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Morphological plasticity of barnacle feeding legs and penises

by

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ABSTRACT

One important source of phenotypic variation on which natural selection can act is developmental plasticity (the capacity of a single genotype to produce different environment-dependent forms). Therefore, studies of how the environment influences development can facilitate our understanding of how natural selection acts to yield phenotypic evolution. Using the Pacific barnacle (*Balanus glandula* Darwin), I explored how functionally independent appendages (the legs and unusually long penises of barnacles) respond to widespread spatial and temporal variation in water velocity and conspecific density. Through field surveys, reciprocal transplant experiments, and histological sectioning, I show that barnacle legs and penises appear remarkably well adapted to spatial and temporal variation in water velocity. Building on past work on leg form variation, I show that penises from exposed shores were shorter than, stouter than, and more than twice as massive for their length, as those from nearby protected bays (this effect holds true for artificially inflated penises as well). A transplant experiment confirmed that most of this variation in penis and leg form variation was due to developmental plasticity. Penises and legs of barnacles from an exposed shore also had thicker cuticle, and muscles with greater cross-sectional area (and shorter sarcomeres) compared to those from a protected shore. Form variation was consistent with numerous predictions from engineering theory suggesting that barnacles show dramatic, complex and likely adaptive variation in leg and penis form among sites that differ dramatically in water velocity. Additional experiments showed evidence for and against developmental limits to plasticity in barnacles. A transplant experiment identified an important (and asymmetrical) developmental limit to leg-length response time – likely mediated by food limitation – while a field survey showed that developmental coupling does not restrict adaptive plastic responses of legs and penises to multiple conflicting cues (conspecific density and water velocity). Finally, a two-year survey of natural populations revealed the first evidence that barnacles also change leg form seasonally. Together these results

contribute valuable information on the mechanisms of phenotypic change. This research also sheds light on the circumstances that allow decoupling of developmental processes to produce novel combinations of characters on which natural selection can act.

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CHAPTER I. GENERAL INTRODUCTION

Organisms interact intimately with their physical environment, and with other organisms that share it. This environment may vary from one place to another and over time. Although independently these observations may seem uncontroversial, understanding how developing organisms interact with a variable environment has far-reaching implications for ecology and evolution that have only begun to be explored. In this thesis, I examine some of the consequences of these phenotype-environment interactions that lie at the interface between ecology and evolution using a well-studied group of crustaceans: intertidal barnacles that live on rocky shores.

1.1 Organisms are a product of their genes and their growth environment

Developing organisms require continuous input from their internal and external environment. Gene products influence the expression of other genes, cells influence other cells and cell lineages, and the external environment provides the developmental resources and many important cues required for the entire process (West-Eberhard 1989, Gilbert 2001). As a direct consequence of this interdependence between genotype and phenotype, changes to the environment often produce changes in the form or behaviour of organisms. This capacity for a single genotype to produce multiple environment-determined phenotypes is known as phenotypic plasticity. Although such plasticity was once treated as merely noise that confounded the study of evolution (Falconer 1952), it is now recognized as an important process capable of being shaped by (Dudley and Schmitt 1996), and influencing (Price et al. 2003), natural selection and subsequent evolution (Agrawal 2001, West-Eberhard 2003).

The range of phenotypes that a single genotype can produce across a range of environments is known as the reaction norm (Johansen 1911), a term popularized by Schmalhausen (1949). A reaction norm can be thought of as a genotype's *capacity* for plasticity. Importantly, plasticity can evolve because not all individuals respond to the environment in the same way. In other words, reaction norms may vary among individuals. For example, in a study of wild radish (Agrawal et al. 2002), defensive chemicals known as glucosinolates were measured in individual families where half the plants in each family were subjected to herbivory and half were not. Assays showed significant variation in defensive compounds among families after herbivory, but no variation among families in the absence of herbivores. In other words, genetic variation existed in the slope of the reaction norm (i.e., the capacity for plasticity varies among families). If this variation in glucosinolate production among families translates to variation in fitness (because relatively undefended plants are attacked more often, for example), then this should provide the necessary variation for the evolution of greater herbivore-induced resistance. In more general terms, the adaptive plasticity hypothesis suggests plasticity should evolve where it maximizes fitness in variable environments (Dudley and Schmitt 1996).

Hundreds of examples of adaptive plasticity now exist across a wide variety of taxa and experimental systems, and plastic response may involve a combination of changes in behaviour, physiology, and form (Tollrain and Harvell 1999, Pigliucci 2001, West-Eberhard 2003). While some changes can occur almost instantaneously (e.g., many changes in behaviour), others require considerably more time (e.g., changes in size or shape). Furthermore, in addition to variation in their relative speed of response, types of plasticity can differ considerably in their reversibility; some plastic responses are triggered during certain stages of development and become fixed later in life (e.g. polyphenisms like the castes in social insects; West-Eberhard 2003), while at the other end of the spectrum, most (but not all) changes in behaviour (Sih et al. 2004) and some changes in form (Piersma and Drent 2003) are fully reversible throughout adulthood.

