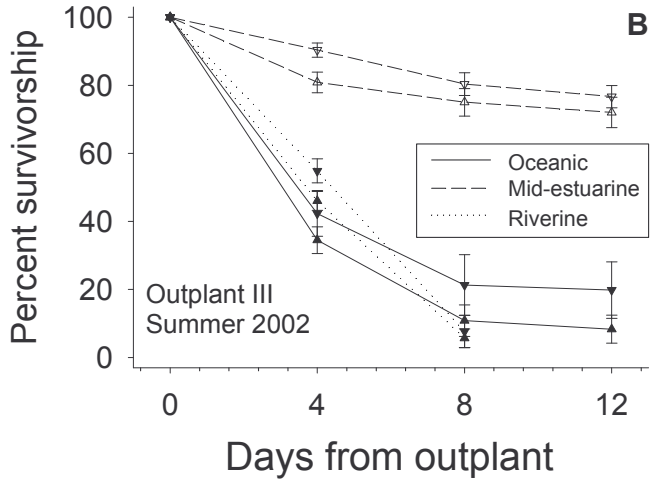
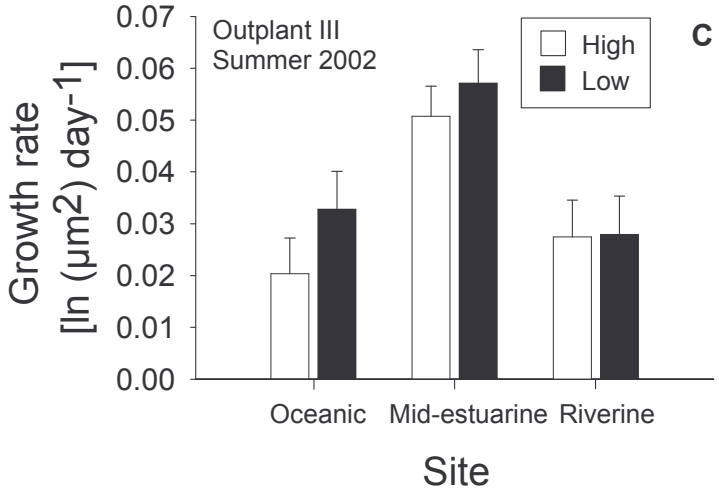


Figure 7. Natural log transformed growth rates of *Balanus glandula* juveniles outplanted along an estuarine gradient for A) spring 2002, B) summer 2002, C) spring 2003, and D) summer 2003. High, mid, and low refer to intertidal heights with equivalent submergence times used during all experiments. Each bar represents the mean of six panels; error bars are 1 standard error. See Table 2 for statistics. Lines below graph C represent non-significant differences of intertidal height on growth rate from Tukey post-hoc analysis ($p > 0.05$).

Figure 8. Air temperature profiles (A), percent survivorship (B), and natural log transformed growth rate (C) of juvenile *Balanus glandula* resulting from an acute thermal stress event in summer 2002 (outplant III). Symbols in graph B represent relative intertidal height; triangle up = high and triangle down = low. Each data point for graphs B and C represents the mean of six panels; error bars are ± 1 standard error. * denotes the acute thermal event in graph A.


Outplant III
Summer 2002
A
Oceanic
Mid-estuarine
Riverine
Air temperature (°C)
30
28
26
24
22
20
18
16
14
12
10
8
0
4
8
12
Days from outplant


B
Percent survivorship
100
80
60
40
20
0
Oceanic
Mid-estuarine
Riverine
Outplant III
Summer 2002
0
4
8
12
Days from outplant


C
Outplant III
Summer 2002
High
Low
Growth rate
[ln (µm2) day-1]
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
Oceanic
Mid-estuarine
Riverine
Site

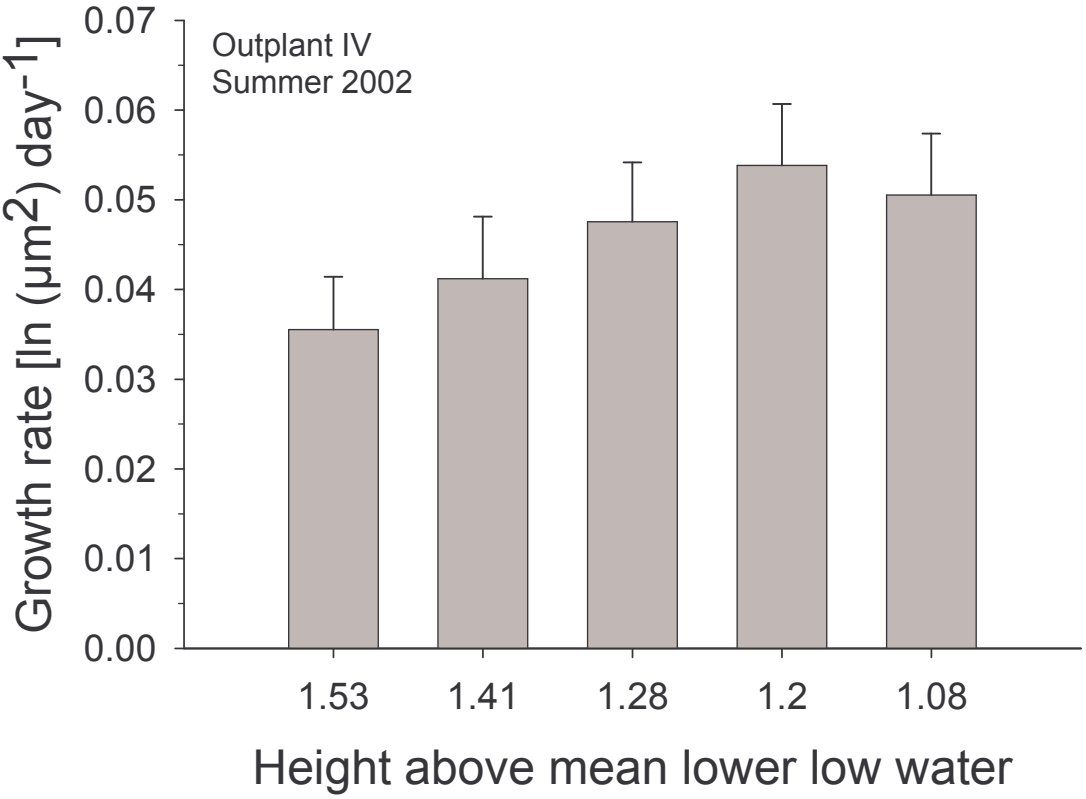


Figure 9. The effect of intertidal height on natural log transformed growth rates of outplanted *Balanus glandula* juveniles during summer 2002 (outplant IV). Each data point represents the mean of six panels; error bars are 1 standard error.

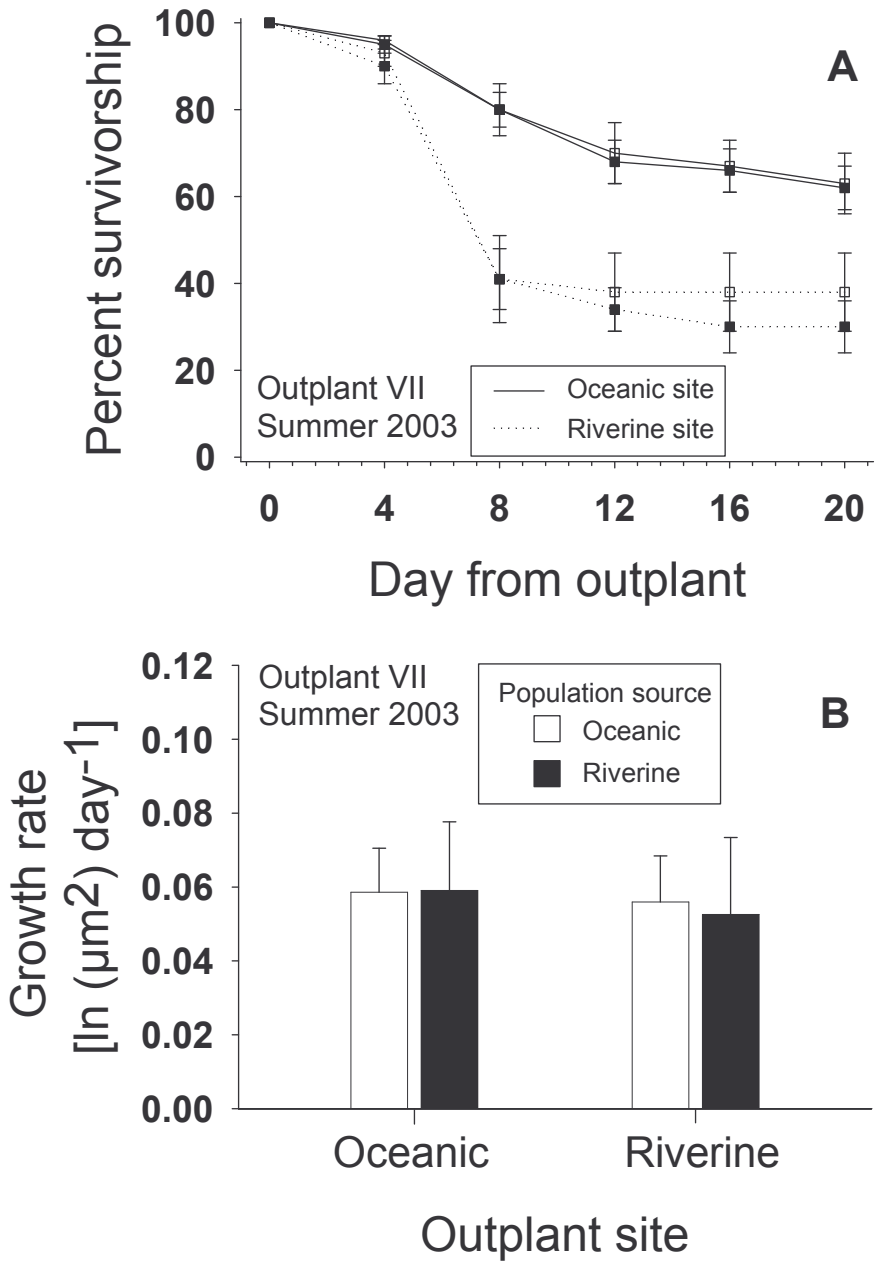


Figure 10. Percent survivorship (A) and natural log transformed growth rates (B) of juvenile *Balanus glandula* reciprocally transplanted between the oceanic and riverine sites. In graph A, symbols represent population source; open squares = oceanic population and filled squares = riverine population. Each symbol for graphs A and B represents the mean of six panels; error bars are ± 1 standard error. See Tables 3 and 4 for statistics. Data presented in this figure was contributed by Abby Darrah.

Table 2. Nested ANOVA on the effect of location and height on growth rate. Location and Height = fixed factors; Panel = random factor (see Fig. 7).

Source	ss	df	Mean-square	*F*-ratio	*P*
Outplant I – Spring 2002					
Location	0.12	2	0.0606	230.28	0.000
Panel (location)	0.01	15	0.0008	2.85	0.000
Error	0.10	370	0.0003		
Outplant II – Summer 2002					
Location	0.01	2	0.0072	5.06	0.021
Panel (location)	0.02	15	0.0014	3.94	0.000
Error	0.18	509	0.0004		
Outplant V – Spring 2003					
Location	0.22	2	0.1116	106.40	0.000
Height	0.03	2	0.0145	13.87	0.000
Location X Height	0.03	4	0.0067	6.43	0.000
Panel (location X height)	0.05	45	0.0010	4.74	0.000
Error	0.31	1422	0.0002		
Outplant VI – Summer 2003					
Location	0.33	2	0.1669	31.72	0.000
Height	0.32	1	0.3208	60.97	0.000
Location X Height	0.01	2	0.0067	1.28	0.291
Panel (location X height)	0.16	30	0.0052	6.51	0.000
Error	0.68	844	0.0008		

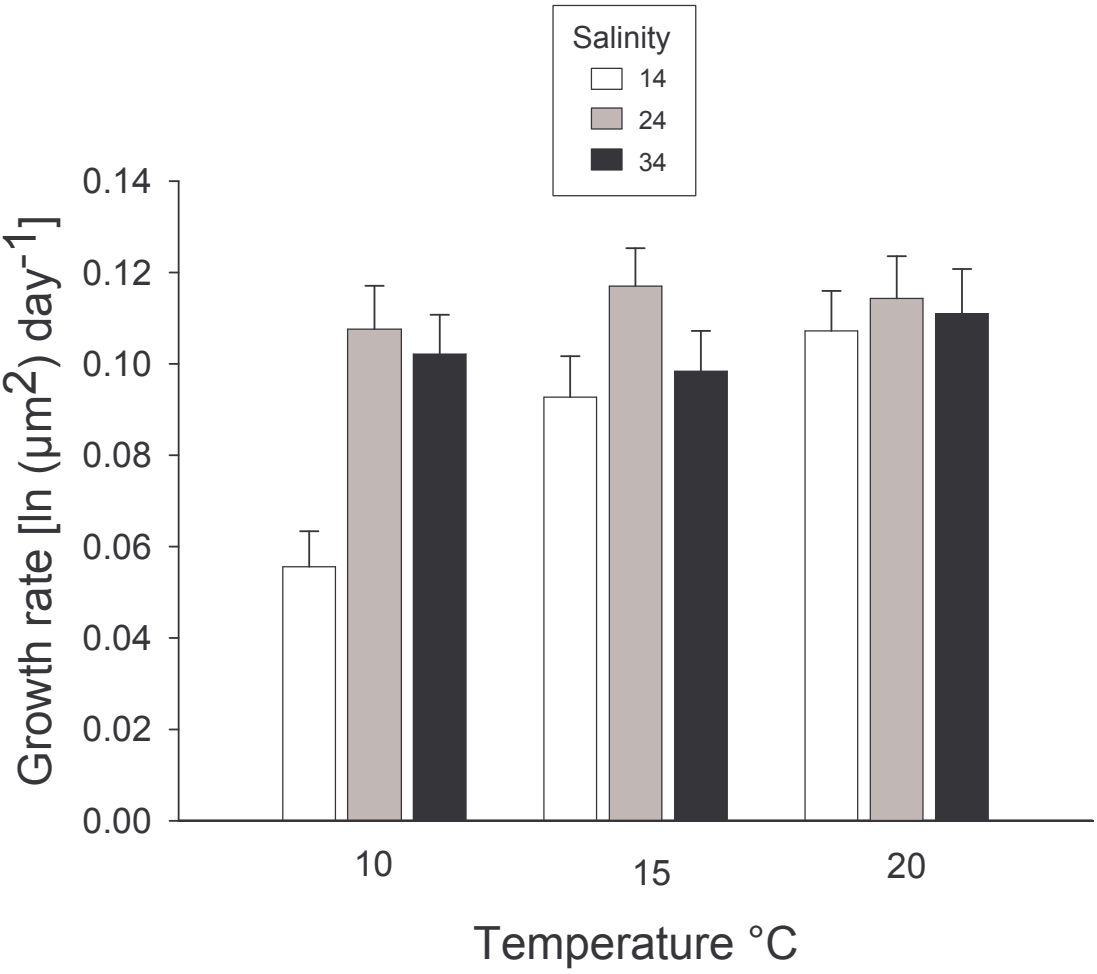


Figure 11. Natural log transformed growth rates of *Balanus glandula* juveniles from a laboratory experiment testing the effects of temperature and salinity. Each bar represents the mean of five panels; error bars are 1 standard error. See Table 5 for statistics.

Table 3. Repeated measures ANOVA from outplant VII on the effect of source and location on survivorship for juvenile *Balanus glandula* for 4, 8, 12, 16, and 20 days from outplant (see Fig. 10A).

Source*	ss	df	Mean-square	*F*-ratio	*P*
Between subjects					
Source	0.02	1	0.020	0.22	0.647
Location	2.26	1	2.255	24.98	0.000
Source X location	0.01	1	0.010	0.11	0.744
Error	1.81	20	0.090		
Within Subjects					
Time	3.44	4	0.859	154.00	0.000
Time X source	0.01	4	0.002	0.35	0.659
Time X location	0.46	4	0.115	20.58	0.000
Time X source X location	0.01	4	0.001	0.26	0.722
Error	0.45	80	0.006		

*The assumption of sphericity (Mauchly's test) was violated. The degrees of freedom were multiplied by the Greenhouse-Geisser Epsilon (0.3959) and an adjusted *P*-value was calculated. The adjusted *P*-values are reported.

Table 4. Nested ANOVA from outplant VII on the effect of source and location on growth rate. Source and Location = fixed factors; Panel = random factor (see Fig. 10B).

Source	ss	df	Mean-square	*F*-ratio	*P*
Source	0.0034	1	0.0034	0.57	0.128
Location	0.0009	1	0.0009	0.15	0.433
Source X location	0.0004	1	0.0004	0.06	0.615
Panel (source X location)	0.1166	20	0.0058	4.05	0.000
Error	0.8992	624	0.0014		

Table 5. Nested ANOVA on the effect of Temperature and Salinity on growth rate. Temperature and Salinity = fixed factors; Panel = random factor (see Fig. 11).

Source	ss	df	Mean-square	*F*-ratio	*P*
Temperature	0.122	2	0.0610	22.93	0.000
Salinity	0.191	2	0.0954	35.87	0.000
Temperature X salinity	0.121	4	0.0301	11.32	0.000
Panel (temperature X salinity)	0.096	36	0.0027	7.98	0.000
Error	0.455	1366	0.0003		

Discussion

Mortality of larvae in the plankton can be very high (Morgan 1995). To the potentially small percentage surviving the larval stage and settling, the next challenge is surviving through the juvenile stage, where mortality rates are also high (Gosselin and Qian 1996, Gosselin and Qian 1997, Hunt and Scheibling 1997). The purpose of this study was to examine the performance (survivorship and growth) of an intertidal barnacle (*Balanus glandula*) during the early juvenile stage at three estuarine sites with different environmental conditions. This study demonstrates that along an environmental gradient over the spatial scale of an estuary, juvenile performance of *B. glandula* varied within the distributional range of the adults. I proposed three alternative hypotheses, which stated performance of juvenile *B. glandula* would be highest at either the (1) riverine site, (2) mid-estuarine site, or (3) oceanic site. This study demonstrated that juvenile performance was lowest at the riverine site and highest at the mid-estuarine site. I will use the environmental data collected at each of the sites, in context with some of the published processes that control barnacle survivorship and growth, to explain the patterns observed in this study.

Juvenile performance along an environmental gradient

Juveniles had poorer performance at the riverine site compared to the other sites in five of six outplant experiments; performance at the riverine site was not highest. One interpretation that may explain the lower survivorship and growth rates during spring 2002 and 2003, compared to summer 2002, was salinity. During the spring, due to

rainfall, barnacles at the riverine site experience low salinity. *Balanus glandula* adults are tolerant of salinities as low as zero for at least 72 hours (Bergen 1968). Although no data exists describing the low salinity tolerance of juveniles, my laboratory results indicate juvenile *B. glandula* can survive at a salinity as low as 14 for at least twenty days. Although low salinities are tolerated, behavioral modifications do occur when *B. glandula* is exposed to low salinities. For example, when salinities are physiologically stressful, barnacles respond by closing their opercular valves and remaining closed (Barnes and Barnes 1957). Cirral activity in adult *B. glandula* stops at salinities below approximately 8 (Newman 1967, Bergen 1968). In a preliminary experiment, I observed a cessation of cirral activity in juvenile *B. glandula* at 10. Since juveniles during both spring outplants experienced salinities below 10 for extended periods (Fig. 2), they presumably spent time with their feeding apparatus retracted and ceased feeding. Decreased feeding time and potential starvation, in concert with elevated levels of osmotic stress, may explain some of the decreased survivorship. Furthermore, the cessation of feeding may explain the observed low growth rate of the surviving individuals. For example, the cessation of feeding during periods of low salinity and a subsequent decrease in growth rate was observed in the mussel *Mytilus edulis* (Seed and Suchanek 1992). Therefore, I suggest that when juvenile *B. glandula* experience extended periods of low salinity their survivorship will be low.

The survivorship of juvenile *B. glandula* at the riverine site varied annually and seasonally. Although a similar pattern of survivorship and growth between sites was observed in spring 2002 and spring 2003, survivorship was distinctly lower in spring

2003. The lower survivorship in 2003 may in part be explained by exposure of the juveniles to salinities of approximately zero during the first week of the experiment (Fig. 2C); both the magnitude and duration of exposure to salinities below 10 was lower and longer in spring 2003 than spring 2002. In contrast to the spring outplants and the summer 2003 outplant, during summer 2002 favorable conditions were present for high survivorship and growth rates at the riverine site. Disregarding the effect of intertidal height for now, an explanation as to why the summer 2003 survivorship and growth had elements of a spring pattern is uncertain. The variability in survival, but lack of an obvious difference in the salinity profiles of summer 2002 and 2003, suggests that salinity alone was not the only factor driving juvenile mortality. Mortality may have been due to desiccation and predation.

Overall, juvenile performance was highest at the mid-estuarine site. Perhaps higher survivorship was due to an interaction between physiological stress and site-specific differential predation. Connell (1975) suggests there may be an interaction between physiological stress and predation, where the combination of tolerable stress in conjunction with reduced predation could potentially create conditions favorable for a successful recruitment into a population. The abundance of micro-predators may have varied along the environmental gradient. Along the length of an estuary, the abundance and composition of species changes with distance from the oceanic inlet (Little 2000). When harsh or extreme environmental conditions are present, the abundance of predators and subsequent predation rates tend to be low (Connell 1975, Underwood and Denley 1984, Menge and Sutherland 1987). Environmental conditions at the mid-estuarine site

were within the tolerance range of *B. glandula*, but may have been outside the range of potential predators. Barnes (1999) has summarized numerous examples of cirripede predators, including flatworms. I did not quantify the presence or absence of predators, however, flatworms were observed multiple times on panels retrieved from the oceanic site and were never observed at the mid-estuarine site. Of the juvenile *B. glandula* that did not survive, no obvious difference in cause of mortality was observed between outplant sites. The interaction between differential predation and physiological stress may potentially explain why more barnacles survived at the mid-estuarine versus the oceanic site.