Perhaps some of the most striking responses are changes in body form associated with anti-predator defense or resource acquisition. For example to defend against would-be predators, many adult (Appleton and Palmer 1988, Edgell et al. 2008) and larval (Vaughn 2007) gastropods develop thicker shells with narrower apertures, tadpoles grow larger tails, (Relyea 2002), plants and animals alter the form of their offspring (Agrawal et al. 1999), and some echinoderms can even clone themselves (Eaves and Palmer 2003, Vaughn and Strathmann 2008). Traits involved in resource acquisition are also highly plastic. Plants alter stem, leaf, and root form, to seek out more-concentrated resources (van Kleunen and Fischer 2005), and crabs grow larger and stronger claws (Smith and Palmer 1994) and shorebirds grow larger more muscular gizzards (Piersma and Drent 2003) to crack harder gastropod prey.

1.2 Conditions required for phenotypic plasticity to evolve

Despite the ubiquity and obvious benefit of plasticity in variable environments, not all traits are plastic. As a result considerable debate persists over the factors that favour or limit its evolution (DeWitt 1998, Auld et al. 2009). For plasticity to evolve, a number of conditions must be met (Via and Lande 1985, Pigliucci 2001). Here I limit my discussion to adaptive changes in body form known as morphological plasticity, although these conditions should apply to all plastic traits.

First, for developmental plasticity to be adaptive, no single phenotype can be optimal in all environments encountered. In other words, trade-offs must exist that favour different body forms in different environments. Such tradeoffs are common, and often result from inherent biomechanical 'design' limitations sometimes combined with trade-offs involving the allocation of costly or limited material (Vogel 2003). The crusher claws in decapods and many other crustaceans offer a nice example of some such limitations. To capture mobile prey, claws should be fast, whereas to crush the hard shells of many gastropods, claws should be strong. However, due to how muscles and levers function (Warner and Jones 1976), claws face an inherent trade-off between strength and speed.

Another such example comes from the feeding nets of filter-feeding animals, which face important trade-offs between filter efficiency and drag force. Imagine a feeding fan as a sieve held in the direction of flow and containing many holes. A sieve with small holes (or a sieve with a larger area) will capture more particles from the water, but must also resist greater drag forces imposed on it as water passes through (Denny 1988). Therefore, in slow-moving water where drag is expected to be low, a large sieve with small holes should capture more food, while in high flow, a smaller sieve with larger holes should be favoured (both because a small sieve with large holes should experience less drag, and because more particles are flowing past the fan as water velocity increases). Indeed, the feeding fans of larval black files (Zhang 2006), bryozoans (Okamura and Partridge 1999), and intertidal barnacles (Arsenault et al. 2001) all possess smaller feeding structures on exposed shores, likely to cope with this inherent design tradeoff.

Again, similar to the crab claws mentioned earlier, building a fan that is robust *and* efficient is possible, but requires the allocation of more material to resist the increased drag force imposed by higher water velocity.

Second, organisms must encounter environmental variation at a scale that is relevant to them. In short, the time-course of environmental variation must be predictable, and occur on a similar time-scale as the time-course of a possible plastic response (Gabriel et al. 2005, Gabriel 2006). Consider a simple example of a small annual plant living in the forest understory. Plants commonly exhibit plastic responses to light levels, changing leaf and stem form to adjust to variation in solar irradiance (Callaway et al. 2003). However, understory plants likely experience variation in solar irradiance at multiple temporal scales and may only respond to some aspects of this variation. At the extremes, light levels may vary considerably on the scale of seconds, as overstory plants move with the wind, and on the scale of centuries as overstory trees grow from seedlings to canopy giants. However, these scales are not relevant to the plants in question because at one extreme, morphological responses clearly take more than a few seconds to develop, while at the other, century-scale variation is not relevant on the timescale of the lifespan of an individual plant. More generally, for plasticity to be effective, a balance must be reached between speed of response, and cost of production; slow responses risk a subsequent environmental change rendering the adaptation obsolete before it is fully developed, while fast responses may entail a higher energetic cost (Padilla and Adolph 1996, Gabriel et al. 2005). Furthermore, similar arguments can be made for patterns of spatial variation in organisms that disperse as juveniles; patterns of spatial variability depend on the scale at which they are experienced by the organism.