Warmer water temperatures at the mid-estuarine site, compared to the oceanic site, during both spring and summer outplant experiments may have increased the stress tolerance of juvenile *B. glandula*. Exposure of juveniles to elevated water temperatures may have had an acclimating effect, increasing their tolerance of subsequent aerial thermal stress. Experimental studies have shown that acclimation to elevated temperatures can raise the upper thermal tolerance of an organism. For example, acclimation to a higher temperature increased the thermal tolerance of three species of porcelain crabs (Stillman 2000). Even a brief thermal shock of 37 °C for one hour increased the percent of Pacific oysters surviving a thermal shock of 44 °C two weeks after the initial shock (Clegg et al. 1998). Therefore, the juveniles outplanted at the mid-estuarine site may have, through the physiological process of thermal acclimation, increased their tolerance to acute thermal stress or other environmentally mediated stressors. In other words, the juveniles at the oceanic site may have been less tolerant to

thermal and desiccation stress during low tide emersion and consequently experienced higher mortality.

Why growth rates at the mid-estuarine site were higher is uncertain. In the laboratory experiment, at temperatures between 10 – 20 °C and salinity between 24 – 34, no difference in growth rates were observed. Therefore, the higher water temperatures at the mid-estuarine site, compared with the oceanic site, do not appear to have caused an increased growth rate. Higher growth rates have been observed for barnacles in locations with higher concentrations of chlorophyll-*a* (Bertness et al. 1991, Sanford and Menge 2001). Since surface chlorophyll-*a* concentrations were lowest at the mid-estuarine site in three of four outplant experiments, this seems an unlikely explanation. In addition, high flow rates will result in higher growth rates (Leonard et al. 1998). For example, a decrease in the growth rate of *Semibalanus* (*Balanus*) *balanoides* was observed when flow was experimentally reduced in the field with baffles (Crisp 1960). The higher observed growth rates observed by Crisp were most likely due to the increased particle (i.e., food) availability caused by an increased flow rate. Maximum water velocity at the oceanic site and the riverine site is approximately 1 ms^{-1} (Juza 1995, Roegner and Shanks 2001). During panel retrieval or deployments, the surface velocity was not obviously higher at the mid-estuarine site (M. Berger, pers. obsv.). Therefore, a higher flow rate seems an unlikely explanation as well.

At all three sites examined in this study, survivorship and growth rates were the least variable at the mid-estuarine site within and between seasons. In fact, no obvious

pattern of seasonality was observed between spring and summer at the mid-estuarine site despite varying patterns of salinity and temperature between seasons.

Lower juvenile performance was usually observed at the oceanic site, compared to the mid-estuarine site. Therefore, the alternative hypothesis that performance would be the highest at the oceanic site can be rejected. This result is surprising, as one would expect that a species predominantly distributed on the open coast would perform best in conditions similar to the open coast. None of the environmental parameters measured at the oceanic site would suggest conditions were unfavorable; in fact just the opposite. As previously discussed, potentially higher predation rates and a lower tolerance to stress may have contributed to the lower survivorship observed at the oceanic site.

Acute climate induced stress event

In the intertidal, where conditions fluctuate between marine and terrestrial, acute climate induced stress events can occur that dramatically decrease survivorship. Climatic conditions can change over a temporal scale of hours to days. Desiccation is thought to be a major factor causing mortality of recently settled intertidal organisms (Foster 1971b, Denley and Underwood 1979). Exposure to a sub-lethal high temperature may intensify the effects of desiccation and consequently increase mortality (Wolcott 1973, Gosselin and Chia 1995). During the course of outplant III, temperatures reached almost 30 °C during midday. Subsequently, low survivorship was observed at the oceanic and riverine sites. If this outplant or a hypothetical settlement event had occurred four days later, the

percent of juveniles surviving could potentially have been greater. Wethey (1986) also noted a dramatic temporal variation in juvenile mortality over the scale of days.

The effect of intertidal height on juvenile performance

The probability of an intertidal juvenile barnacle surviving can be a complex interaction between tidal cycle and intertidal height, with respect to time of day. During summer 2003 (outplant VI), a strong effect of intertidal height was observed, such that survivorship was lower for juveniles higher in the intertidal. Minchinton and Scheibling (1993) observed a similar effect on recently settled *Semibalanus balanoides*, where survivorship decreased as intertidal height increased. In contrast, over a 42-day period, intertidal height did not affect the survivorship of juvenile *B. glandula* (Gosselin and Qian 1996). These results, in conjunction with the results of this study, suggest that the effect of intertidal height on survivorship is temporally variable. Examination of the tide amplitude data during outplant VI (summer 2003) suggests a reasonable explanation for the observed height effect. Juveniles were outplanted during a neap tide, when low tides occurred during the midday. During the first three days of the outplant, the higher intertidal height was only submerged during the high high tide, while the lower intertidal height was submerged during both the high high tide and the low high tide. Survival is typically higher for intertidal barnacles when shading or seeps are present (Hatton 1938, Denley and Underwood 1979, Wethey 1984). Effectively, juveniles at the lower height were able to briefly take refuge from the desiccating effects of the midday sun via submergence during the low high tide. This result suggests that a small variation in the

timing of settlement over a scale of days or the vertical height at which settlement occurs could dramatically affect the number of juveniles surviving and subsequently recruiting into the population. Why the effect of height over time was more pronounced at the oceanic site than the other two sites is uncertain.

In addition to the effects of a horizontal environmental gradient on growth, intertidal organisms have to contend with a vertical gradient. Relative intertidal height had a strong effect on the growth rates of juveniles. I observed a significant decrease in the growth rate of juvenile *B. glandula* as intertidal height increased. Similar results from field experiments have been observed for other barnacle species (Moore 1934, Barnes and Powell 1953, Bertness et al. 1991, Benedetti-Cecchi 2000). Further, growth rates of adult mussels have been reported to decrease as intertidal height increased (Seed and Suchanek 1992). The inverse relationship between growth rate and tidal height suggests that submergence time appears to be driving increased growth rates at lower intertidal heights. I should note that in all the outplant experiments where height was a factor, only during outplant VI (summer 2003) did an interaction between tidal cycle and intertidal height occur. During all other experiments, the highest set of panels was submerged during both the high high and high low tide. A longer submergence time equates to a longer available feeding period. Using tide height data (1.53 – 1.41 and 1.41 – 1.28 meters above MLLW) from outplant IV as an example, I estimated that juveniles had approximately seven percent more time available for feeding with each 12.5 centimeter decrease in intertidal height over the 20-day experimental period. Ritz and Crisp (1970) suggested that as an adaptation to less available feeding time, *B. balanoides* fed more

rapidly at higher intertidal heights than lower intertidal heights. If an increased rate of feeding did occur at higher intertidal heights over the 20-day duration of this study, the effects were not noticed.

Local adaptation

The observed variation in survivorship and growth could be due to genetic differentiation as a result of different selective forces upon the respective populations at either end of the environmental gradient. Circulation patterns within an estuary can retain barnacle larvae (Bousfield 1955, DeWolf 1973) and potentially maintain isolated populations. In addition, populations can also be genetically modified if post-settlement mortality selects for location specific traits (Brind'Amour et al. 2002, Drouin et al. 2002). In the summer of 2003 (outplant VII), the population source (e.g., oceanic or riverine) did not significantly contribute to any of the variation observed in survivorship or growth. If genetic differences do exist between the two populations, they were not manifested by variation in survivorship or growth. Local adaptations to thermal stress of recently settled barnacles occurs when estuarine flushing time is long, but not when flushing time is short (Bertness and Gaines 1993). When flushing time is long, larval retention can maintain genetically isolated populations (Gaines and Bertness 1992). A short flushing time of approximately three days has been estimated in the South Slough Estuary (Juza 1995). Therefore, a larval period of approximately 8 to 15 days for *B. glandula* (Strathmann et al. 1981, Emlet unpublished obsv.) is more than sufficient time for mixing between the two populations to occur.

Conclusions

In conclusion, the pattern of increased performance at the mid-estuarine site stands out. The results from this study support the model Connell (1975) presented that suggests intermediate physical conditions can facilitate the successful recruitment of an individual into a population. Future work should examine differential predation on juvenile barnacles along an estuarine gradient. Also further exploration into the costs and benefits of settlement at a mid-gradient location should be explored.

In contrast to high juvenile performance at the mid-estuarine site, the reproductive output of *B. glandula* along the environmental gradient was highest at the oceanic end of the estuary (see Chapter III). This presents an interesting trade-off with respect to choice of settlement site. The optimal location for juvenile survivorship and growth was the mid-estuarine site, whereas the optimal location for reproduction was the oceanic site. This trade-off should be further evaluated between sites by examining the total reproductive output over an individual's life span, the age of reproduction, and the fitness of the offspring produced at both sites.

BRIDGE I

Chapter II demonstrated that performance (survivorship and growth) of juvenile *Balanus glandula* was affected by estuarine location. The optimal site for survival and growth was the mid-estuarine site, while the least suitable site was the riverine end of the estuary. Assuming the barnacle survives the juvenile stage, the next challenge is surviving to reproductive maturity. Once reproductive, similar process that influenced the survival and growth of the juvenile, will influence the reproductive output of the adult. Chapter III addresses the influence of location along an estuarine gradient on the reproductive output of *B. glandula*.

Chapter III

REPRODUCTIVE ECOLOGY OF THE BARNACLE *BALANUS GLANDULA* ALONG AN ESTUARINE GRADIENT

Introduction

Habitat-specific environmental conditions influence the reproductive patterns of marine invertebrates (Rutherford 1977, Etter 1989, Stewart-Savage 2001, Yund and Stires 2002). Typically the reproductive output of an organism decreases in habitats that are considered stressful or food limited. The mussel *Mytilus edulis*, when experiencing physiologically stressful conditions in the laboratory and field has a lower reproductive output than mussels experiencing non-stressful conditions (Bayne et al. 1978, Bayne et al. 1983, Thompson 1984). The reproductive output of the barnacle *Semibalanus balanoides* was higher in a bay with abundant food than the open coast where food was less abundant (Bertness et al. 1991).

Reproductive output, the number of offspring produced per individual over time (Lewis and Chia 1981, Page 1984, Hines 1978), is typically used as a comparative measure between species; however the term has also been used as a comparative measure between individuals from different habitats (Page 1984). The term reproductive output is often interchanged with reproductive effort in the literature. Reproductive effort is defined as the amount of energy or resources that an individual devotes towards

reproduction over interval (Stearns 1976, Page 1984). Reproductive output will be the basis of this study.

Invertebrate organisms display a variety of reproductive strategies with respect to the timing of reproduction. At one extreme, some species are reproductive over a very short time span, while at the other end, some species are reproductive year round (Barnes 1989, Hadfield and Strathmann 1996). A reproductive season is not necessarily fixed for a species, and in fact, can vary with environmental conditions (reviewed in Giese and Kanatani 1987, Sastry 1975). The flexibility of being reproductive across multiple seasons may be an adaptation to increase the probability of successful recruitment in an environment that is highly variable (Hadfield and Strathmann 1996).

Reproductive output in benthic marine organisms, including barnacles, is the result of a complex process involving the potential interaction of multiple environmental variables such as temperature, salinity, and food availability (Vernberg and Vernberg 1972, Sastry 1975, Vernberg 1981, Barnes 1989). When temperatures rise above a critical point, barnacle species with a boreo-arctic to temperate distribution will not reproduce (Barnes 1963, Crisp and Patel 1969, Barnes and Stone 1973, Page 1983). For example, the percentage of populations of *Balanus glandula* and *Elminius modestus* brooding was lower at warmer sites than at cooler sites (Hines 1978, O'Riordan and Murphy 2000).

In estuarine habitats where exposure to reduced salinity occurs, a decrease in reproductive activity can occur (reviewed in Barnes 1989). Barnes and Barnes (1968) observed a lower number of brooded embryos in *Balanus improvisus* at a site with a low

salinity of 6 compared to a site with a high salinity of 30. Although a low salinity environment may not preclude barnacles from reproducing, low salinity can be lethal to developing embryos. In a variety of barnacle species, including *Balanus glandula*, embryonic development was halted due to cellular lysing when embryos were exposed to salinities below 15 - 20 (Crisp and Costlow 1963, Bergen 1968, Barnes and Barnes 1974).

Food abundance is another factor contributing to variation in barnacle reproduction. An adequate supply of food is required before a number of barnacle species become reproductively active (Patel and Crisp 1960). A delay in ovarian maturation, the formation of egg lamellae, and subsequent brooding occurs when food is limiting (Barnes and Barnes 1967, Barnes and Barnes 1975, Page 1983). A delay in reproduction can decrease the number of broods produced per year (Hines 1978), which will ultimately affect the total reproductive output.

An additional aspect of barnacle reproductive biology to consider is embryo size. Brooded embryos go through a series of developmental stages, before being released as swimming nauplii larvae (Groom 1894, Anderson 1994). Developing embryos increase in volume, especially during limb formation (Crisp 1987). Changes in embryo size can also be environmentally mediated. Embryos exposed to a lower temperature during development are larger than embryos exposed to warmer temperatures (Patel and Crisp 1960). Crisp (1987) suggested that as temperature increases, so do the metabolic rate of the developing embryo, thus resulting in a higher consumption of energetic reserves contained in the egg yolk that results in a smaller embryo.

The barnacle *Balanus glandula* is an ideal organism to study reproductive patterns along an environmental gradient. This species is commonly found in bays and estuaries (Morris et al. 1980), where temperature, salinity, and food availability can vary over a spatial scale of kilometers. In the South Slough Estuary, where this study was conducted, *B. glandula* can cover approximately 100 percent of the available substrate, well into the oligohaline region of the estuary (M. Berger, pers. obsv.). In addition, gonad maturity and reproductive status can easily be determined, as *Balanus glandula* is hermaphroditic and broods it's offspring for approximately one month (Hines 1978, Anderson 1994).

The goal of this study was to observe the seasonal reproductive patterns in the barnacle *Balanus glandula* along an estuarine gradient. With increasing distance from the oceanic inlet, conditions within the estuary become less marine-like, increasing the potential for environmentally mediated stress; environmental stress may in turn affect reproductive output. The following questions were addressed: (1) How does location along an estuarine gradient affect the reproductive output of *B. glandula*? (2) How does estuarine location affect gonad maturity? (3) Does embryo size vary as a function of estuarine location or season?

Methods

Adult barnacles of *Balanus glandula* were sampled at four sites distributed along an estuarine gradient in the South Slough Estuary, Charleston, Oregon, USA (Fig. 1). Sites were located at the oceanic end, mid-estuarine, and riverine end of the salinity gradient within the estuary. At the riverine end of the gradient, where the South Slough

Estuary splits into two arms, a site in each arm was sampled. The rationale for sampling two riverine sites was to partially address issues of pseudoreplication by examining reproductive patterns from two separate riverine sites.

On a monthly basis between April 2002 and July 2003, fifty adult *Balanus glandula* were collected from wooden pilings at each of three sites: oceanic, mid-estuarine, and riverine site of the Winchester arm (WI) (Fig. 1). Collection at the riverine site on the Sengstacken arm (SE) occurred monthly between October 2002 and June 2003. Specimens were randomly collected by placing a 10 cm^2 quadrat with a removable clear acetate sheet over the piling; the acetate sheet contained a series of fifty dots. The specimen nearest to a dot was collected. Only specimens with a rostro-carinal diameter greater than 5.0 mm were considered for collected, because Hines (1976) reported *B. glandula* larger than 5.0 mm were consistently mature. Additional collection criteria included collecting specimens that were not crowded or hummocked, but were adjacent to another individual so copulation could occur. Specimens were collected at an intertidal height with equivalent submergence times (see Chapter II). As a reference, the collection height at the oceanic site was 1.41 m above mean lower low water.

Following collection, the specimens were returned to the lab and dissected. The length of each barnacle's shell along the rostro-carinal axis was recorded. The presence or absence of ripe yolky ovarian tissue was recorded. Egg lamellae were removed from the mantle cavity and placed in a 1.0 % (w/v) protease solution (Sigma, P-5380) for 2 - 3 hours at room temperature to separate the individual embryos. Following separation, the embryos were fixed in a 4 % formalin solution. The prosoma was removed and the

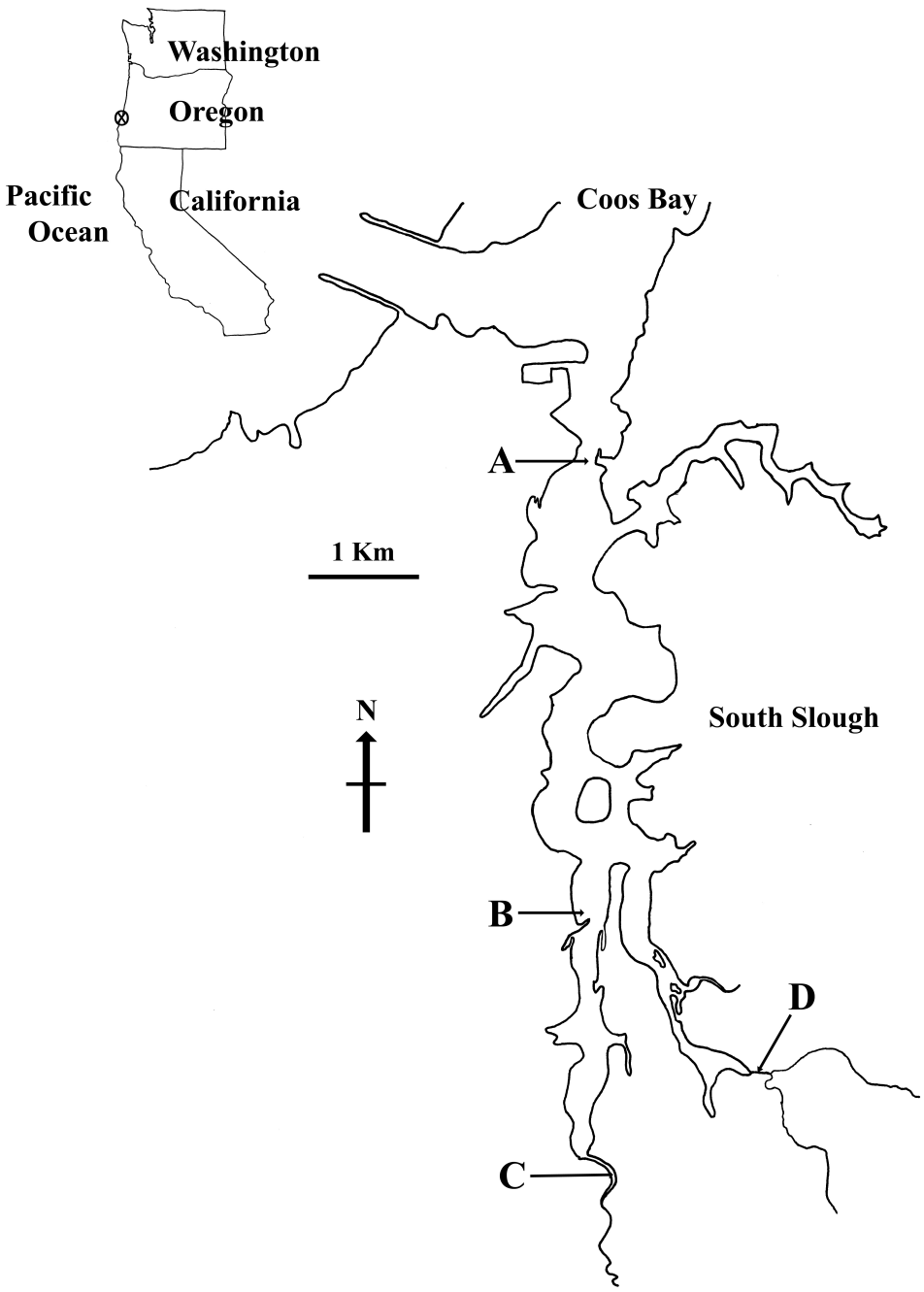


Figure 1. Location of study sites within the South Slough Estuary, Charleston, Oregon, USA. The three study sites are labeled as A) oceanic, B) mid-estuarine, C) riverine Winchester arm (WI), and D) riverine Sengstacken arm (SE). See inset for an approximate location along the west coast of the United States.

presence or absence of a sperm filled seminal vesicle was recorded; ovarian tissue, mantle tissue, and opercular muscles were not removed with the prosoma. The prosoma was dried to a constant weight at 60 °C for approximately ten days and then the dry mass was measured to the nearest 0.1 mg.

The total number of embryos per individual was estimated by diluting the fixed embryos in 50 ml of seawater and then counting the number of embryos in eight 1.0 ml subsamples. Twenty embryos were then randomly selected for length and width measurements (to the nearest 0.01 mm). Using the length and width measurements, embryo volume was estimated as a prolate spheroid using the following equation:

$$\text{volume} = 4/3\ \pi l w^2,$$

where l is the length along the major radius and w is the width along the minor radius (Patel and Crisp 1960, Lucas and Crisp 1987).

Salinity and water temperature were continuously monitored throughout the sampling period at the oceanic, mid-estuarine, and WI riverine site (A, B and C). Salinity and water temperature were recorded with a YSI model 6600 data logger at the oceanic and riverine site, and a YSI model 6000 data logger at the mid-estuarine site. In addition, measurements of chlorophyll-*a* concentrations were determined by collecting three replicate surface water samples during high tide at each site on a monthly basis. The water samples were filtered onto glass fiber filters (1.2 μm retention) and analyzed for chlorophyll-*a* concentration following the method of Parsons et al. (1984).

Analysis

To statistically test the overall effect of location within the estuary on the population parameter of proportion brooding, I pooled samples for each site to get a proportion for the population that was brooding over the entire sampling period (April 2002 - July 2003) for each site. A chi-square analysis on multiple proportions was performed to test the null hypothesis that the proportions between sites were not different (Zar 1984). To determine which proportions (collection sites) were different, a post-hoc test to compare multiple proportions was performed (Zar 1984). An identical analysis was performed to test the effect of estuarine location on the population parameters of proportion with filled seminal vesicles and proportion with well-developed ovaries. Since the second riverine site (SE, site D) was not sampled from the start of the study, it was not included in this statistical analysis.

To test for seasonal effects between the study sites on the proportion brooding, proportion with filled seminal vesicles, and proportion with well-developed ovaries, samples were grouped into two seasons as: dry (May 2002 – November 2002) and rainy (December 2002 - May 2003). Salinity at the WI riverine site was used as the grouping criteria for season. Months with mean salinities greater than 20 were considered the dry season and months with salinities below 20 the rainy season. During the dry season, salinity is relatively high because freshwater input is low, whereas during the rainy season, salinity sharply decreases along the estuarine gradient due to a high freshwater input. A chi-square analysis on multiple proportions for each season was performed,

followed by a post-hoc test to compare multiple proportions (Zar 1984). The SE riverine site was not included in the seasonal analysis.

Because embryos are brooded for 22 days (19 °C) to 29 days (12 °C) (Hines 1978), environmental conditions one month prior to the sampling date should influence the percent of the population brooding more than the coincident conditions should. Preliminary analysis indicated there was not a significant correlation between environmental variables (temperature, salinity, and chlorophyll-*a*) and the percent brooding during the same month. Therefore, to examine the effect of temperature, salinity, and chlorophyll-*a* on the percent of the population brooding, I used the mean monthly data from the month prior to barnacle collection dates. These data were regressed on the percent of the population brooding.

To quantify site-specific differences in individual fecundity, the total number of embryos per individual was normalized to dry body mass (Barnes and Barnes 1968). Because the number of embryos increases linearly as a function of mass of a barnacle (Barnes and Barnes 1956), normalization to body mass is necessary and appropriate with populations that vary in individual size.

Because fifty individuals were collected each month at each site, and the frequency of brooding ranged from seventy-eight percent to zero percent, it was not possible to estimate individual fecundity for all sample sites and dates. Preliminary data analysis consisted of performing a series of three two-way ANOVAS that compared the effects of collection site and collection time between (1) the oceanic and mid-estuarine site, (2) the oceanic and WI riverine site, and (3) mid-estuarine and WI riverine site. A

Bonferoni correction for multiple comparisons was used to adjust the significance level (Zar 1984). Because sample collection was spread out over the period of thirteen months, the most parsimonious approach was to pool the samples across sample dates and examine the number of brooded embryos per individuals for the whole 13 month period.

Samples were pooled from monthly collections during April 2002 to April 2003 for each site. A one-way ANOVA was performed to test for site-specific effects of fecundity, followed by a Tukey post-hoc test to examine specific differences between sites. A natural log transformation was used on the data prior to analysis, to meet the assumption of homoscedasticity. Although the assumption of normality was violated, ANOVAS are robust to departures from normality (Sokal and Rohlf 1995, Underwood 1997).

To test for changes in embryo volume over time, I used a nested ANOVA design with individual nested in collection date. Only embryos in the early phase of development, up to stage seven (when six or more yolk cells are present) as described by Crisp (1954), were analyzed because embryo volume increases when limb formation begins (stage eight) (Crisp 1987). A preliminary analysis indicated there was not a significant effect of collection site on embryo volume (ANOVA, $F_{2,54} = 3.07$, $p = 0.055$), so samples were pooled across site to increase the sample size. A Tukey post-hoc analysis was performed to examine significant differences between collection dates. Although the assumption of homoscedasticity was met, the assumption of normality was violated.

Results

Physical site characteristics

Seasonal patterns of salinity and water temperature were observed in the South Slough Estuary during the 16 months of this study. During April through July 2002 salinity decreased along the estuarine gradient with distance from the oceanic inlet (Fig. 2). During August through November of 2002, the estuary had a strong marine influence with relatively high salinity at all sites. In December a strong salinity gradient emerged as distance from the oceanic inlet increased. Water temperature followed a seasonal pattern with warmer temperatures for the mid-estuarine and riverine sites in the late spring through summer and cooler temperatures in the fall and winter (Fig. 3). The annual variation in water temperature was higher at the mid-estuarine and riverine sites than the oceanic site. Maximum mean monthly water temperatures were 17 and 20 °C in June at the mid-estuarine and riverine sites, respectively, while the water temperature at the oceanic site reached a maximum of 13 °C during June. In late fall and winter (November 2002 – March 2003), the water temperature at the all three sites was relatively similar. For example, in December, the mean monthly water temperature was 11.4, 10.1, and 9.5 °C at the oceanic, mid-estuarine, and riverine sites, respectively.

Concentrations of chlorophyll-*a* were highly variable over spatial and temporal scales; however, a couple of patterns do merit mention. In three out of the eleven sample dates, concentrations of chlorophyll-*a* were significantly higher at the oceanic site than the mid-estuarine or riverine sites (Tukey HSD, $p < 0.05$) (Fig. 4). A trend was also

observed for lower chlorophyll-*a* concentrations at the mid-estuarine site than the oceanic or riverine sites.

Reproductive patterns along an environmental gradient

Overall, the percent of the *Balanus glandula* brooding varied significantly by location in the estuary ($\chi^2 = 153.8$, df = 2, $p < 0.001$). The percent brooding was significantly higher at the oceanic site (40 %) than the mid-estuarine site (28 %) ($q_{0.05, \infty, 3} = 14.52$, $p < 0.001$), and the percent brooding at the Winchester (WI) riverine site (12 %) was significantly lower than the mid-estuarine site ($q_{0.05, \infty, 3} = 21.9$, $p < 0.001$).

When the data is pooled into a dry (May 2002 – November 2002) and rainy season (December 2002 – May 2003), site has a significant effect on the percent of *B. glandula* brooding during both seasons. During the dry season, more *B. glandula* were brooding at the oceanic site (35 %) than the mid-estuarine site (10 %) ($q_{0.05, \infty, 3} = 34.62$, $p < 0.001$) and the WI riverine site (9 %) ($q_{0.05, \infty, 3} = 37.04$, $p < 0.001$) (Fig. 5A). The mid-estuarine and riverine sites were not significantly different during the dry season ($q_{0.05, \infty, 3} = 2.49$, $p > 0.2$). During the rainy season, the percent of *B. glandula* brooding at the riverine site (16 %) was significantly less than at the oceanic site (40 %) ($q_{0.05, \infty, 3} = 13.28$, $p < 0.001$) and the mid-estuarine site (37 %) ($q_{0.05, \infty, 3} = 11.42$, $p < 0.001$) (Fig. 5A). The oceanic and mid-estuarine sites were not significantly different during the rainy season ($q_{0.05, \infty, 3} = 1.84$, $p > 0.2$).

The three estuarine sites had different patterns regarding the percent of the population brooding (Fig. 5A). Except during November and December, the oceanic

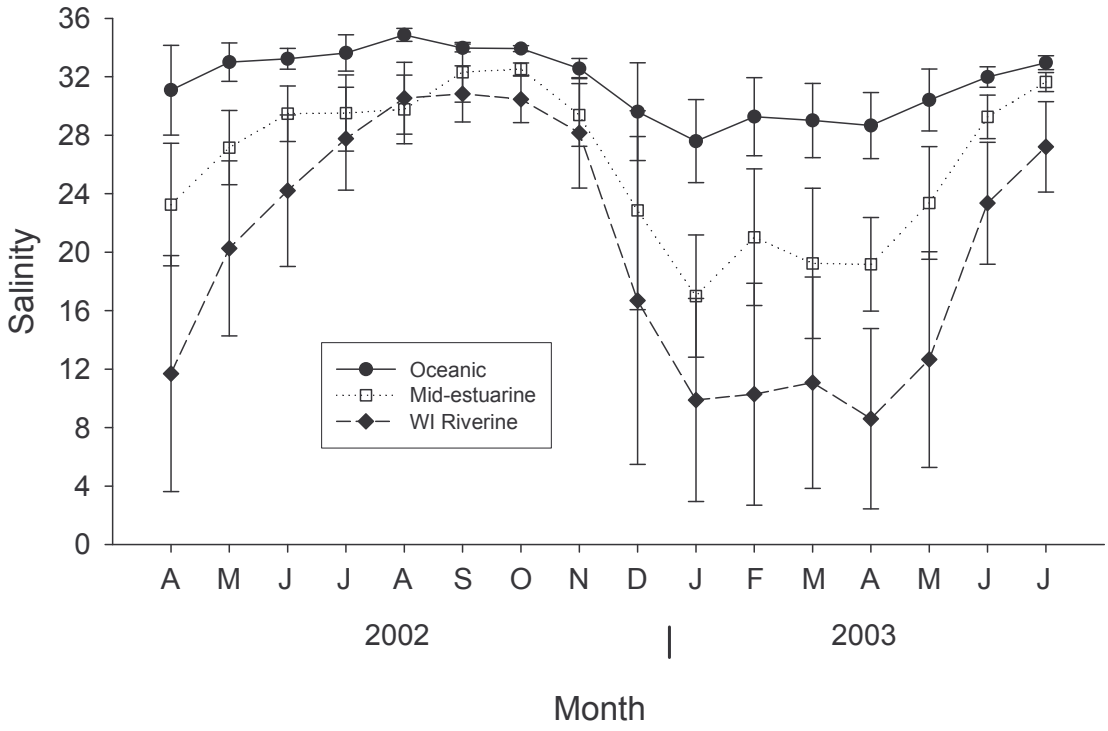


Figure 2. Mean monthly salinity profiles at the collection sites along an estuarine gradient. Error bars are ± 1 standard deviation. Data points represent salinity measured during submergence at the collection sites.

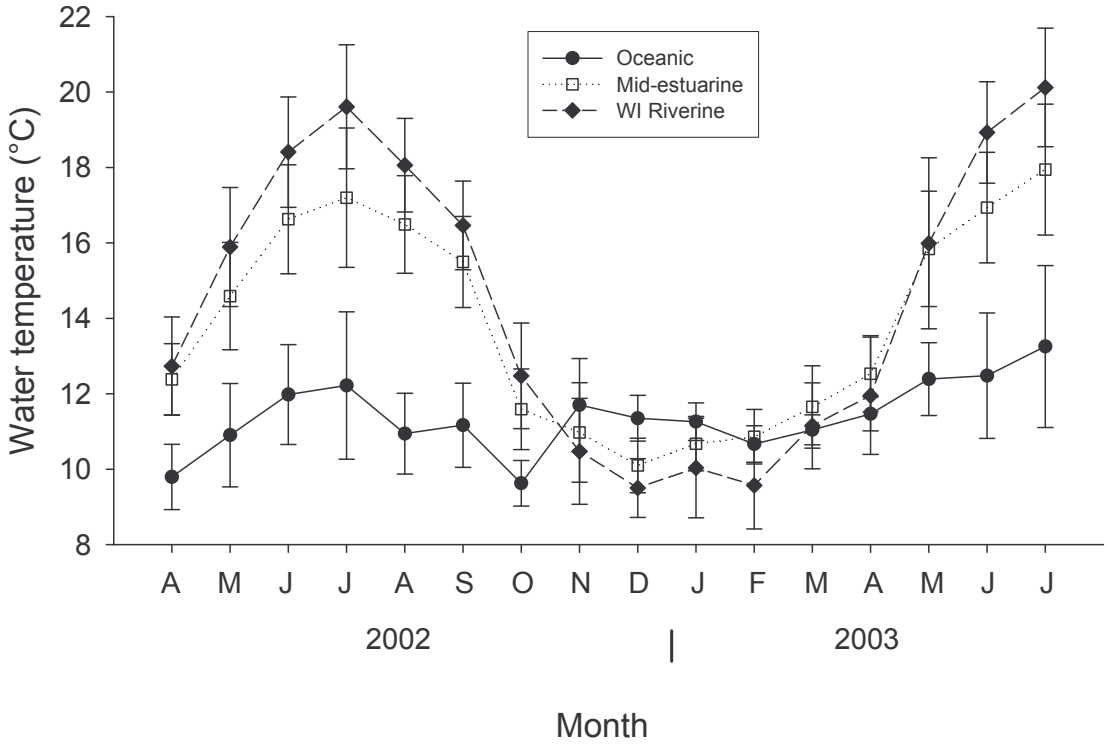


Figure 3. Mean monthly water temperature profiles at the collection sites along an estuarine gradient. Error bars are ± 1 standard deviation.

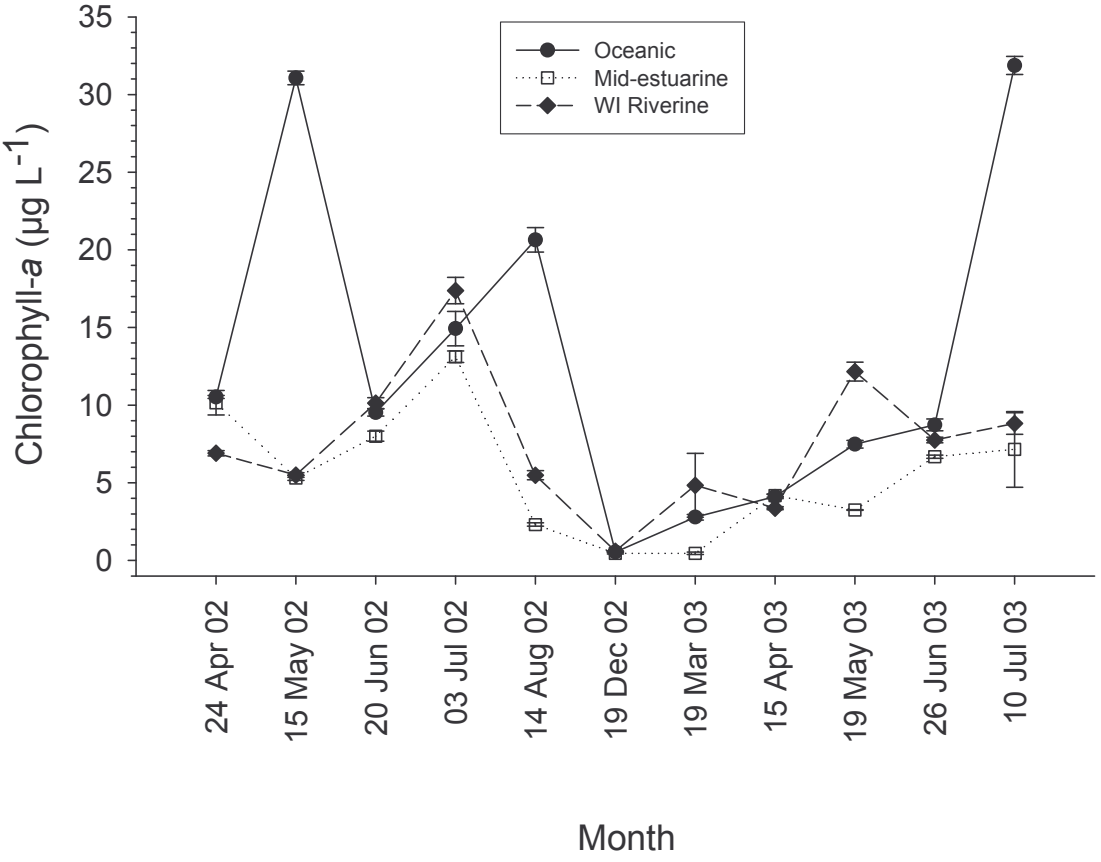


Figure 4. Surface chlorophyll-*a* concentration at three collection sites along an estuarine gradient. Data points represent a mean of three replicate samples; error bars are ± 1 standard error.

population had a relatively high percentage brooding throughout the study. The percent brooding at the mid-estuarine site decreased in April through August 2002, with a slight peak in September. No brooding was observed in November and December, followed by an increase to a peak in March 2003. Interannual variation in the percent brooding at the mid-estuarine site was observed between April – July 2002 and the same period in 2003. In 2002, the pattern of percent brooding at the mid-estuarine site was similar to the pattern observed for the WI riverine site, while in 2003 the percent brooding was similar to the oceanic site. At the WI riverine site, three successively smaller peaks in brooding were observed during May, July, and September 2002, respectively. Less than five percent of the population was brooding at the riverine site between October 2002 – January 2003, followed by a peak in February and March 2003. The percent brooding at the riverine site steadily decreased to less than five percent in July 2003. There was no obvious difference overall between the WI and SE riverine sites.

Overall, location in the estuary had a significant effect on the percent of the population with sperm filled seminal vesicles when pooled across the duration of this study ($\chi^2 = 147.04$, df = 2, $p < 0.001$). The percent with filled seminal vesicles was significantly higher at the oceanic site (92 %) than the mid-estuarine site (75 %) ($q_{0.5, \infty, 3} = 25.71$, $p < 0.001$) and the percent with filled seminal vesicles was significantly lower at the WI riverine site (67 %) than the mid-estuarine site ($q_{0.05, \infty, 3} = 10.35$, $p < 0.001$).

Seasonal (i.e., dry and rainy) effects were also observed in the percent of the population with sperm filled seminal vesicles. During the dry season, more *B. glandula* had sperm filled seminal vesicles at the oceanic site (90 %) than the mid-estuarine site

(63 %) ($q_{0.05, \infty, 3} = 36.38$, $p < 0.001$) and the WI riverine site (61 %) ($q_{0.05, \infty, 3} = 39.24$, $p < 0.001$) (Fig. 5B). The mid-estuarine and riverine sites were not significantly different during the dry season ($q_{0.05, \infty, 3} = 2.93$, $p > 0.05$). During the rainy season, more *B. glandula* had sperm filled seminal vesicles at the oceanic site (93 %) than the mid-estuarine site (89 %) ($q_{0.05, \infty, 3} = 3.99$, $p < 0.025$) and more had sperm filled seminal vesicles at the mid-estuarine site than the WI riverine site (80 %) ($q_{0.05, \infty, 3} = 6.11$, $p < 0.001$) (Fig. 5B). There was no obvious difference between the WI and SE riverine sites.

Location in the estuary had a significant effect on the percent of the *B. glandula* population with yolky ovaries when pooled across the duration of this study (Fig. 5C) ($\chi^2 = 433.20$, df = 2, $p < 0.001$). The percent of the population with ripe ovaries decreased along the estuarine gradient as distance from the oceanic inlet increased. The percent with yolky ovaries was significantly higher at the oceanic site (65 %) than the mid-estuarine site (28 %) ($q_{0.5, \infty, 3} = 41.93$, $p < 0.001$), which was significantly higher than the WI riverine site (16 %) ($q_{0.05, \infty, 3} = 16.63$, $p < 0.001$).

The seasonal pattern during the dry season was similar to the pooled pattern with the higher percentage of ripe ovaries at the oceanic site (86 %), than the mid-estuarine site (43 %) ($q_{0.5, \infty, 3} = 53.38$, $p < 0.001$) and a higher percentage of ripe ovaries at the mid-estuarine than the WI riverine site (18 %) ($q_{0.5, \infty, 3} = 85.02$, $p < 0.001$). The pattern during the rainy season was similar to the dry season with a higher percentage of ripe ovaries at the oceanic site (36 %), than the mid-estuarine site (21 %) ($q_{0.5, \infty, 3} = 8,02$, $p < 0.001$) and a higher percentage of ripe ovaries at the mid-estuarine than the WI riverine site (12 %) ($q_{0.5, \infty, 3} = 6.17$, $p < 0.001$).

A cyclic trend in the percent of the population with ripe ovaries was observed at all sites; however, as previously mentioned the magnitude did vary between sites. At the oceanic site, a steady increase in the percent with ripe ovaries occurred from April – October 2002. From October 2002 - March 2003, the percent with ripe ovaries decreased, and then increased steadily until July 2003. A pattern similar to the oceanic site was observed at the mid-estuarine site, however, after decreasing to less than five percent in February 2003, the percent with ripe ovaries never increased over 15 percent between February – July 2003. A cyclic pattern was observed at the WI riverine site with an increase from March – July 2002, a peak during August – December 2002, then a decrease to below ten percent from January – March 2003, followed by an increase in April. There was no obvious difference between the WI and SE riverine sites.

Of the environmental variables measured, only water temperature was significantly correlated with percent brooding. Water temperature significantly explained 14 percent of the variation in percent brooding (Linear regression, $t = -2.63$, $p = 0.012$, $R^2 = 0.138$). When the water temperature exceeded 13 °C, the percent brooding was less than 25 percent, with the exception of one datum point (Fig. 6A). Neither salinity (Linear regression, $t = -0.33$, $p = 0.74$, $R^2 = 0.003$) (Fig. 6B) nor chlorophyll-*a* (Linear regression, $t = 0.27$, $p = 0.79$, $R^2 = 0.003$) (Fig. 6C) significantly explained variation in percent brooding.

The mass-specific fecundity data were initially analyzed as a function of collection site and collection month (Fig. 7, Table 1). The effect of site on mass-specific fecundity was significant when the oceanic site was compared to the mid-estuarine site

and when the oceanic site was compared to the WI riverine site; however, there was no effect of site between the mid-estuarine and riverine site. The only significant effect of collection time was observed from the comparison between the oceanic and WI riverine sites. A significant collection site by time interaction was observed from the comparison between the oceanic and WI riverine sites (Table 1). Interestingly, if the two-way ANOVA analysis is performed between the oceanic and riverine sites with the April 2003 data excluded, the collection site by time interaction is no longer significant following a Bonferoni correction for multiple tests (ANOVA, $F_{1,7\ \alpha = 0.017} = 2.27$, $p = 0.030$). A graphical analysis of the mass-specific fecundity data did not bring to light any obvious interaction patterns (Fig. 7). Although sample size is small in some months, mass-specific fecundity data was collected from most of the sites throughout the sampling period. Therefore, any concern about introducing an artifact due to a site by time interaction should be alleviated.

When samples were pooled across sample date from April 2002 to April 2003, location in the estuary had a significant effect on the fecundity of *B. glandula* (ANOVA, $F_{2,271} = 12.25$, $p = 0.000$). Specifically, mass-specific fecundity was approximately 28 percent higher at the oceanic site than the mid-estuarine (Tukey HSD, $p = 0.000$) and WI riverine sites (Tukey HSD, $p = 0.002$) (Fig. 8). There was no significant difference in mass-specific fecundity between the mid-estuarine and WI riverine sites (Tukey HSD, $p = 0.86$).