A third requirement for the evolution of adaptive plasticity is that development must be able to produce adaptive plastic responses upon which selection may act. The generation of phenotypic variation is at the core of evolution via natural selection, yet variation in one trait may sometimes limit variation in another (Pigliucci and Preston 2004, Sih et al. 2004, Brakefield 2006), a fact noticed as far back as Darwin (Darwin 1872). Specifically, for plastic traits, an adaptive response of one trait may limit the range of plastic responses in another, if the two traits share components of developmental pathways or share developmental resources (Moran 1992). Although studies of developmental coupling among plastic traits are rare (Neufeld 2011), at least one study suggests they do exist (Gianoli and Palacio-Lopez 2009). In sum, that adaptive phenotypic plasticity is so common suggests that many of these conditions are often met, while the absence of plasticity in many traits where it would seem beneficial suggests important constraints restrict the evolution of adaptive plasticity in variable environments.

1.3 Experimental system

Rocky shores are an ideal testing ground for studying the role of developmental plasticity in shaping ecological and evolutionary interactions. Wave-exposed shores represent one of the most extreme, stressful, and variable environments on earth. When the tide is out, organisms must be able to tolerate extreme air temperatures, hypoxia, and solar radiation (Harley and Helmuth 2003). When the tide is in, organisms must perform all the necessary functions of life while resisting the extreme forces imposed by breaking waves (Denny 2006). Water velocity varies by over two orders of magnitude over multiple spatial scales from centimeters (O'Donnell and Denny 2008) to kilometers (Denny et al. 2003), and over time as waves break and recede, between waves, and seasonally due to the severity and frequency of wave-generating storms. Furthermore, spatial wave-force variation can be quantified inexpensively (Bell and Denny 1994), and a wide network of buoys records and disseminates offshore wave height hourly throughout the year for the quantification of seasonal patterns. Remarkably, despite this extreme spatial and temporal variation in water velocity, a diverse assemblage of organisms thrives under these conditions. This variation in wave force not only affects the interactions among species, but also the form of many algae (Koehl 1999) and invertebrates (Palumbi 1984, Trussell 1997). Collectively, the combination of widespread environmental variation and a diverse assemblage of well-studied organisms inhabiting this dynamic environment make rocky shores ideal for studying how common patterns of selection shape the evolution and maintenance of morphological plasticity in variable environments.

Barnacles are one group that is particularly successful on rocky shores, attaining large populations throughout the world. Barnacles are an ideal group to test the role of variable environments on the evolution of morphological plasticity. First, their ecology, life-history, and form have been well-studied as far back as Darwin (1854), so much is already known about their basic biology and ecology. Second, they inhabit a highly variable environment at multiple scales, and individuals of a single species often span a wide range of water velocities (Anderson 1994). Third, barnacles are sessile and can easily be moved around by taking advantage of their habit of settling on mussel shells and small stones. Fourth, barnacles are well-known to be plastic in multiple morphological traits (Lively 1986, Bertness et al. 1998, Arsenault et al. 2001). Past work on a common Pacific barnacle *Balanus glandula* has shown that leg form varies dramatically and predictably with maximum wave force (Arsenault et al. 2001), although there appears to be limits to such variation at extreme velocities (Li and Denny 2004). Furthermore, leg form variation is common, occurring in multiple species inhabiting rocky shores (Marchinko and Palmer 2003). In at least one species this leg form variation is due largely to reversible developmental plasticity that is retained through adulthood (Marchinko 2003). Fifth, feeding legs are not the only structure possessed by barnacles that must interact with extreme and variable flows. Most barnacles are hermaphrodites, and to reproduce they extend unusually long penises that sometimes reach out up to 8 times their body length (Darwin 1854) to find and fertilize distant mates. The existence of multiple structures that must extend into flow to perform different functions allows one to ask an additional set of questions around how similar patterns of selection shape functionally independent appendages. Collectively, these characteristics make barnacles an ideal system to study the evolution of reversible plasticity in variable environments.

1.4 Overview of data chapters

My first goal, addressed in **Chapter II**, was to determine how functionally independent appendages (the legs and penises of barnacles) respond to similar wave conditions. Specifically, collaborating with Rich Palmer, I showed that penises of an intertidal barnacle (*Balanus glandula* Darwin) from wave-exposed shores were shorter than, stouter than, and more than twice as massive for their length, as those from nearby protected bays. In addition, penis shape variation was tightly correlated with maximum velocity of breaking waves. Finally, a field experiment showed that barnacles transplanted to a wave-exposed shore produced dramatically shorter and wider penises than counterparts moved to a protected harbour, confirming that most of this variation in penis form was due to developmental plasticity.