Figure 5. Monthly reproductive patterns of A) percent brooding, B) percent with filled seminal vesicles, and C) percent with yolky ovaries in *Balanus glandula* along an estuarine gradient. Each data point represents a sample size of 50 individuals.

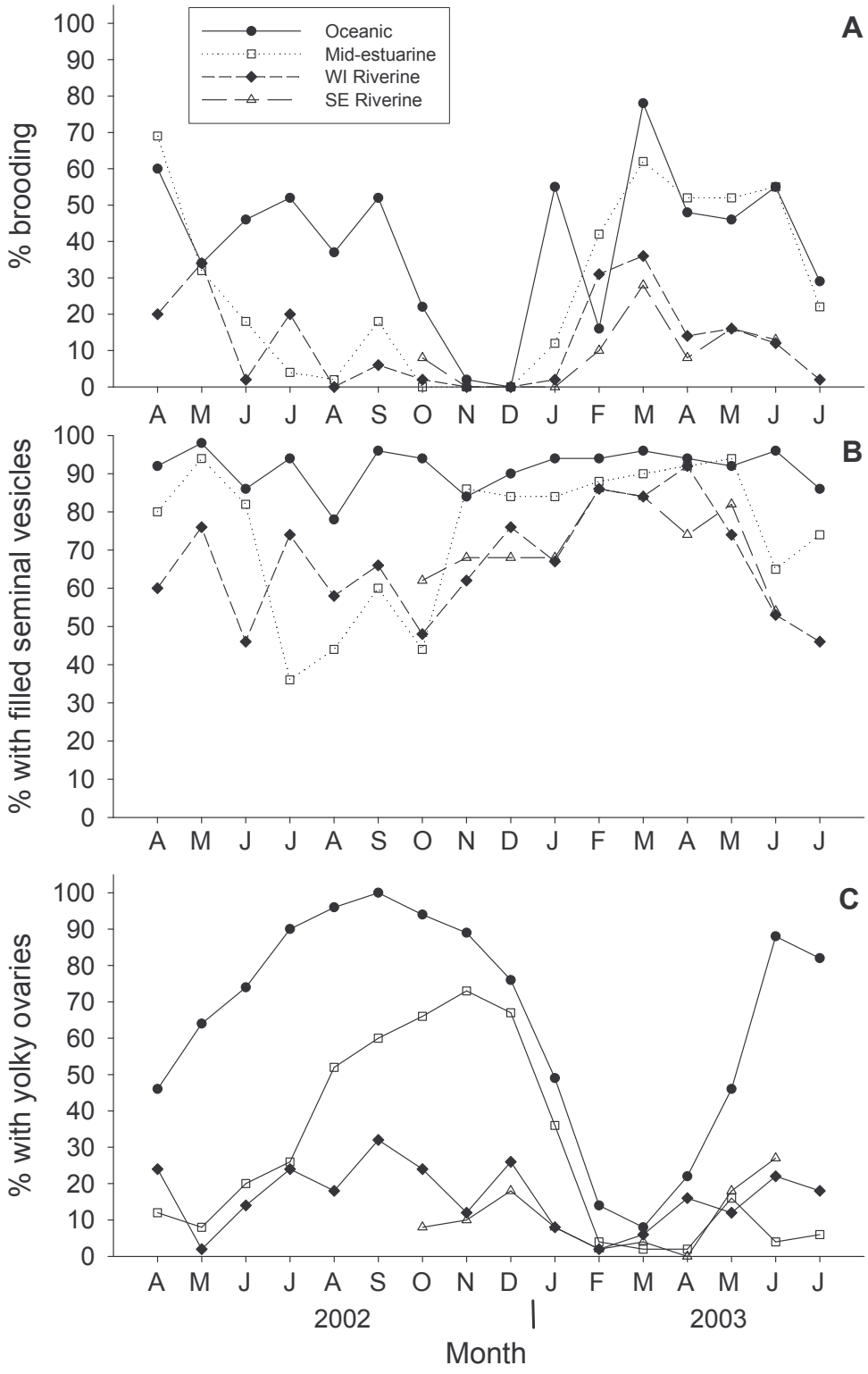
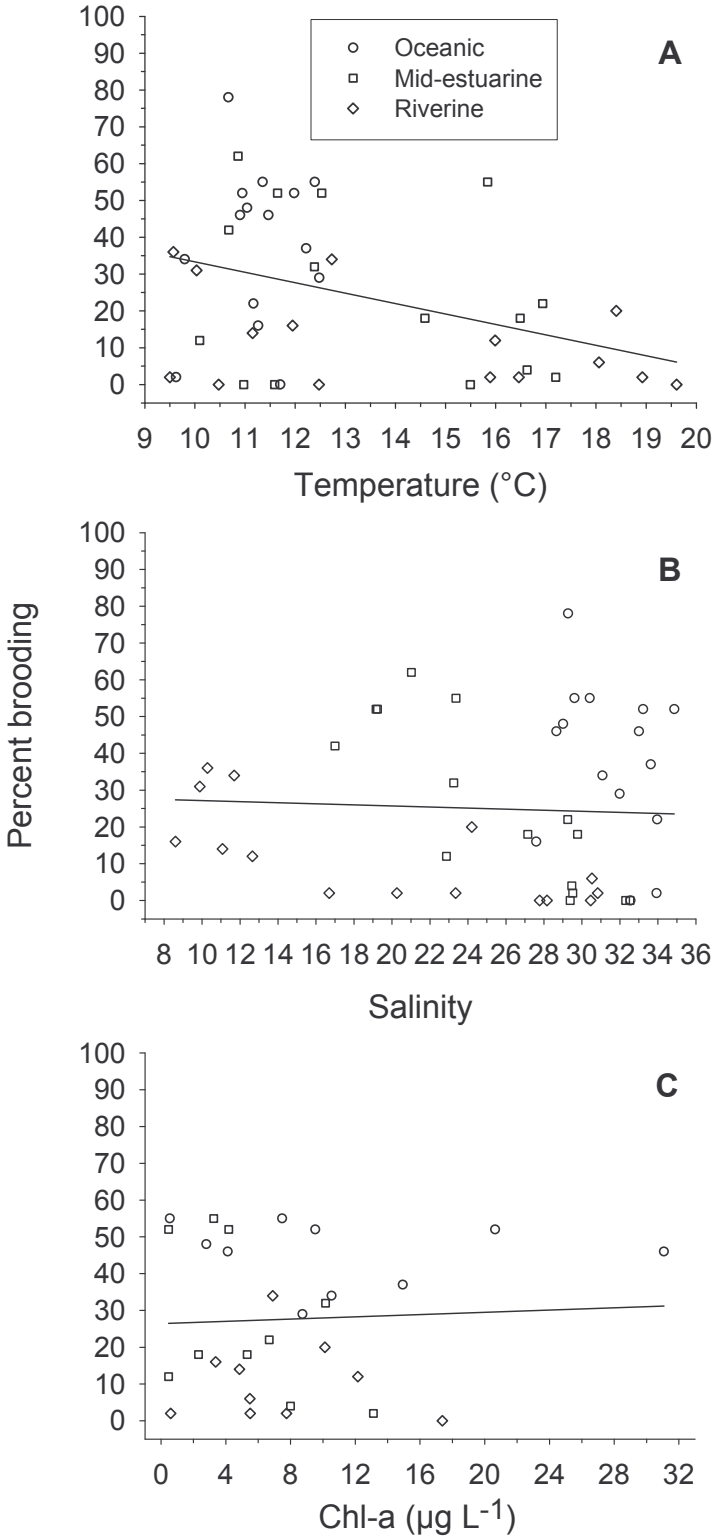

Oceanic
Mid-estuarine
WI Riverine
SE Riverine
A
B
C
% brooding
% with filled seminal vesicles
% with yolky ovaries
100
90
80
70
60
50
40
30
20
10
0
A M J J A S O N D J F M A M J J
2002
2003
Month

Figure 6. Relationship between percent brooding and A) mean monthly water temperature, B) mean monthly salinity, C) and chlorophyll-*a* concentrations in *Balanus glandula*. Temperature, salinity, and chlorophyll-*a* values were offset one month prior to the percent brooding. Lines represent the linear regression through all data points at all sites.


Oceanic
Mid-estuarine
Riverine
A
Temperature (°C)
B
Salinity
C
Chl-a (µg L-1)
Percent brooding

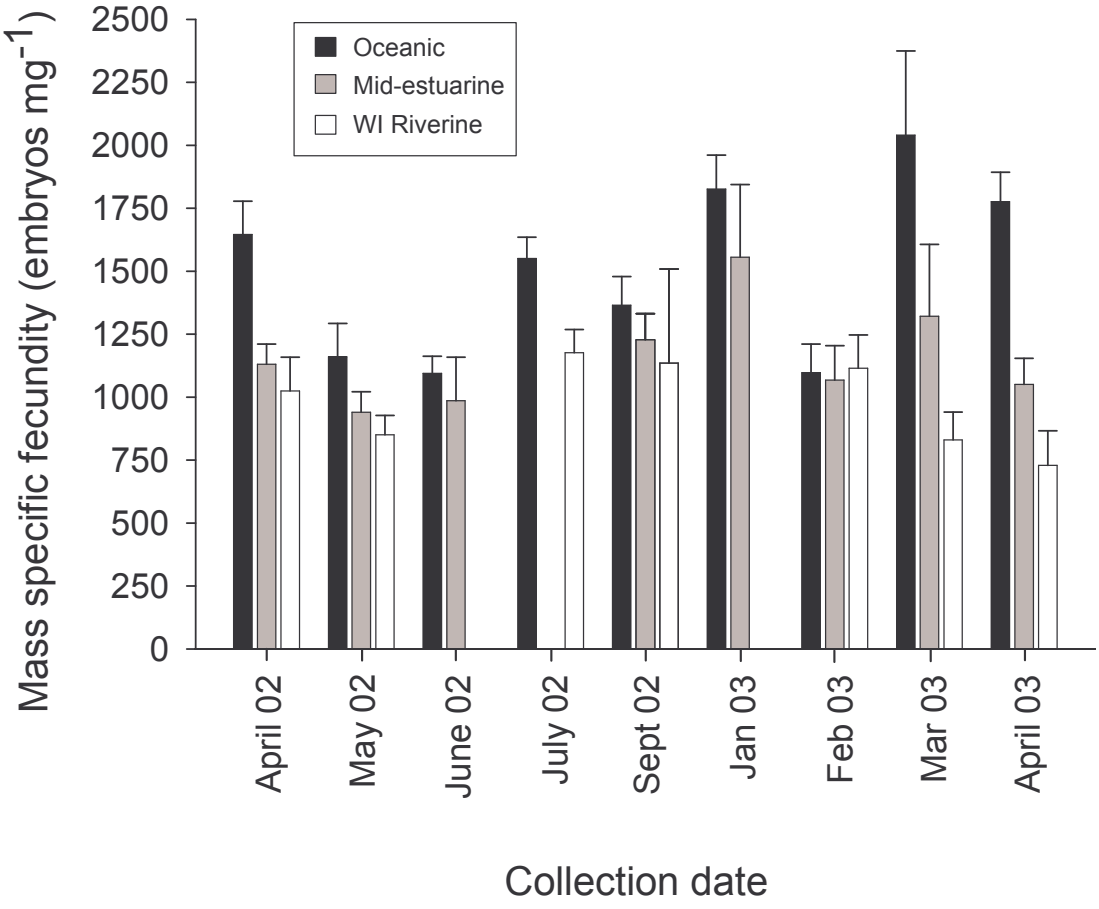


Figure 7. Mass-specific fecundity in *Balanus glandula* collected at three sites along an estuarine gradient. Each bar represents the mean of replicate samples; sample size varies depending on collection site and date (see methods section for an explanation). Error bars are 1 standard error. See Table 1 for statistics.

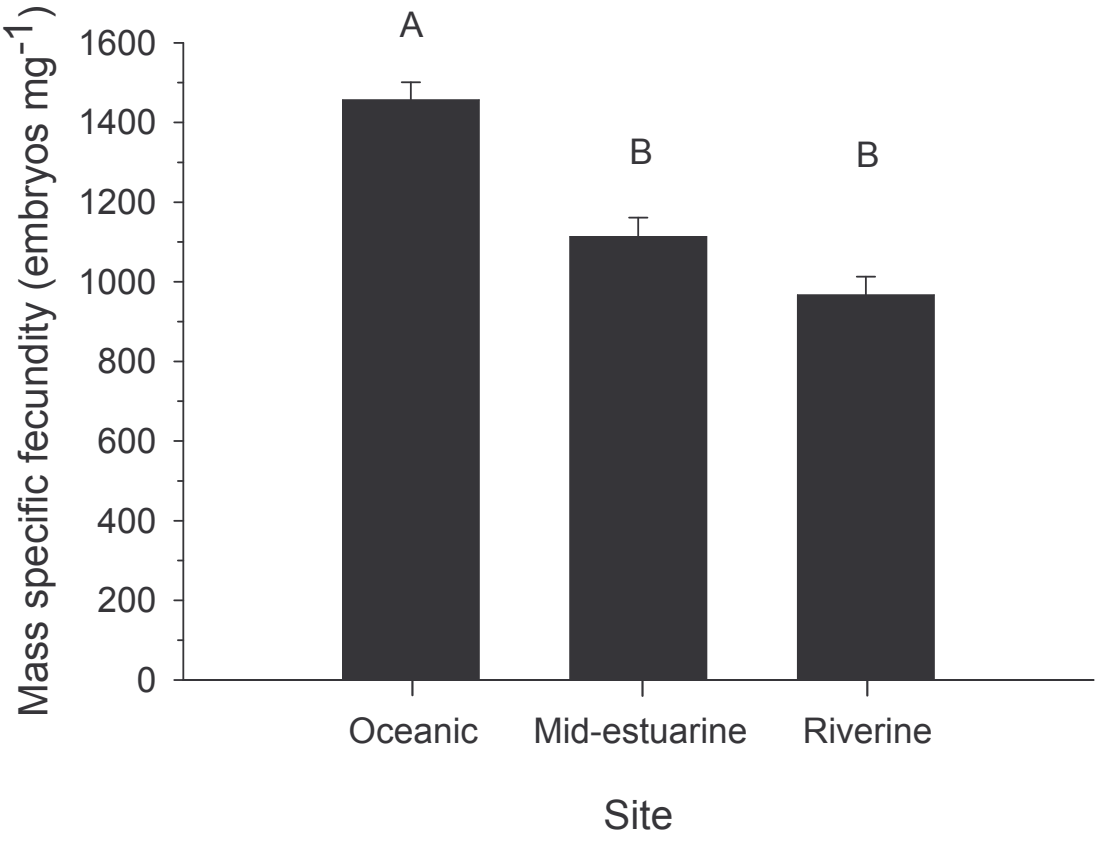


Figure 8. Mass-specific fecundity in *Balanus glandula* collected at three sites along an estuarine gradient. Samples were collected in April 2002 - April 2003 and then pooled together. Each bar represents the mean of replicate samples; n = 221 at the oceanic site; n = 119 at the mid-estuarine site; n = 68 at the WI riverine site. Error bars are 1 standard error. Different letters above bars represent significant differences from a Tukey post-hoc analysis ($p > 0.5$).

Table 1. Two-way ANOVA on the effect of collection site and date on mass-specific fecundity in *Balanus glandula*. A Bonferoni correction for multiple comparisons was used to adjust the significance level ($\alpha = 0.017$).

Source	ss	df	Mean-square	*F*-ratio	*P*
Oceanic vs. mid-estuarine					
Site	4.14	1	4.14	24.33	0.000
Date	5.20	8	0.65	3.83	0.000
Site x Date	2.64	8	0.33	1.94	0.054
Oceanic vs. WI riverine					
Site	3.51	1	3.51	23.20	0.000
Date	2.31	8	0.29	1.91	0.059
Site x Date	3.94	8	0.49	3.26	0.002
Mid-estuarine vs. WI riverine					
Site	0.04	1	0.04	0.21	0.649
Date	1.70	8	0.21	1.06	0.392
Site x Date	1.43	8	0.18	0.89	0.527

Reproductive output

An index of reproductive output over the period from April 2002 to April 2003 was calculated for the oceanic, mid-estuarine, WI riverine sites. Reproductive output was calculated from the product of the mass-specific fecundity (number of embryos mg^{-1} dry body mass) and the estimated number of broods per year (Barnes and Barnes 1968, Hines 1978, Page 1984) (Table 2). The number of broods per year was estimated from the equation $y = f(t/d)$, where y is the estimated number of broods, f is the proportion of the population with broods (oceanic = 0.39; mid-estuarine = 0.24; WI riverine = 0.13), t is the time period being examined (395 days), and d is the development time for a brood (Hilgard 1960, Lewis and Chia 1981, Page 1984). An estimate of 30 days for a brood cycle was used (Hines 1978). Reproductive output decreased as distance from the oceanic inlet increased (Table 2). Reproductive output at the oceanic site was

approximately two times higher than the mid-estuarine site and approximately four times higher at the WI riverine site.

Table 2. Estuarine location, mass-specific fecundity (number of embryos individual^{-1} mg^{-1} dry body mass), estimated number of broods (see text), and reproductive output (product of fecundity and number of broods) for *Balanus glandula* during April 2002 to April 2003.

Estuarine location	Mass-specific fecundity	Estimated number of broods	Reproductive output
Oceanic	1457	5.1	7431
Mid-estuarine	1113	3.2	3562
WI riverine	966	1.7	1642

Embryo volume

The volume of embryos during the early stages of development varied significantly as a function of collection month (ANOVA, $F_{8,126} = 21.51$, $p = 0.000$). During spring 2002, embryo volume significantly decreased between April and May (Tukey HSD, $p = 0.045$) and embryo volume remained relatively lower throughout June, July, August, and September (Fig. 9). Embryo volume was significantly higher during the winter months of January and February 2003 than any of the other months sampled during this study. In comparison to embryo volume during winter, by April 2003 embryo volume had decreased to the volume observed during April 2002 (Tukey HSD, $p > 0.99$). Water temperature significantly explained only 10.5 percent of the variation in embryo volume (Linear regression, $t = -17.7$, $p = 0.00$, $R^2 = 0.105$).

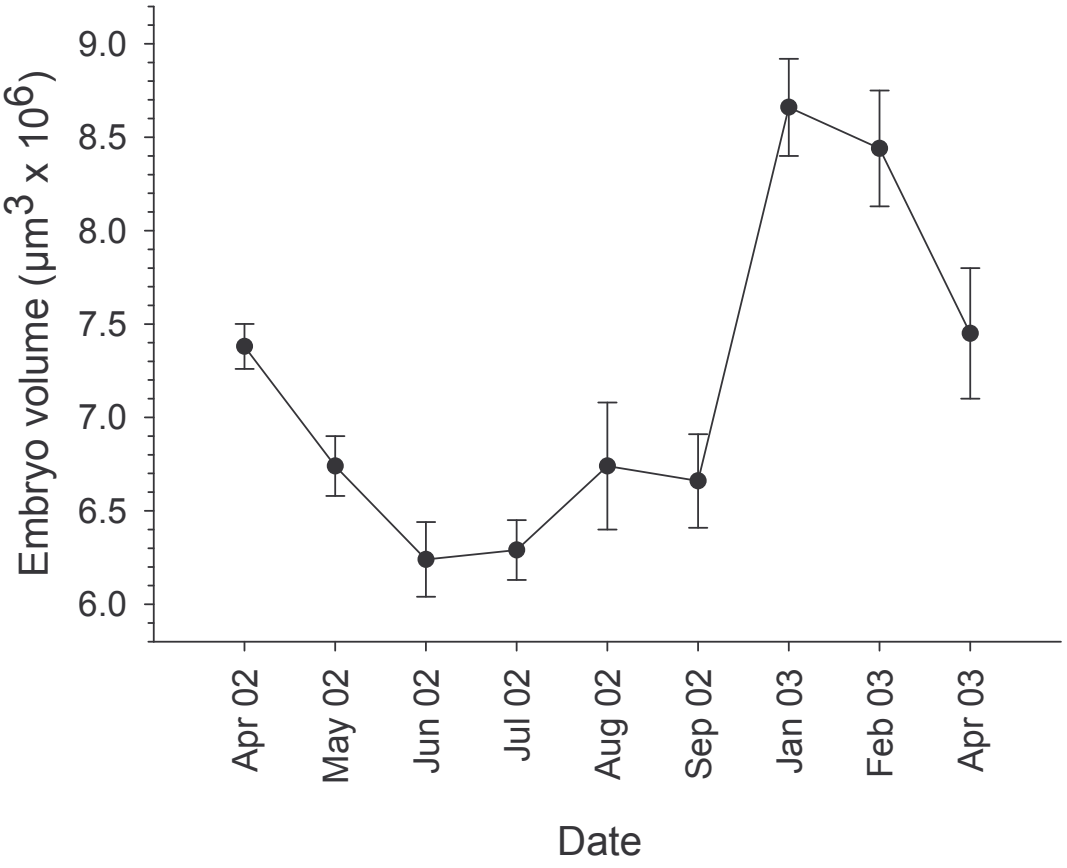


Figure 9. Seasonal changes in embryo volume in *Balanus glandula*. Samples were pooled across collection site. Error bars represent ± 1 standard error.

Discussion

This study investigated variation in the reproductive output of *Balanus glandula* distributed along an estuarine gradient. The reproductive output of a barnacle is affected by factors such as temperature, salinity, and food availability; these three factors all varied along the estuarine gradient. The reproductive output of *B. glandula* decreased as distance from the oceanic end of the estuary increased. During April 2002 to April 2003, reproductive output of *B. glandula* at the oceanic site was 2.1 times higher than the mid-estuarine site and 4.5 times higher than at the riverine site. Over a spatial scale of kilometers, the reproductive patterns of this population changed dramatically as a function of estuarine location.

In general, reproductive output decreases with environmental stress (Menge 1974, Bayne et al. 1978, Bayne at al. 1983, Fletcher 1984, Thompson 1984). For example, reproductive output was lower in a population of the barnacle *Pollicipes polymerus* exposed to fresh water runoff than a population without runoff exposure (Lewis and Chia 1980). Additionally, in the barnacles *P. polymerus* and *Chthamalus fissus*, the reproductive output increased as intertidal height decreased (Page 1984). As intertidal height increased, exposure to thermal and desiccation stress increased. These conditions along a vertical gradient are analogous to conditions along a horizontal gradient within an estuary. Physiological stress increased with distance from the oceanic end and reproductive output decreased in *B. glandula*.

The reproductive patterns of *B. glandula* along the estuarine gradient were examined, during the dry and rainy seasons. Reproduction varied with season. During the dry season, the percent brooding and percent with sperm filled seminal vesicles at the mid-estuarine site and WI riverine site were similar. However, during the rainy season, the percent brooding at the mid-estuarine site was similar to the oceanic site, and percent brooding at the oceanic and mid-estuarine sites were higher than at the WI riverine site. No seasonal effects were observed for the percent of the population with ripe ovaries.

Two arms of the South Slough Estuary were studied to determine if the patterns that occurred at one riverine site were similar to the patterns at another riverine site. Although some variation in the percent brooding at the two riverine sites occurred during the seasons examined, no obvious trends indicated that the percent brooding differed between the two riverine sites.

Which environmental factors may cause the observed variation in reproduction of *B. glandula* and why does reproductive output decrease along the estuarine gradient? Three potential factors that can affect reproduction, and vary along an estuarine gradient, are temperature, salinity, and food abundance (reviewed in Barnes 1989). I will examine these three factors in the context of published data to explore what may have caused the decrease in reproductive output along the South Slough estuarine gradient.

Water temperature did appear to play a role in the regulation of reproduction in *B. glandula*; as water temperature increased, the percent of the population brooding decreased. Interestingly, with the exception of one outlier, above 13 °C the percent brooding decreased to less than 25 percent. High temperatures can delay the timing of

reproduction or reduce the reproductive output in barnacles with a boreo-arctic to temperate distribution (Barnes 1963, Tighe-Ford 1967, Crisp and Patel 1969, Hines 1978, Page 1984). For example, a lower percentage of a population of *B. glandula* at a warm water outfall was brooding than in a population at a cool water site (Hines 1978). Further, in a laboratory experiment the percentage of individuals brooding was higher in *B. glandula* maintained at 11 °C than at 20 °C (Hines 1978). Unlike *Semibalanus balanoides*, which will not reproduce above approximately 10 °C (Tighe-Ford 1967), *B. glandula* has the potential to reproduce at higher temperatures, but at a reduced percentage of the population. Brooded embryos were observed in a southern population of *B. glandula* at La Jolla, California, during the winter months when the average monthly water temperature ranged between 14 and 16 °C (Barnes and Barnes 1956). Page (1983) attributed the decrease in reproduction in the barnacle *Pollicipes polymerus* above a critical temperature to a reallocation of resources due to an increase in metabolism. Potentially a similar increase of metabolism in *B. glandula* at higher temperatures (above 13 °C), coupled with physiological stress in an estuarine environment, could cause a reallocation of resources away from reproduction. Relatively warmer temperatures at the mid-estuarine site during the dry season than the rainy season may in part account for seasonal difference in the percent of the population brooding. Although there is no absolute certainty in assigning causality to temperature, an increase in water temperatures is correlated with a reduction in reproductive output (Fig. 6A).

There was no correlation between salinity and the percent of the population brooding. Published data on the effect of salinity on barnacle reproduction is limited. In one study, the percent of a population of the barnacle *Elminius modestus* brooding was similar in a marine site and a site with low salinity (O'Riordan and Murphy 2000). Peak reproduction in *B. glandula* at the riverine site occurred during February and March, when salinity was the low. Low salinity may reduce reproductive output by reducing the amount of time available for feeding. When the salinity is below 8, *B. glandula* will close the opercular valves and stop feeding (Barnes and Barnes 1957, Newman 1967, Bergen 1968). If the time available for feeding is reduced, nutrient storage and reproductive output may be affected.

Based on the limited chlorophyll-*a* data collected in this study, there was not a significant relationship between the percent of the populations brooding and chlorophyll-*a* concentrations, despite higher levels at the oceanic site on occasion. Due to limited data, the lack of a significant correlation between chlorophyll-*a* and percent brooding should be interpreted with caution. Many studies on barnacle reproduction suggest that food availability is an important factor regulating reproductive output (Patel and Crisp 1960, Barnes and Barnes 1967, Barnes and Barnes 1968, Barnes and Barnes 1975, Page 1984; however see Cimberg 1981). For example, on the Oregon coast, the reproductive output of *B. glandula* was higher at sites with higher than lower primary productivity (Leslie et al., in preparation). Therefore, the *a priori* expectation would be for food availability to be a limiting factor in reproduction. In the South Slough Estuary, Roegner and Shanks (2001) demonstrated that ocean water entering the estuary is generally higher

in chlorophyll-*a* than estuary water. Therefore, higher reproductive output at the oceanic site may in part be due to more abundant food. In addition, a decrease in the percent of ripe ovarian tissue along the estuarine gradient suggested that food may have been a limiting factor as distance from the oceanic end increased. When food is abundant ovarian tissues are built up (Barnes et al. 1963, Barnes and Barnes 1967, Wu and Leavings 1978). Hines (1978) estimated that ovarian tissue in *B. glandula* stored enough yolky material for at least three broods. The lower percent of the population with ripe ovarian tissue at the riverine site than the oceanic site suggested that less food was available at the riverine end of the estuary.

The phytoplankton particle size and quality is another issue that should be addressed. Measurements of chlorophyll-*a* are an integration of all phytoplankton species, with no differentiation of particle size above the pore size of the filter used. Perhaps chlorophyll-*a* did not provide an accurate measure of food availability. The phytoplankton species assemblage changes along the South Slough estuary (Hughes 1997). Centric diatoms are more abundant at the oceanic end, while pennate diatoms and cryptomonads are more abundant at the riverine end of the estuary. Possibly variation in the food source along the estuarine gradient may influence reproductive output of *B. glandula*.

Embryo volume

Balanus glandula embryo size varied during April 2002 to April 2003. Embryo size in a number of barnacle species varied as a function of latitude and more importantly

as a function of temperature (Patel and Crisp 1960, Barnes and Barnes 1965, Barnes and Barnes 1968, Crisp 1987). Patel and Crisp (1960) demonstrated that barnacle embryo size decreased as temperature increased. As temperature increased, the embryonic metabolic rate increased leading to a higher consumption of energy reserves and decreased embryo volume (Crisp 1987, Lucas and Crisp 1987). During late spring and summer, water temperature was higher at the mid-estuarine and riverine sites than the oceanic site (Fig. 3). Higher water temperatures should have increased the metabolic rate of developing embryos and consequently a reduction in embryo size should have been observed. However, no site-specific differences were observed and since only ten percent of the variance in embryo size was explained by temperature, it seems apparent that other factors were involved in regulating embryo size. Air temperature may have potentially played a role in embryo size. The air temperature is warmer in the summer and cooler in the winter. However, without air temperature data, the role of air temperature can not be explored further.

There does appear to be a relationship between successive broods and embryo size; starting in January, embryo size decreased after each brood. However, if embryo size was decreasing, the expectation would be to maximize the reproductive output by increasing the number of embryos (Stearns 1992). An obvious seasonal change in number of brooded embryos was not observed; however, due to small sample sizes, variation in number of embryos over time may not have been resolvable. Further work should examine the relationship between the number of brooded embryos and embryo size. Although no differences in embryo size were detected along the estuarine gradient,

future work should also examine the energy content of the developing embryos to determine if embryo quality varies along an estuarine gradient.

Conclusions

In summary, dramatic differences in the reproductive output of *B. glandula* along an estuarine gradient were observed; potential contributions to the larval pool were not equal from populations living along an environmental gradient. It is not known whether the quality of larvae produced from each site was equivalent. Future work should examine larval quality as a function of estuarine location. The site-specific inequality in reproductive output was driven by site-specific environmental conditions. The most likely factors causing variation in reproductive output were temperature and food availability.

BRIDGE II

Chapters I and II examine the life-history parameters of juvenile survivorship and growth, and adult reproductive output along a horizontal estuarine gradient in *Balanus glandula*. These life-history parameters are direct measures of relative performance from sites along a horizontal gradient that experience different environmental conditions. These measures indicate an end result of exposure to habit-specific conditions, but they do not indicate the current physiological condition of an organism. Chapter IV examines the physiological response of *Balanus glandula* to thermal stress in a series of controlled laboratory experiments and examines specimens collected from the field.

Chapter IV

THE HEAT-SHOCK RESPONSE OF THE BARNACLE *BALANUS GLANDULA* TO THERMAL STRESS

Introduction

Intertidal organisms inhabit an interface between an aquatic and a terrestrial habitat. These organisms are exposed to extreme physical conditions during low tides (Newell 1979, Lewis 1978). Body temperature of intertidal organisms can exceed the temperature of the surrounding air and on a regular basis approach sub-lethal limits (Helmuth 1999, Helmuth and Hofmann 2001, Tomanek and Sanford 2003). For example, intertidal porcelain crabs exist right on the boundary of their physiological limits to thermal stress (Stillman and Somero 2000). In addition, exposure to sub-lethal temperature can intensify the deleterious effects of desiccation due to aerial emersion (Wolcott 1973, Gosselin and Chia 1995). One specific physiological adaptation to residing in a stressful habitat, like the intertidal, is the upregulation of molecular chaperones (Feder and Hofmann 1999, Hochachka and Somero 2002).

In response to environmental stress, molecular chaperones, such as heat-shock proteins, are expressed by organisms. Potentially any type of stress that results in protein denaturation, including thermal, desiccation, osmotic, and hypoxic stress, can induce the expression of molecular chaperones (Lindquist 1986, Feder and Hofmann 1999). It

should be noted that organisms maintain constitutive background levels of molecular chaperones as well. Molecular chaperones rescue and prevent the aggregation of damaged proteins, thereby helping to conserve the pool of existing proteins from irreversible damage (Parsell and Lindquist 1993, Buchner 1996, Fink 1999). When a protein is irreversibly damaged, ubiquitin, a low molecular weight protein, binds to the damaged protein, marking it for degradation by cytoplasmic proteases (Hershko and Ciechanover 1992, Hochstrasser 1995). Although energetically expensive, the expression of molecular chaperones can be viewed as an adaptation to maintain existing protein pools during periods of acute and chronic stress (Somero 2002, Hofmann et al. 2002).

Since the early 1990's, ecological physiologists have been examining the variation of molecular chaperone expression and induction in intertidal organisms (reviewed in Feder and Hofmann 1999, Hofmann et al. 2002). The term heat-shock protein (hsp) refers to a large grouping of molecular chaperones that function in normal cellular processes and are also upregulated during periods of stress (Morimoto et al. 1994). The quantification of heat-shock protein expression has become a molecular measure to examine the physiological response of an organism to stress in an ecological context. When exposed to elevated temperatures, invertebrates physiologically respond by upregulating the expression of heat-shock proteins. The upregulation of heat-shock proteins can vary in magnitude of the response, threshold of induction, and the range over which induction occurs; none are mutually exclusive (Feder 1999). Differential expression of heat-shock proteins have been observed in the intertidal as a function of vertical height (Hofmann and Somero 1995, Roberts et al. 1997, Halpin et al. 2002),

shading (Tomanek and Sanford 2003), and substrate angle (Helmuth and Hofmann 2001). The premise is that as habitat conditions become more stressful from longer aerial exposure or increased solar irradiation, the magnitude of hsp expression increases. Although elevated levels of heat-shock proteins suggest an attempt to remediate thermally induced protein denaturation, some irreversible protein damage can occur. In studies measuring the level of ubiquitin conjugate, the amount of irreversibly damaged proteins increases as the level of stress increases in both field and laboratory studies (Hofmann and Somero 1996, Buckley et al. 2001, Halpin et al. 2002).

The heat-shock response is apparently plastic and can be adjusted based on the past experience of an organism. Gene expression and subsequent patterns of protein expression display a level of plasticity in response to environmental variables (Pigliucci 1996, 2001). For example, the induction temperature, at which hsp expression is turned on, can be adjusted by long term seasonal acclimatization or short term acclimation in some species (reviewed in Hofmann et al. 2002). The induction temperature is higher for summer versus winter acclimatized mussels and fish (Dietz and Somero 1992, Roberts et al. 1997, Buckley et al. 2001). In addition, endogenous levels of constitutive and induced isoforms of hsp70 in *Mytilus* spp. are higher in warmer months and lower in cooler months (Roberts et al. 1997, Chapple et al. 1998). Laboratory acclimation to elevated temperatures will also raise the induction temperature of heat-shock proteins (Dietz 1994, Buckley and Hofmann 2002, Tomanek and Somero 1999). A plastic heat-shock response may be an adaptation to the physically variable environment of the intertidal zone.

In recent years, a series of influential studies have been published on the physiological response of rocky intertidal organisms to ecologically relevant environmental stress (Sanders et al. 1991, Sharp et al. 1994, Hofmann and Somero 1995, Tomanek and Somero 1999, Tomanek and Sanford 2003). Most of this research has focused on the response of molluscs and little attention has been paid to other abundant intertidal organisms. For example, no studies have investigated the heat-shock response of the barnacle *Balanus glandula*, a ubiquitous and important component of the rocky intertidal zone. *Balanus glandula* is an ideal study organism because it is sessile, very abundant, and has a broad distribution that includes mid and high intertidal locations (Newell 1979, Morris et al. 1980). Examining organisms from multiple taxonomic groupings is necessary to gain a complete understanding of the physiological response to ecologically relevant stress in intertidal habitats.

This study investigated the heat-shock response of *Balanus glandula* to thermal stress, by addressing the following questions: (1) Does *B. glandula* display a heat-shock response and if so, at what temperature does *B. glandula* begin to synthesize heat-shock proteins in response to thermal stress? (2) Do measurements of endogenous and induced hsp70 measure a similar heat-shock response in *B. glandula*? (3) Do specimens experiencing different thermal environments in the intertidal zone display evidence of physiological stress? (4) Does the molt-cycle affect the heat-shock response? (5) Is the heat-shock response of *B. glandula* plastic with respect to thermal acclimation? (6) Does the heat-shock response vary between summer and winter?

Methods

Collection site

Unless otherwise noted, adult specimens of *Balanus glandula* were collected from the intertidal zone at the mouth of the South Slough Estuary in Charleston, Oregon, USA (43° 20.4′ N, 124° 19.4′ W). The barnacles were carefully removed from pilings under the Charleston bridge or collected from an adjacent cobble field.

Endogenous hsp70 analysis (Western-blot immunochemical assay)

Tissue preparation and Western blot analysis of heat-shock protein (hsp70) was modified from methods outlined by Hofmann and Somero (1995). Samples were homogenized in 70 µl of buffer containing 50 mmol L^{-1} Tris- HCL, pH 7.6, 2 mmol L^{-1} EDTA, 5 mmol L^{-1} iodoacetamide, and 1 mmol L^{-1} phenylmethylsulfonylfluoride (PMSF). The samples were centrifuged at 16,000 *g* for 10 minutes. An aliquot was removed from the centrifuged supernatant to determine the protein concentration using a Coomassie Blue protein assay (BioRad). The remaining supernatant was diluted 1:1 (v/v) with SDS sample buffer (50 mmol L^{-1} Tris- HCL, pH 6.8, 10 % glycerol, 2 % 2-mercaptoethanol, and 10 % SDS), heated to 100 °C for five minutes, and centrifuged at 16,000 *g* for 30 seconds. An aliquot of the homogenate was stored at -80 °C for ubiquitin conjugate analysis. In preparation for gel electrophoresis, the supernatant that was not frozen was diluted 1:1 with Laemmli sample buffer (62.5 mmol L^{-1} Tris-HCL, pH 6.8, 10 % glycerol, 5 % 2- mercaptoethanol, 2 % SDS, and 0.1 % Bromophenol Blue) and heated for three minutes at 100 °C. Protein was electrophoretically separated by loading 10 µg

of total protein per sample on a 7.5 % SDS-polyacrylamide gel. A preliminary experiment indicated that 10 μg of total protein was within the linear range of the detection procedure. In addition, a positive control of known concentration was loaded on each gel. Following electrophoresis, proteins were transferred to a nitrocellulose membrane (0.45 μm, Schleicher & Schuell) with a Mini Trans-Blot Cell (BioRad). The transfer buffer consisted of 25 mmol L^{-1} Tris base, 192 mmol L^{-1} glycine, and 20 % methanol. The blot containing the transferred proteins was dried in an oven at 50 °C for 20 minutes.

For the Western blot analysis, the membrane was blocked for one hour in phosphate buffered saline (PBS) containing 5 % nonfat dried milk and then rinsed three times in PBS with 0.1 % Tween-20. The blot was incubated for 1.5 hours in a primary antibody solution (primary antibody (anti-hsp70, rat monoclonal, Affinity Bioreagents MA3-001) diluted 1:2,500 in a solution of PBS with 2 % nonfat dried milk, 20 % fetal calf serum, 0.02 % thimerosal, and 1 mmol L^{-1} PMSF), washed three times in PBS with 0.1 % Tween-20, and then incubated for 30 minutes with a secondary antibody (rabbit anti-rat, Vector AI-4000) diluted 1:2,000 in blocking solution. The blot was then washed three times in PBS with 0.1 % Tween-20, incubated for one hour with protein-A horseradish peroxidase conjugate (BioRad, 170-6522) diluted 1:5,000 in blocking solution, and washed three times in PBS with 0.3 % Tween-20; followed by washing three times in PBS with 0.1 % Tween-20. To visualize the proteins, an enhanced chemiluminescence detection method (Amersham, ECL reagents) was used, followed by exposing the membrane to X-ray (Kodax X-OMAT) film for 5 – 30 seconds. The

exposed X-ray film was scanned and the optical density of each visualized band was quantified using Gel-Pro Analyzer software (Media Cybernetics, 2000). When multiple gels were compared within an experiment, all bands were normalized to the positive control on each gel so relative comparisons could be made.

Ubiquitin conjugate analysis

Ubiquitin conjugate detection was modified from the methods of Hofmann and Somero (1995). Previously homogenized samples were thawed and diluted to a concentration of 5 μg ml^{-1} in a saline solution (150 mmol L^{-1} NaCl with 0.01 % SDS). Samples and a positive control were loaded in triplicate 100 μl volumes onto a presoaked nitrocellulose membrane (0.45 μm, Schleicher & Schuell) in a dot blot vacuum apparatus (BioRad) and allowed to gravity feed through the membrane. Each well was washed three times with 500 μl of Tris-buffered saline (TBS) under light vacuum, and then the membrane was dried at 50 °C for 20 minutes.

The immunochemical assay consisted of blocking the membrane for one hour in PBS with 5 % nonfat dried milk, incubating for 1.5 hours with a polyclonal rabbit anti-ubiquitin conjugate primary antibody (provided by Dr. Gretchen Hofmann) diluted 1:2,500 in blocking solution, and then incubating for one hour with protein-A horseradish peroxidase conjugate (Vector Industries, PI-1000) diluted 1:5,000 in blocking solution. Between incubations, the membrane was washed three times in PBS with 0.1 % Tween-20; in addition, a triplicate wash in PBS with 0.3 % Tween-20 was performed following

final incubation. Visual detection and analysis was identical to methods previously outlined for hsp70 Western blotting.

Metabolic labeling induction assay

The two right and two left depressor muscles (*e.g.* scutorum rostralis and scutorum lateralis) were dissected in a cold room at 10 °C from *B. glandula* specimens with a basal diameter greater than 15 mm (see Hoyle and Smyth 1963). Immediately following dissection, the muscle fibers were placed in a barnacle "Ringer's" solution consisting of 486 mmol L^{-1} NaCl, 20 mmol L^{-1} $CaCl_2$, 12 mmol L^{-1} $MgCl_2$, 10 mmol L^{-1} $NaHCO_3$, 10 mmol L^{-1} glucose, and 8 mmol L^{-1} KCl (modified from Hoyle and Smyth 1963). The depressor muscle bundles were assigned to one of four temperature treatments of 10, 23, 28, or 33 °C in a temperature controlled chamber for 2.5 hours. This dissection procedure allowed the exposure of one individual to four temperatures. Following the thermal stress period, muscle tissue was incubated in 500 µl of Ringer's solution with 50 µCi of ^{35}S labeled amino acid mixture (Perkins Elmer, NEG-772) at 10 °C for 4 hours. After incubation, samples were washed three times in Ringer's solution, frozen with liquid nitrogen, and stored at -20 °C for two weeks.

Frozen samples were homogenized in 30 µl of buffer containing 32 mmol L^{-1} Tris- HCL, pH 6.8, 2 % SDS, and 1 mmol L^{-1} PMSF. Samples were heated at 100 °C for five minutes and then centrifuged at 16,000 *g* for ten minutes. To determine the total protein concentration labeled with ^{35}S, 3 µl of homogenate were pipetted onto GF/A filters (Whatman) and allowed to dry. Filters were washed with 10 % trichloroacetic acid

(TCA) for ten minutes, and then washed twice in 5 % TCA, followed by an ethanol rinse. Filters were dried at room temperature and incorporated ^{35}S was measured in a scintillation counter.

For electrophoresis, the samples were thawed, diluted 1:1 with Laemmli sample buffer, and then heated for three minutes at 100 °C. Each sample was loaded onto a 7.5 % SDS-polyacrylamide gel using an equal amount of protein that incorporated the ^{35}S label. The concentration of labeled protein loaded into the gels varied between experiments, but never within an experiment; typically 60,000 counts minute^{-1} were loaded, but in a few cases it was necessary to load 40,000 or 50,000 counts minute^{-1}. After electrophoresis, the gels were fixed for one hour in a solution containing 60 % distilled water, 30 % methanol, and 10 % acetic acid. Gels were then dried under vacuum and exposed to X-ray film (Kodak X-OMAT) with an intensifying screen (Kodak BioMax Transcreen LE) for 12 hours (60,000 counts minute^{-1}) at -80 °C. The lower counts minute^{-1}was compensated for by increasing the X-ray film exposure time to 14.5 hours (50,000 counts minute^{-1}) and to 18 hours (40,000 counts minute^{-1}). The exposed film was scanned and a recognizable band in the 70 kDalton and 90 kDalton regions of each gel was quantified using Gel-Pro Analyzer software (Media Cybernetics, 2000). The proteins (heat-shock proteins) quantified in this series of experiments will be referred to by the range of their molecular mass (e.g., proteins in the 70 – 80 kDalton region are referred to as hsp70).

Laboratory experimental procedures to manipulate endogenous hsp70 and ubiquitin conjugate levels

A series of three laboratory experiments were performed to examine the heat-shock response of *B. glandula* using an immunochemical assay to measure levels of endogenous (i.e., constitutive and induced) hsp70 and ubiquitin conjugate. During experiment I, *B. glandula* were collected and then maintained in a flowing seawater table for two weeks at an average temperature (± 95%CI) of 9.2 ± 0.1 °C. Experimental temperature manipulations were performed by placing live barnacles in temperature controlled chambers at 11, 20, and 28 °C for six hours. To mimic conditions in the intertidal zone during low tide, barnacles were not submerged in water, but the humidity was maintained at 100 percent. Immediately following the thermal incubation period, the prosoma of five barnacles from each temperature treatment were dissected, frozen with liquid nitrogen, and stored at -80 °C for hsp70 analysis.

A second experiment was aimed at examining endogenous levels of hsp70 over a 16 hour period after thermal incubation. During experiment II, *B. glandula* were collected and then maintained in a seawater table for one week at an average temperature of 11.2 ± 0.1 °C. Experimental temperature manipulations were the same as those described in experiment I, but the duration was reduced to five hours. Immediately after thermal incubation, barnacles were placed in seawater at 11 °C. At 0, 2, 4, 10, and 16 hours after thermal incubation was terminated, the prosoma of five barnacles from each temperature treatment were dissected, frozen with liquid nitrogen, and stored at -80 °C for hsp70 and ubiquitin conjugate analysis.

During experiment III, *B. glandula* were collected and then maintained in a seawater table for two weeks at an average temperature of 12.0 ± 0.1°C. Experimental temperature manipulations were performed by placing the live barnacles in temperature controlled chambers at 14 and 34 °C for 8.5 hours. The barnacles were not submerged in water, but the humidity was maintained at 100 percent. Immediately following thermal incubation, barnacles were placed in seawater at 14 °C. At 0, 2, 4, 10, and 16 hours after thermal incubation was terminated, the prosoma of five barnacles from each temperature treatment were dissected, frozen with liquid nitrogen, and stored at -80 °C for hsp70 and ubiquitin conjugate analysis.

Endogenous hsp70 and ubiquitin conjugate levels in the field

Two field experiments were performed to examine the endogenous levels of hsp70 in *B. glandula*. In the first experiment, *B. glandula* were collected on equal sized cobbles from the high and mid intertidal zone at 1.7 and 0.4 meters above mean lower low water (MLLW), respectively; the mid intertidal site was partially shaded by a thin layer of dried algae (*Ulva* sp). The mean surface temperature (± 95%CI) on three cobbles encrusted with *B. glandula* at the time of collection was recorded at 29.5 ± 0.5 °C in the high intertidal and at 23.7 ± 0.1 °C in the mid intertidal after an approximate aerial exposure time of eighteen and three hours, respectively. In the second experiment, equal size cobbles covered with barnacles were collected from the high intertidal zone (1.7 m above MLLW) to measure hsp70 levels from *B. glandula* on the warmer upper surface compared to the cooler underside of the cobbles. The mean surface temperature on four

cobbles was 29.0 ± 0.8 °C on the upper surface and 24.7 ± 0.8 °C shaded underside. In both experiments, barnacles were immediately returned to the lab following collection, where they were allowed to recover for 4 hours in a flowing seawater table at 13.6 °C. Following the recovery period, the prosoma was removed from each barnacle in a cold room at 13.5 °C, immediately frozen with liquid nitrogen, and stored at -80 °C for hsp70 and ubiquitin analysis.

Relationship between endogenous hsp70 levels (immunochemical assay) and thermally induced protein synthesis (metabolic labeling)

A Western blot immunochemical assay and a metabolic labeling assay were used to compare the relationship between endogenous hsp70 expression and thermally induced hsp70 expression. Specimens were collected, returned to the laboratory, and prepared for thermal incubation within 12 hours of collection. The methods outlined in the metabolic labeling induction assay section were used with the following modifications. Only incubation temperatures of 10 and 28 °C were used. After incubation, one of each depressor muscle was immediately frozen with liquid nitrogen and stored at -80 °C for immunochemical hsp70 analysis. The two remaining depressor muscles were incubated with a ^{35}S labeled amino acid mixture according to the procedures outlined in the metabolic labeling induction assay.

Effect of molt-cycle on temperature induced protein expression

To examine the effect of molting on the heat-shock response, winter acclimatized *B. glandula* were collected, returned to the lab, and examined within 12 hours. The molt stage was determined (Davis et al. 1973) and only individuals in interecdysis (stage C) or proecdysis (stage D) were prepared for a metabolic labeling thermal induction assay.

Acclimation and acclimatization experiments

To examine the effect of acclimation and acclimatization during winter, *B. glandula* were collected during December 2001 and January 2002. The specimens collected in December were acclimated at 10 °C in a flowing seawater table for seven weeks. At the end of the acclimation period, metabolic labeling induction assays were performed. To test the effect of thermal stress on field acclimatized barnacles, metabolic labeling assays were performed within 12 hours of collecting the January 2001 specimens. Data from NOAA recording stations in Charleston, OR (station 9432780) and Cape Arago lighthouse, Charleston, OR (station CAR03) were used to calculate mean (±95%CI) water and air temperatures, respectively. One week prior to collecting specimens for field acclimatization, *B. glandula* experienced a mean water temperature of 8.2 ± 0.3 °C and a mean air temperature of 10.0 ± 0.1 °C. Specimens were collected during a neap tide series.

To examine the effect of acclimation and acclimatization during the summer, *B. glandula* were collected on pilings in the intertidal at the mouth of the South Slough Estuary, Charleston, Oregon, USA and from a rocky intertidal bench on the open coast in

Sunset Bay State Park, Charleston, Oregon, USA during August 2003; the two collection sites are separated by approximately six km. The barnacles were returned to the laboratory and acclimated in aquaria at 10, 16, or 22 °C for eight weeks. During the acclimation period, the water was changed every four days and the barnacles were fed the diatom *Skeletonema costatum* in excess. Within 12 hours of collection, metabolic labeling induction assays were performed to test the effect of thermal stress on field acclimatized barnacles. All other metabolic labeling experiments were performed as soon as possible following the acclimation period. Data, one week prior to collection, from a local weather station and a CTD maintained by the National Estuarine Research Reserve System in Charleston, OR were used to calculate average water and air temperatures, respectively. Mean (±95%CI) air and water temperatures at the Sunset Bay population were calculated from a NOAA weather station at Cape Arago Lighthouse, Charleston, OR (station CAR03) and the Port Orford Buoy (station 46015), respectively. One week prior to collecting specimens for field acclimatization, *B. glandula* at the Charleston site (CH) experienced a mean water temperature of 14.7 ± 0.2 °C and a mean air temperature of 14.9 ± 0.9 °C. *Balanus glandula* at the Sunset Bay site (SB) experienced a mean water temperature of 15.0 ± 0.3 °C and a mean air temperature of 14.3 ± 0.3 °C. Specimens were collected during a neap tide series.

Statistical Analysis

All data examined in this study were analyzed with both Systat (version 9.0, SPSS Inc. 1999) and Statistica (version 6.0, Statsoft 2002). The assumptions of normality and

homoscedasticity were tested with a Komogorov-Smirov test with Lilliefors option and Cochran's C test, respectively. The assumption of normality was violated a few times. However, ANOVAS are robust to departures from normality (Sokal and Rohlf 1995, Underwood 1997). All repeated measures ANOVAS were subjected to Mauchly's test of sphericity. Violations of sphericity were interpreted by using the Greenhouse-Geisser epsilon to adjust the appropriate degrees of freedom.

To test the effects of incubation temperature on endogenous hsp70 and ubiquitin conjugate levels, separate ANOVAS were performed. All data were natural log transformed prior to analysis. In experiment I, a one-way ANOVA with temperature as a fixed factor was performed. In experiment II and III, a two-way ANOVA with temperature and time as fixed factors was performed independently for endogenous hsp70 levels and ubiquitin conjugate.

To determine differences between endogenous hsp70 and ubiquitin levels in field collected barnacles, separate t-tests were performed. Data were natural log transformed for endogenous hsp70 levels in both field experiments prior to analysis.

In the experiment examining the relationship between endogenous hsp70 and temperature induced protein synthesis, paired t-tests were separately performed for each method to test for differences between the control and heat-shock temperatures.

Because muscle tissue from a single individual barnacle were tested across all incubation temperatures in all the metabolic labeling experiments, a repeated measures ANOVA was performed with incubation temperature as the repeated measure. The analysis was performed separately for hsp70 and hsp90. Data were natural log

transformed prior to testing for the effect of molt cycle (fixed). In winter and summer acclimation experiments, the incubation temperatures of 23, 28, and 33 °C were normalized to the mean of the 10 °C control treatment so comparisons could be made between acclimations (Tomanek and Somero 1999). In both acclimation experiments, all data were natural log transformed prior to analysis. Type of acclimation, which includes field acclimatization and laboratory acclimation treatments, was treated as a fixed factor.

Results

Endogenous hsp70 and ubiquitin conjugate levels in the laboratory

Immediately following thermal incubation during experiment I, levels of endogenous hsp70 in *Balanus glandula* were not significantly higher at 20 and 28 °C compared to the control temperature of 10 °C (ANOVA, $F_{2,12} = 0.40$, $p = 0.68$) (Fig. 1). When hsp70 levels were measured over a 16-hour period after thermal incubation during experiment II, there was no significant increase of hsp70 levels from exposure to elevated temperatures of 20 and 28 °C compared to the control temperature of 10 °C (ANOVA, $F_{2,72} = 0.93$, $p = 0.40$) (Fig. 2A). During experiment II, levels of hsp70 did significantly vary over time across all treatments (ANOVA, $F_{5,72} = 3.70$, $p = 0.005$), such that levels increased to a maximum six hours after thermal incubation ended. When the incubation temperatures was raised to 34 °C in experiment III, significantly higher endogenous hsp70 levels were observed in the 34 °C treatment compared to the 14 °C control (ANOVA, $F_{1,48} = 8.10$, $p = 0.006$) (Fig. 3A). Over the course of the recovery period

during experiment III, endogenous hsp70 levels did not vary significantly (ANOVA, $F_{5,48} = 0.84$, $p = 0.53$).

Levels of ubiquitin conjugate were measured in experiments II and III to determine if irreversible protein damage occurred in response to thermal stress during laboratory experiments. Ubiquitin conjugate levels did not significantly vary as a result of incubation temperature (ANOVA, $F_{2,72} = 1.42$, $p = 0.25$) or over time (ANOVA, $F_{5,72} = 0.35$, $p = 0.88$) throughout the entire 16-hour recovery period following thermal incubation during experiment II (Fig. 2B). Ubiquitin-conjugate levels also did not significantly vary as an effect of temperature (ANOVA, $F_{1,48} = 0.84$, $p = 0.36$) or over the course of recovery (ANOVA, $F_{5,48} = 0.80$, $p = 0.55$) after exposure to 34 °C for 8.5 hours during experiment III (Fig. 3B).

Endogenous hsp70 levels in the field

Endogenous levels of hsp70 from specimens of *B. glandula* collected on cobbles from the high and mid intertidal zones were not significantly different ($t = 1.54$, $p = 0.17$) (Fig. 4A). However, a trend can be observed such that the mean hsp70 level was greater in the high intertidal zone than the mid. In addition, there was no significant difference between ubiquitin conjugate levels in *B. glandula* collected from the high and mid intertidal zone ($t = -0.42$, $p = 0.68$) (Fig. 4B). In the second field experiment, endogenous hsp70 levels in *B. glandula* attached to the warmer top and cooler underside of the cobbles were not significantly different ($t = -1.40$, $p = 0.22$). Similar to the

previous field measurements, a trend toward higher mean levels was observed on the warmer topside of the cobbles (Fig. 5).

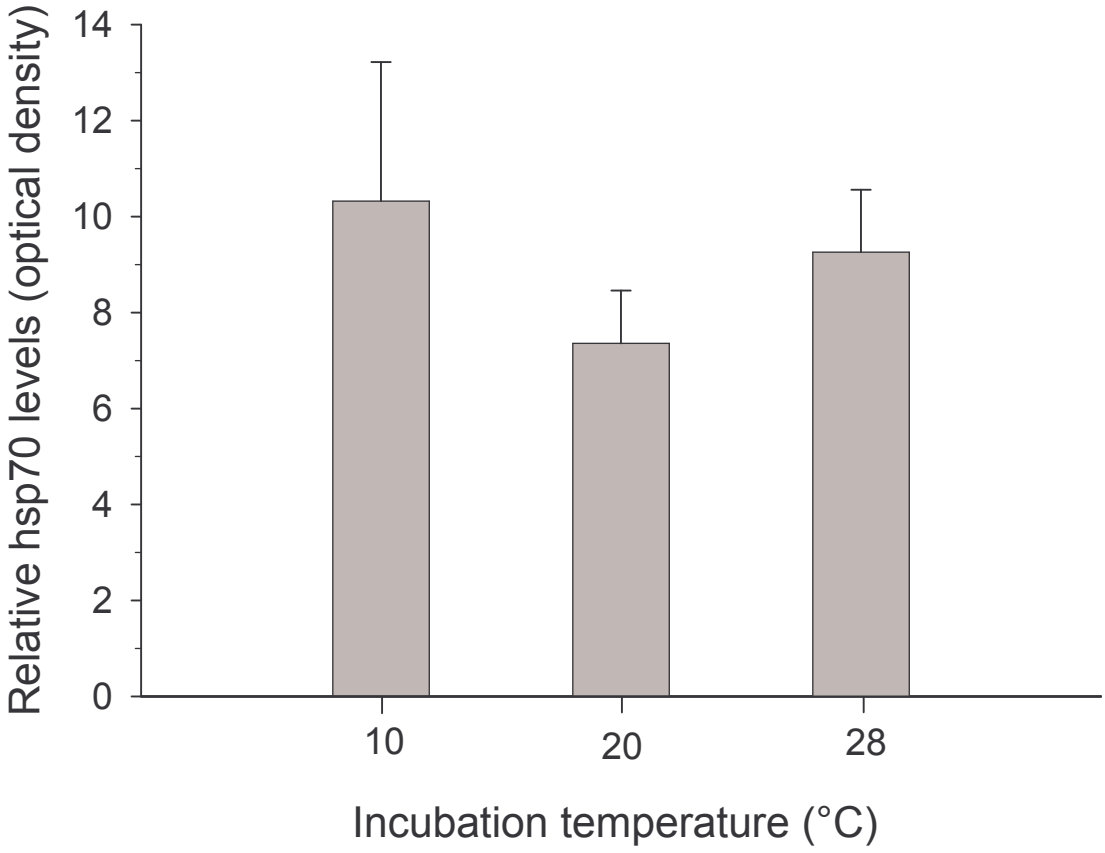


Figure 1. Endogenous levels of hsp70 in *Balanus glandula* from a laboratory experiment examining the effect of thermal stress. Each bar represents the mean of five individuals; error bars are 1 standard error.

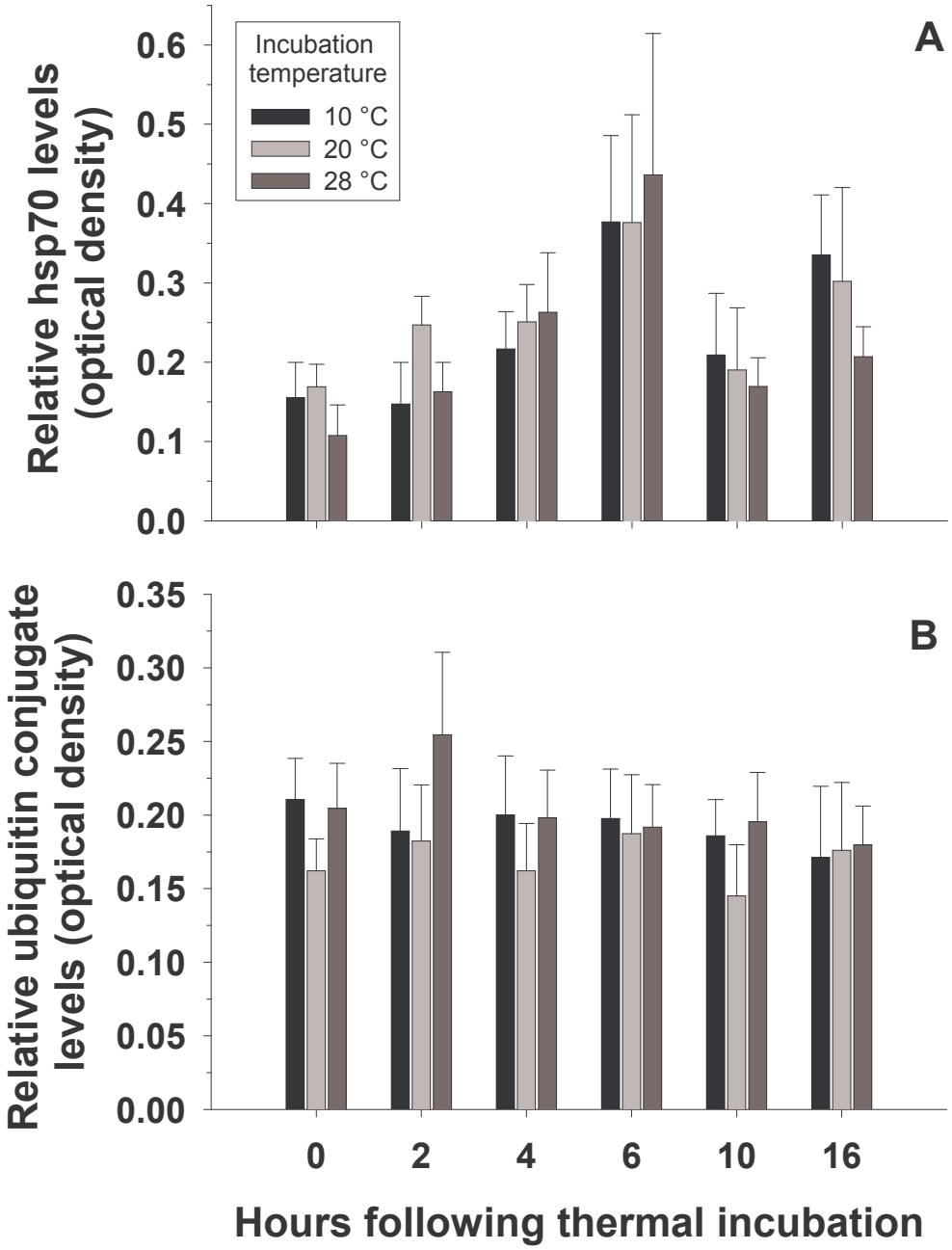


Figure 2. Levels of (A) endogenous hsp70 and (B) ubiquitin conjugate in *Balanus glandula* following recovery from thermal incubation. Each bar represents the mean of five individuals; error bars are 1 standard error.

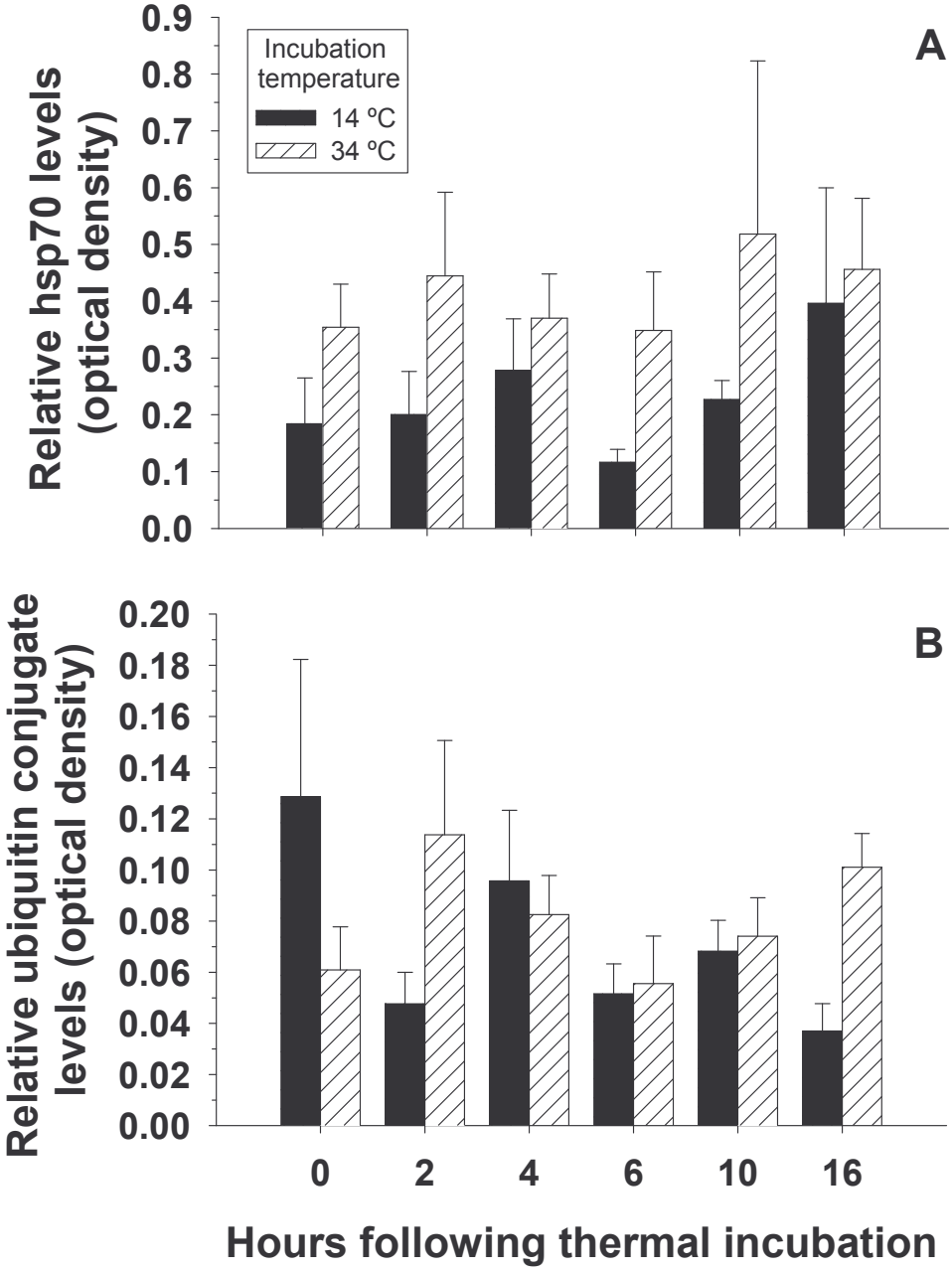


Figure 3. Levels of (A) endogenous hsp70 and (B) ubiquitin conjugate in *Balanus glandula* following recovery from thermal incubation. Each bar represents the mean of five individuals; error bars are 1 standard error.

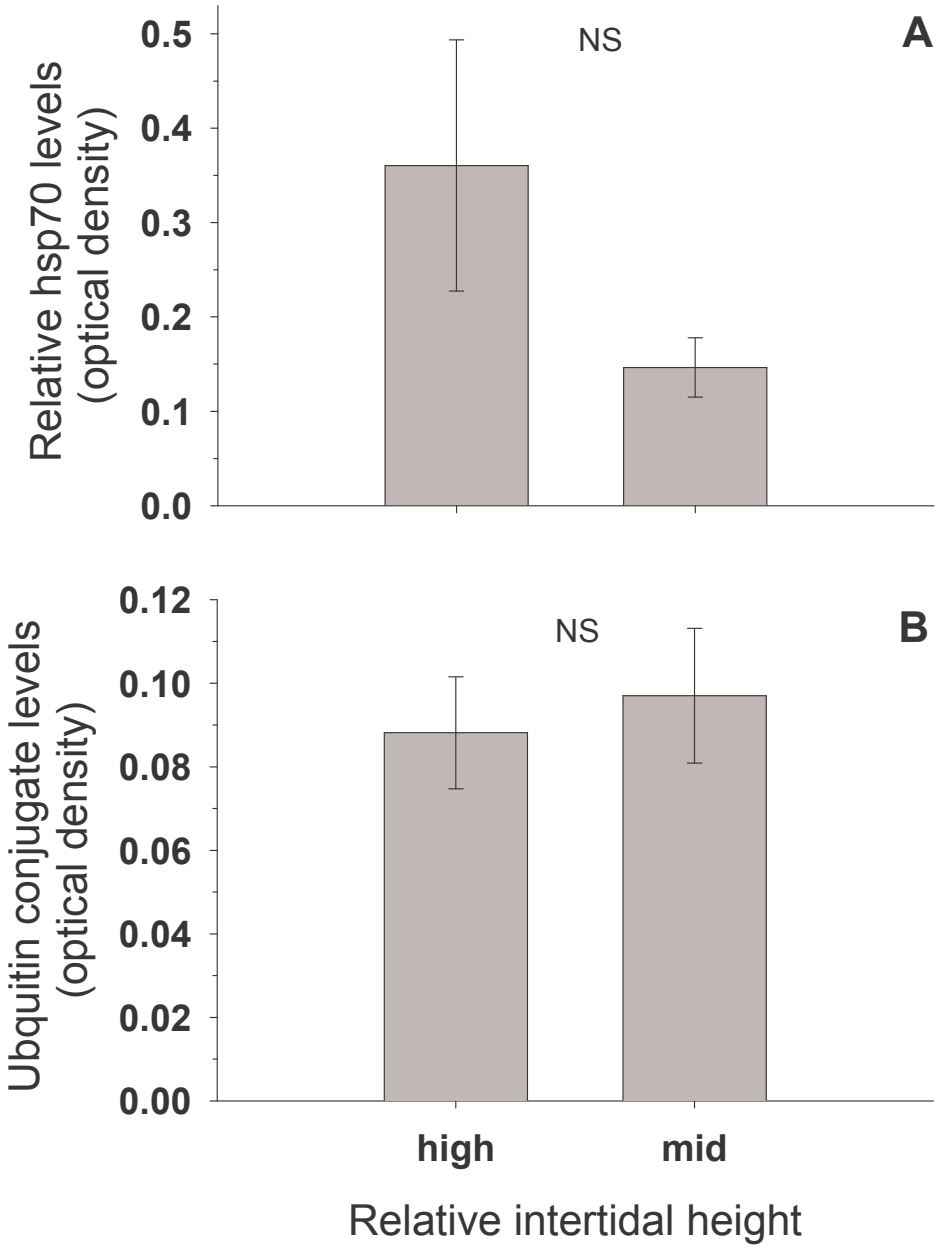


Figure 4. Endogenous levels of (A) hsp70 and (B) ubiquitin conjugate from *Balanus glandula* collected in the high and mid intertidal. Each bar represents the mean of seven individuals; error bars are 1 standard error. NS refers to not significant ($p > 0.05$).

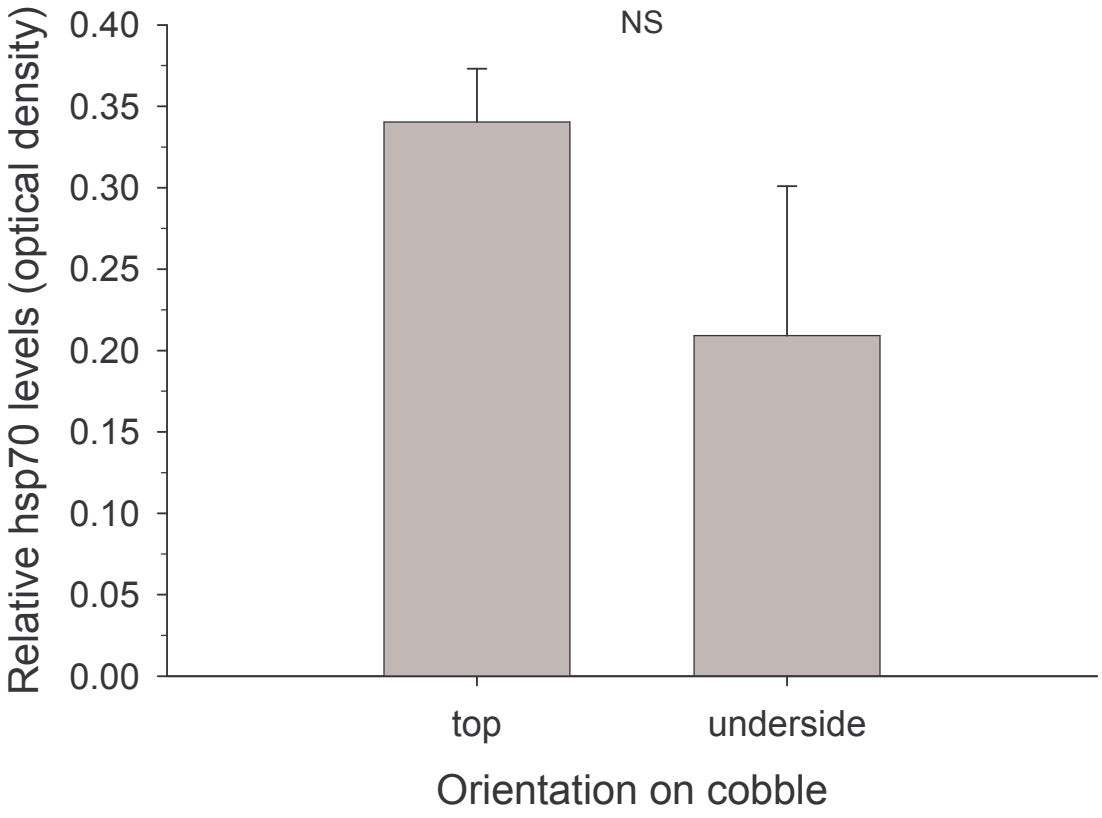


Figure 5. Endogenous levels of hsp70 from *Balanus glandula* collected on the top and underside of cobbles in the intertidal. Each bar represents the mean of six individuals from the topside and five from the underside; error bars are 1 standard error. NS refers to not significant ($p > 0.05$).

Relationship between endogenous hsp70 levels (immunoassay) and thermally induced protein synthesis (metabolic labeling)

Isolated muscle tissue from the same individual revealed a disparity between endogenous hsp70 levels and temperature induced protein synthesis (Fig. 6). Levels of endogenous hsp70 incubated at 10 and 28 °C were not significantly different from each other ($t = 1.05$, $p = 0.34$) (Fig. 6A). However, levels of induced protein in the 70 kDa region were significantly higher at 28 °C than at the 10 °C control temperature ($t = -4.36$, $p = 0.003$) (Fig. 6B).

Molt cycle effects on temperature induced protein expression

An experiment was performed to test for differences between *B. glandula* in an interecdysis and proecdysis (molting) stage. Molt stage had no significant effect on either hsp70 or hsp90 protein levels across incubation temperatures (Table 1, Fig. 7). A significant increase in hsp70 levels was observed as incubation temperature increased (Table 1, Fig. 7A). Levels of hsp90 also significantly varied over incubation temperature (Table 1). However, unlike the hsp70 pattern where levels continued to increase as incubation temperature increased, hsp90 levels reached a maximum at 28 °C and then decreased at 33 °C (Fig. 7B).

Acclimation and acclimatization experiments

Field acclimatized *B. glandula* in the winter were compared to 10 °C acclimated *B. glandula* to examine the heat-shock response during winter. Levels of hsp70 and

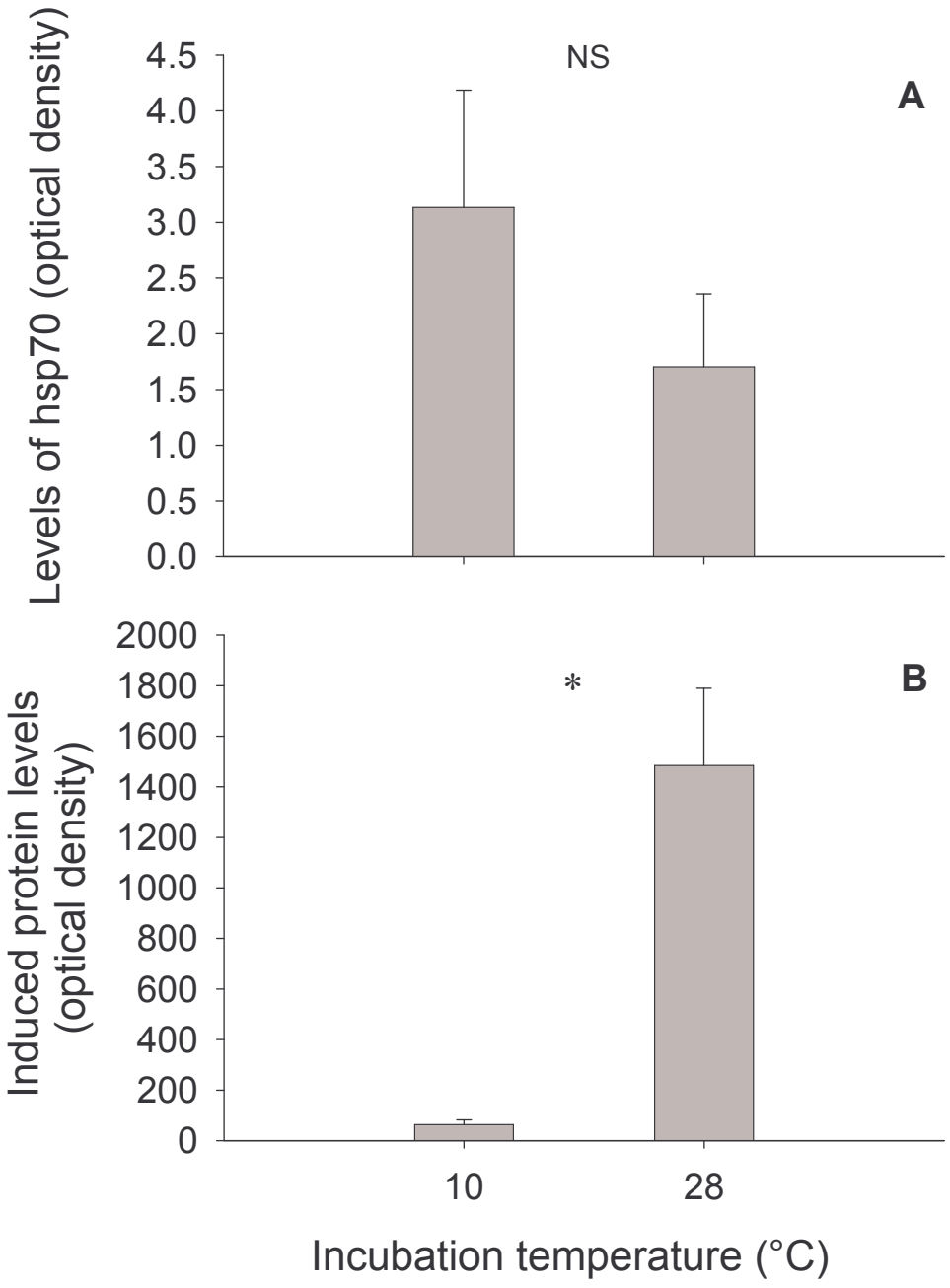


Figure 6. Comparison of (A) endogenous levels of hsp70 measured via immunochemical assay and (B) temperature induced protein synthesis with a molecular mass in the 70 kDa region (hsp70) in isolated muscle tissue from *Balanus glandula*. Each bar represents the mean of six individuals in graph A and eight individuals in graph B; error bars are 1 standard error. * = a significant difference ($p < 0.05$) and NS = not significant.

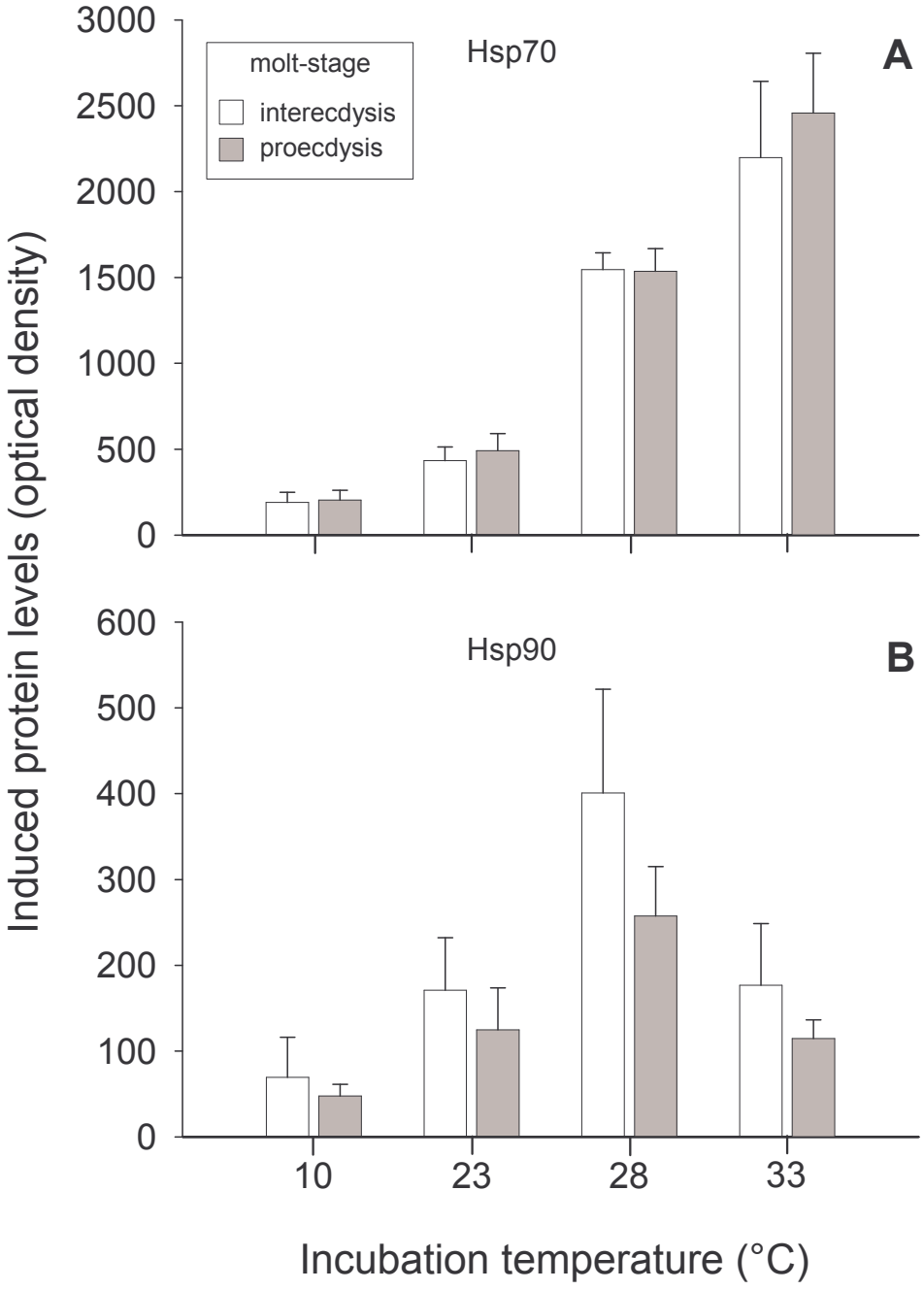


Figure 7. The effect of molt-stage on temperature induced protein synthesis in isolated muscle tissue from *Balanus glandula* for (A) hsp70 and (B) hsp90. Each bar represents the mean of six individuals; error bars are 1 standard error. See table 1 for statistics.

Table 1. Repeated measures ANOVA on the effect of molt-stage on temperature induced hsp70 and hsp90 levels in *Balanus glandula* (see Fig. 7).

Source*	ss	df	Mean-square	*F*-ratio	*P*
Hsp70					
Between subjects					
Molt stage	0.816	1	0.816	0.984	0.35
Error	8.300	10	0.830		
Within subjects[1]					
Temperature	61.132	3	20.377	20.337	0.000
Temperature x molt-stage	0.735	30	0.245	0.245	0.74
Error	30.060		1.002		
Hsp90					
Between subjects	0.003	1	0.003	0.001	0.97
Molt stage	28.079	10	2.808		
Error					
Within subjects[2]					
Temperature	46.058	3	15.353	10.783	0.001
Temperature x molt-stage	7.024	3	2.341	1.645	0.22
Error	42.712	30	1.424		

* The assumption of sphericity (Mauchly's test) was violated in all repeated measures ANOVAs. The degrees of freedom were multiplied by the Greenhouse-Geisser Epsilon and an adjusted *P*-value was calculated. The adjusted *P*-values are reported. Greenhouse-Geisser Epsilon values for within subjects: [1]0.5275 and [2]0.6006.

hsp90 were not significantly different between *B. glandula* that were field acclimatized and acclimated to 10 °C, despite a trend of higher protein levels for barnacles acclimated to 10 °C (Table 2, Fig. 8). Protein synthesis did significantly vary across temperature for hsp70 and hsp90 (Table 2, Fig. 8). As temperature increased, hsp70 levels increased and reached a maximum at 28 and 33 °C (Fig. 8A). Levels of hsp90 reached a maximum between 23 to 28 °C and decreased at 33 °C (Fig. 8B).

The effects of acclimatization and acclimation on the heat-shock response were examined on two populations of *B. glandula* during the relatively warmer summer months in contrast to winter. Levels of hsp70 in the Charleston (CH) population

significantly increased as incubation temperature increased (Table 3, Fig. 9A). In the CH population, hsp70 levels significantly varied as a result of the type of acclimation treatment (e.g., field acclimatization and laboratory acclimation) *B. glandula* experienced (Table 3). Specifically, barnacles acclimated to 16 °C and 22 °C displayed higher levels of hsp70 across all incubation temperatures compared to barnacles that were field acclimatized or acclimated to 10 °C (Fig. 9A). In addition, the field acclimatized *B. glandula* from the CH population displayed patterns of hsp70 synthesis that were similar to the 10 °C acclimation treatment (Fig. 9A).

Although levels of hsp70 in the Sunset Bay (SB) population did significantly increase as incubation temperature increased, levels of hsp70 in the SB population did not significantly vary as a function of acclimation treatment (Table 3, Fig. 9B). However, in the SB population, acclimation did significantly interact over temperature such that levels of hsp70 for *B. glandula* acclimated to 10 °C did not increase as incubation temperature increased, whereas hsp70 levels in all other acclimatization and acclimation treatments did increase as incubation temperature increased (Table 3, Fig. 9B). At an incubation temperature of 28 °C, field acclimatized *B. glandula* from the SB population displayed a pattern similar to the 10 and 16 °C acclimation treatment, but at an incubation temperature of 33 °C the field acclimatized hsp70 level was more like the 22 °C acclimation treatment (Fig. 9B).

Levels of hsp90 did not significantly vary as a function of acclimation temperature or incubation temperature in the CH population; no recognizable patterns were observed (Table 3, Fig. 9C). In contrast, the effect of acclimation on hsp90 levels

did significantly vary in the SB population; a trend for hsp90 levels to decrease as acclimation temperature increased was observed (Table 3, Fig. 9D). However, hsp90 levels in the SB population did not significantly vary across incubation temperatures (Table 3).

Table 2. Repeated measures ANOVA on the effect of acclimation on temperature induced hsp70 and hsp90 levels from *Balanus glandula* collected during the winter (see Fig. 8).

Source	ss	df	Mean-square	*F*-ratio	*P*
Hsp70					
Between subjects					
Acclimation	4.314	1	4.314	2.227	0.16
Error	25.190	13	1.938		
Within subjects*					
Temperature	11.374	2	5.687	5.032	0.036
Temperature x acclimation	0.690	2	0.345	0.305	0.62
Error	29.384	26	1.130		
Hsp90					
Between subjects					
Acclimation	0.762	1	0.762		
Error	12.154	13	0.935	0.816	0.38
Within subjects					
Temperature	10.546	2	0.536	18.697	0.000
Temperature x acclimation	1.072	2	0.282	1.900	0.17
Error	7.332	26			

* The assumption of sphericity (Mauchly's test) was violated for the hsp70 analysis. The degrees of freedom were multiplied by the Greenhouse-Geisser Epsilon (0.5739) and an adjusted *P*-value was calculated. The adjusted *P*-values are reported.

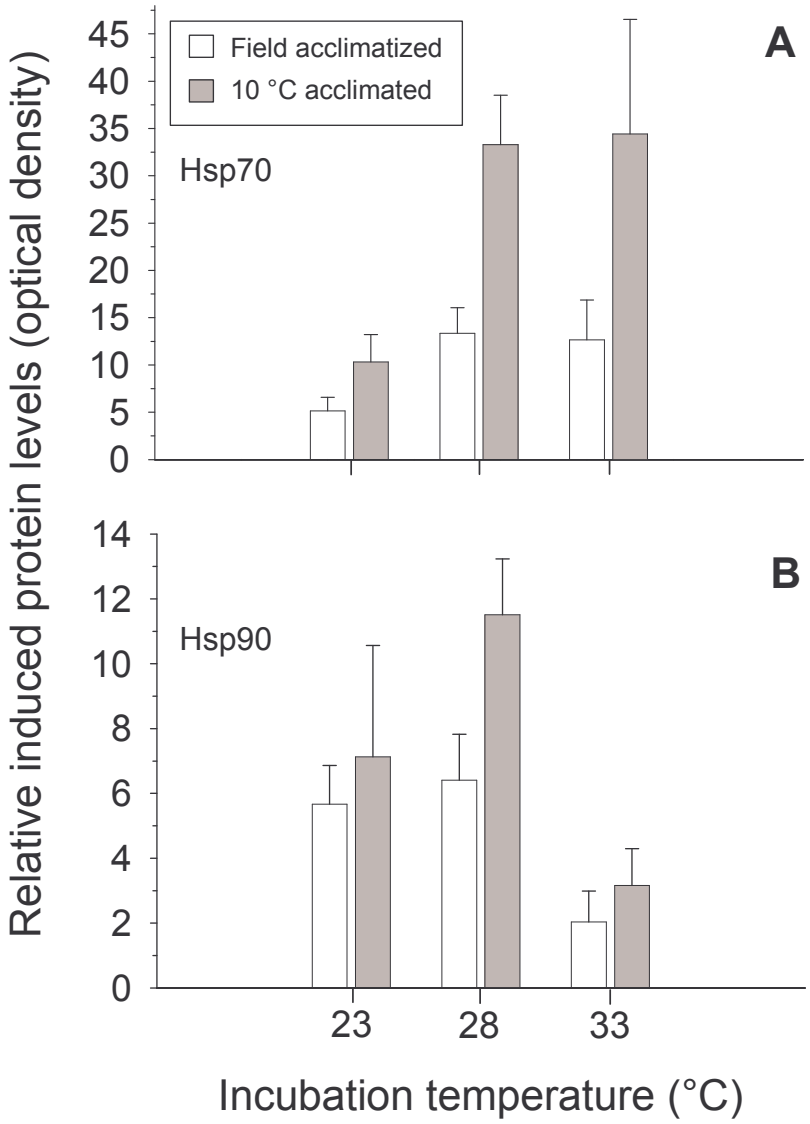


Figure 8. The effect of thermal acclimation on relative temperature induced protein synthesis in isolated muscle tissue from *Balanus glandula* for (A) hsp70 and (B) hsp90. Collections were made during winter 2002. Each bar represents the mean of eight individuals normalized to the 10 °C control treatment. Error bars are 1 standard error. See table 2 for statistics.

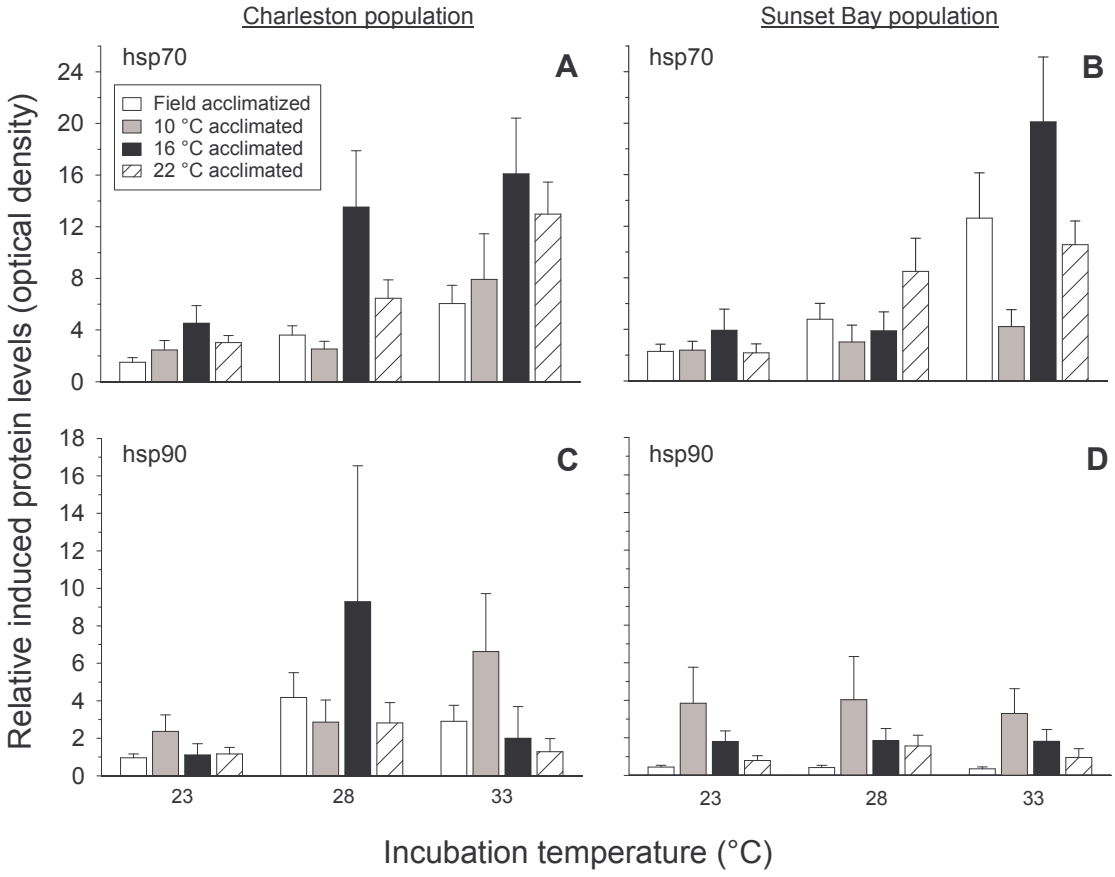


Figure 9. The effect of thermal acclimation on relative temperature induced protein synthesis in isolated muscle tissue from *Balanus glandula*. Thermally induced hsp70 levels in *Balanus glandula* from (A) Charleston population and (B) Sunset Bay population. Thermally induced hsp90 levels in *Balanus glandula* from (C) Charleston population and (D) Sunset Bay population. Each bar represents the mean of eight individuals normalized to the 10 °C control treatment. Error bars are 1 standard error. See table 3 for statistics.

Table 3. Repeated measures ANOVA on the effect of acclimation on temperature induced hsp70 and hsp90 levels from *Balanus glandula* collected during the summer from the Charleston population and Sunset Bay population (see Fig. 9).

Source	ss	df	Mean-square	*F*-ratio	*P*
Hsp70 - Charleston pop.					
Between subjects					
Acclimation	24.742	3	8.247	3.704	0.023
Error	62.344	28	2.227		
Within subjects					
Temperature	27.806	2	13.903	33.763	0.000
Temperature x acclimation	1.001	6	0.168	0.408	0.87
Error	23.060	56	0.412		
Hsp70 - Sunset Bay pop.					
Between subjects					
Acclimation	12.236	3	4.079	1.230	0.32
Error	98.811	28	3.315		
Within subjects					
Temperature	39.011	2	19.506	28.266	0.000
Temperature x acclimation	9.630	6	1.605	2.326	0.045
Error	38.645	56	0.690		
Hsp90 - Charleston pop.					
Between subjects					
Acclimation	30.069	3	10.023	0.480	0.70
Error	584.965	28	20.891		
Within subjects					
Temperature	2.72	2	1.364	0.113	0.89
Temperature x acclimation	134.319	6	22.387	1.847	0.11
Error	678.624	56	12.118		
Hsp90 - Sunset Bay pop.					
Between subjects					
Acclimation	9.218	3	3.073	3.291	0.035
Error	26.142	28	0.934		
Within subjects					
Temperature	0.123	2	0.061	0.551	0.58
Temperature x acclimation	0.360	6	0.060	0.539	0.78
Error	6.243	56	0.111		

Discussion

This study examined the heat-shock response of *Balanus glandula* to determine what ecologically relevant temperatures were physiologically stressful. To address this question, two methods were used: metabolic labeling to examine total induced protein synthesis and an immunochemical assay to examine endogenous hsp70 levels. Since the two techniques produced different outcomes, I will begin by discussing the results from the metabolic labeling technique, followed by a discussion of the immunochemical assay results and the potential limitations of using that specific technique on *B. glandula*.

General physiological response of Balanus glandula to thermal stress

Intertidal organisms regularly experience body temperatures from 20 to 35 °C (Hofmann and Somero 1995, Tomanek and Somero 1999, Buckley et al. 2001). Metabolic labeling experiments in this study have indicated that hsp70 is induced in *B. glandula* at 23 °C, regardless of acclimatization or acclimation, indicating that heat-shock proteins may be upregulated in *B. glandula* on a regular basis. Experimental work with other intertidal organisms such as mussels (Hofmann and Somero 1996), snails (Tomanek and Somero 1999), and limpets (Sanders et al. 1991) indicate that above a critical temperature, levels of hsp70 increase towards a maximum and then decrease to a level where expression stops before reaching a lethal thermal temperature. A similar pattern may exist in *B.* glandula, but further experimentation with incubation temperatures higher than 33 °C are necessary.

In *B. glandula*, the pattern of hsp90 synthesis differed from that of hsp70. Maximum expression of hsp90 in winter collected specimens was between 23 and 28 °C and then decreased at 33 °C (the effects of summer collection are discussed below). Presumably, at 33 °C expression of hsp90 was close to turning off. A difference in the pattern of hsp90 and hsp70 expression is not surprising, as different heat-shock proteins may perform different processes within a cell (Lindquist 1986, Parsell and Lindquist 1993).

Compared to the metabolic labeling experiments, results gathered from immunochemical assay experiments portrayed a different picture of the physiological response of *B. glandula* to thermal stress. The immunochemical assay suggests that, *B. glandula* does not respond to thermal stress until exposed to 34 °C for 8.5 hours. This result conflicts with an observed induction temperature of 23 °C from the metabolic labeling experiments and is perhaps inaccurate. Further, when both the immunochemical assay and metabolic labeling methods were performed on the same individual, an obvious heat-shock response was observed via metabolic labeling, but not via the immunochemical assay. A similar lack of a heat-shock response using an immunochemical assay was observed for the barnacle *Chthamalus fragilis* as well (T. Fitzhenry, pers. comm.).

Immunochemical assays are a very effective method for measuring heat-shock response in a variety of molluscs (Hofmann and Somero 1995, Dahlhoff et al. 2001, Tomanek and Somero 2003). However, it may not be an effective method for measuring the heat-shock response in the arthropod *B. glandula*. In immunochemical assays on

Mytilus edulis, using the same antibody as this study, two obvious hsp70 isoforms (presumably constitutive and induced hsp70 isoforms) are present when measuring endogenous levels (Hofmann and Somero 1995, Halpin et al. 2002). Typically a cell will synthesize both constitutive and inducible isoforms of molecular chaperones (Sanders 1993, Feder and Hofmann 1999). Only one resolvable isoform, potentially consisting of both constitutive and inducible hsp70, was visible in *B. glandula*. Therefore, constitutive levels may have masked over any induced levels. This would explain why a heat-shock response was observed using the immunochemical method only under extreme levels of stress. An alternative explanation is that the antibody used in this study was not specific to the inducible hsp70 isoform. If an immunochemical assay is to be used for future work on *B. glandula*, two-dimensional gel electrophoresis should be used to separate the constitutive from the induced isoform.

Endogenous hsp70 levels in the field

A significant difference in endogenous hsp70 levels was not observed for *B. glandula* from the high or mid intertidal zones and the top versus underside of cobbles. A higher intertidal height is usually more stressful because the intensity of the thermal stress is in part a function of the change in temperature multiplied by the duration of exposure (Hochachka and Somero 2002). The expectation was for higher levels of endogenous hsp70 to be expressed in this presumably more stressful habitat. For example, endogenous levels of hsp70 were high in *Mytilus californianus* collected higher in the intertidal zone than lower (Roberts et al. 1997). A similar pattern of higher hsp70

levels was observed for *M. trossulus* from a more stressful intertidal habitat compared to a subtidal habitat (Hofmann and Somero 1995). In both field experiments, recorded temperatures were high enough to elicit a heat-shock response in the more stressful habitats of the high intertidal zone or cobble topside. As previously discussed, constitutively expressed hsp70 isoforms may have masked over any heat-induced isoforms in the high intertidal and on the top of the cobble.

Irreversible protein damage

Irreversible protein damage was not observed when *B. glandula* was exposed to temperatures as high as 34 °C for 8.5 hours in the laboratory or from specimens exposed to high temperatures in the field. This result is different from other studies that have examined levels of ubiquitin conjugate in intertidal mussels. In multiple cases, an increase in ubiquitin conjugate levels and subsequent irreversible protein damage was observed for *Mytilus spp.* exposed to physiological stressful conditions where a heat-shock response was observed (Halpin et al. 2002, Buckley et al. 2001, Hofmann and Somero 1996, 1995). These results from *Mytilus spp*. suggest that although heat-shock proteins were expressed at elevated levels, some irreversible protein damage occurred. A heat-shock response occurred in *B. glandula* above 23 °C, yet there was no indication of irreversible protein damage. This result implies that *B. glandula* is well adapted to living in a stressful habitat and that an adequate concentration of heat-shock proteins may be present to efficiently remediate protein denaturation. Further work directly correlating

the relationship between hsp expression and irreversible protein damage from both laboratory and field collected specimens are necessary.

Molt cycle effects on temperature induced protein expression

One area of concern is the reorganization that occurs in a crustacean during molting and the potential effect molting may have on induced heat-shock protein expression. During the process of molting in a crustacean, cuticular reorganization, limb regeneration, and protein degradation occurs (Skinner et al. 1992, Hopkins 1993, Roer and Dillaman 1993). Since molecular chaperones have multiple cellular functions (Lindquist 1986, Parsell and Lindquist 1993), the process of molting could potentially elicit an increase in hsp levels, thus confounding the effects of temperature. Injecting the lobster *Homarus americanus* with a molting hormone (20-hydroxyecdysone) produced an increase in the level of hsp90 mRNA expressed in the midgut gland (Chang et al. 1999). Also in *Homarus americanus*, levels of hsp90 mRNA increase in muscles undergoing atrophy during molting, but not in muscles that do not go through atrophy during molting (Spees 2002). I found that the expression of hsp70 and hsp90 varied as a function of temperature, but not as a function of the two molt-stages examined. Therefore, a change in hsp levels in this study was probably affected by only temperature treatments and not by molt-stage.

Acclimation and acclimatization

The effect of thermal acclimation and acclimatization was examined to address the plasticity of the heat-shock response in *B. glandula*. Typically, when acclimation or acclimatization temperature is increased, an upward shift in the induction temperature of heat-shock proteins is observed (Dietz and Somero 1992, Tomanek and Somero 1999, Buckley et al. 2001, Buckley and Hofmann 2002). In *B. glandula*, an upward shift in the hsp induction temperature was not observed at higher acclimation temperatures or in summer acclimatized specimens. In fact, higher levels of hsp70 were expressed by *B. glandula* acclimated to higher temperatures, contrary to the expectation and observation of other organisms. At present an explanation is unclear, with more questions raised than answered. Current models indicate that the regulation of hsp synthesis is controlled by a negative feedback mechanism where heat-shock transcription factor (HSF1) is bound to a heat-shock protein complex (Craig and Gross 1991, Morimoto 1993, Shi et al. 1998). When the hsp begins to actively chaperone denaturing proteins, HSF1 is released, trimerizes, and begins the upregulation of heat-shock proteins. Additional experimentation that simultaneously examines constitutive levels of heat-shock proteins, levels of HSF1, and induced levels of heat-shock proteins may be necessary to completely understand the regulation and expression patterns of the heat-shock response in *B. glandula*.

The heat-shock response of *B. glandula* varied between summer and winter, but only for hsp90 and not hsp70. There was no observable difference in the induction and overall expression pattern of hsp70 in summer compared to winter. However, a

significant effect of incubation temperature on hsp90 levels was observed in winter and not in summer. Why incubation temperature did not effect the expression of hsp90 during summer in both barnacle populations is unclear. Heat-shock proteins have a short half-life, on the order of days (Landry et al. 1982, Chen et al. 1990), so the effects of previous experience (e.g., prior stress) should have been removed by the acclimation process, making acclimated individuals similar across seasons. Therefore, during summer the acclimated individuals should have displayed a pattern of hsp90 synthesis similar to barnacles from the winter acclimation experiment and the experiment examining the molt cycle.

In summary, *B. glandula* responds to thermal stress by upregulating the synthesis of heat-shock proteins. Although some similarities were observed between the heat-shock response of *B. glandula* compared with other intertidal organisms, *B. glandula* generally responds differently to the acclimation process. In addition, lack of any irreversible protein damage suggests that *B. glandula* may be well adapted to life in the high intertidal zone. Further, this study suggests that all intertidal organisms may not respond to stress in a similar manner and that organisms from multiple taxonomic groupings should be examined to complete the story on the physiological response of intertidal organisms to ecologically relevant stress. This study has just begun to investigate the complexities of the heat-shock response in barnacles and has raised more questions than it has answered.

CHAPTER V

GENERAL SUMMARY

This dissertation examined the effect of environmental stress on juvenile survivorship and growth, adult reproductive effort, and adult heat-shock protein expression in the barnacle *Balanus glandula*. Chapter II followed survivorship and growth as a measure of performance in newly settled juvenile barnacles for twenty days at three sites along an estuarine gradient. Experimental outplants were conducted during the spring when a strong estuarine gradient was present, and during the summer when conditions within the estuary had more of a marine influence. Juvenile performance was variable over spatial and temporal scales. Surprisingly, juvenile performance was highest at the mid-estuarine site than all other sites. As predicted, juvenile performance was lowest at the riverine site than all other sites.

The prediction was that as juvenile *B. glandula* experienced increasing levels of stress with distance up the estuary, juvenile performance would decrease. In other words, as a species commonly found on the open coast, *B. glandula* should have performed best at the oceanic end of the estuary and worst at the riverine end of the estuary. Connell (1975) proposed a model that potentially explains the observed pattern of higher performance at the mid-estuarine site. He suggested that there can be an interaction between physiological stress and predation, where the combination of tolerable stress in

conjunction with reduced predation could potentially create favorable conditions for successful recruitment. Although Connell's model was not directly tested, the results in this dissertation are consistent with his model. Future work should examine differential predation on juvenile barnacles along an estuarine gradient and in addition explore the cost and benefits of settlement at a mid-gradient location.

Chapter III examines the reproductive patterns of a natural population of *B. glandula* along an estuarine gradient to understand how reproductive effort varies along that gradient. The results indicate that reproductive effort in *B. glandula* decreases up the estuary. Along the estuarine gradient, from the oceanic to the riverine end, the potential for physiological stress increases, while the abundance of available food decreases. A decrease in reproductive effort may have been due to an increase in physiological stress compounded by a decrease in available food.

Together, the results from Chapter II and III suggest that the optimal location for juvenile survivorship and growth was the mid-estuarine site, whereas the optimal location for reproduction was the more oceanic site. These results present an interesting trade-off with respect to choice of settlement site. This trade-off should be further evaluated by examining rates of adult survivorship and growth, age of reproduction, total reproductive output over an individual's life span, and the fitness of the offspring produced at both sites.

The focus of Chapter IV shifted away from the effects of an estuarine gradient and focused on examining the physiological response of *B. glandula* to thermal stress by measuring patterns of heat-shock protein (hsp) expression. The results suggested that *B.*

glandula physiologically responds to thermal stress by upregulating the expression of hsp70 at 23 °C; a temperature commonly experienced in an intertidal habitat. Although a heat-shock response was observed, there was no evidence to suggest that irreversible protein damage occurred due to elevated temperatures. Unlike the intertidal invertebrates examined to date (e.g., molluscs), acclimation to higher temperatures did not shift the induction temperature of hsp70 to a higher temperature in *B. glandula*. These results suggest that *B. glandula* may be well adapted to life in the high intertidal zone and that the heat-shock response in *B. glandula* may be different than other intertidal invertebrates, such as molluscs.

This research has just begun to explore the heat-shock response in *B. glandula*. Further work on a variety of aspects regarding the heat-shock response is necessary. The relationship between hsp expression and irreversible protein damage from laboratory experiments and field collected specimens should be further explored. Additionally, the mechanism that regulates the heat-shock response should be examined in detail. Simultaneously examining constitutive levels of heat-shock proteins, levels of heat-shock transcription factor, and induced levels of heat-shock proteins are necessary to completely understand the regulation and expression patterns of the heat-shock response.

The unifying theme of this dissertation has been stress. An intertidal invertebrate, such as a barnacle, can experience stress along a horizontal gradient in an estuary or along a vertical gradient in the intertidal. The conditions experienced along these gradients have been shown to effect the growth and survivorship of juveniles and reduce adult reproductive output. Furthermore, a physiological heat-shock response was

observed at temperatures commonly experienced by adult barnacles and their brooded offspring.

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