Given that barnacles adjust penis form and leg form in response to water velocity, the objective of **Chapter III** was to determine more broadly whether development can restrict the direction of plastic responses in penis form and leg form in response to water velocity and conspecific density. Specifically, I compared leg and penis form variation of both isolated and grouped barnacles at multiple low-flow and high-flow sites. Then, using the same data set, I asked whether the direction of concurrent responses of legs and penises to variation in conspecific density and water velocity were consistent with documented adaptive responses from past studies. I showed that penis length and leg length in *B. glandula* varied in parallel with variation in wave exposure but in opposite directions with variation in conspecific density, consistent with adaptive responses of single appendages to single cues.

Next, to address another important question relating to the development of leg form, in **Chapter IV** I measured reciprocal response time-lags and developmental mechanics of appendage form plasticity in *B. glandula*. A ten-week transplant experiment under natural conditions in the intertidal revealed: i) barnacles take longer to change leg form than previously thought, ii) response times depend on transplant direction, and iii) barnacles change leg length through a combined response of a) growing longer leg segments and b) adding more leg segments. These results suggest that resource acquisition under natural conditions may be an important and underappreciated limit on morphological response times in natural systems (energy is necessary for morphological change, yet resources are often limited). Furthermore, the ability of barnacles to change the number of feeding-leg segments over time raises many interesting questions about how such changes occur during development. Finally, a two-year survey of barnacle leg and penis form in natural populations revealed that adult barnacles do change leg and penis form over time, providing the first evidence that barnacles modify appendage form in a manner consistent with adaptation to seasonal variation in wave force.

In earlier chapters, I focus primarily on gross morphology of legs and penises (overall size and shape). In **Chapter V**, in collaboration with Cassidy Rankine (who performed the sarcomere measurements), I explore the mechanical design and material properties of barnacle legs and penises at the level of individual functional components, namely cuticle and muscle. Measurements made from detailed feeding leg and penis histology revealed that barnacles from an exposed shore had feeding legs and penises with thicker cuticle, and leg muscles with shorter sarcomeres, relative to similar-sized barnacles from a nearby quiet bay. Sarcomeres were also much shorter at the base of the feeding legs compared to the tip. Furthermore, artificial inflation of excised penises showed penises stretch five times more in length than in circumference when pressurized, although I found no clear variation in the capacity to stretch among penises of barnacles from sites that differed in wave exposure. Together these results suggest barnacles show dramatic, complex and presumably adaptive variation in leg and penis form among sites that differ dramatically in water velocity.

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CHAPTER II. PRECISELY PROPORTIONED: INTERTIDAL BARNACLES ALTER PENIS FORM TO SUIT COASTAL WAVE ACTION[1]

2.1 Introduction

To cope with a sessile lifestyle and an evolutionary legacy of mandatory internal fertilization (Ruppert et al. 2004), barnacles have evolved a remarkable mechanism for copulating with distant neighbours: penises that can extend up to 8 times their body length (Darwin 1854; Table 1-1). However, as one of very few sessile animals to copulate (Ruppert et al. 2004), barnacles face a delicate tradeoff. Although longer penises greatly increase the number of potential mates – because the searchable area expands as the square of penis length – the benefits of larger penises may be outweighed by increased drag as water turbulence and velocity intensifies, particularly in species that live on wave-exposed shores (see Fig. 2-6 in Discussion).

Curiously, because most free-living barnacles are simultaneous hermaphrodites (Charnov 1987) they could potentially self-fertilize, yet in most species self-fertilization is rare or non-existent (Barnes and Crisp 1956, Furman and Yule 1990). Instead most barnacles reproduce by extending impressively long penises (Table 2-1) to find and fertilize distant mates (Klepal 1990). However, such long penises pose a major challenge because many intertidal barnacle species live under a wide range of wave conditions where water velocities can span up to three orders of magnitude (Denny 1988) and reach extremes of up to 20 ms^{-1} (Helmuth and Denny 2003). Consequently, individuals with penises well suited for mating in quiet waters may be poorly suited for copulating on wave-exposed shores. Indeed, other structures that must extend into flow, like the long feathery feeding legs of barnacles, differ dramatically in form between protected harbours and nearby wave-exposed sites (Arsenault et al. 2001). Furthermore, in one species, these differences in feeding leg form arise primarily due to phenotypic plasticity (Marchinko 2003).

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Therefore, because penises of barnacles likely face similar hydrodynamic constraints to those experienced by feeding legs, we predicted that 1) penis size and shape should vary among sites with different wave-force regimes: penises should be shorter (i.e., smaller) on wave-exposed shores because of greater drag in high flow, and for a given length, penises should be stouter at wave-exposed sites to better resist bending in high flow, and 2) because wave action varies so dramatically in space and time, differences in penis form should arise primarily due to phenotypic plasticity.

2.2 Materials and Methods

2.2.1 Artificial inflation of barnacle penises

To ensure relaxed penis length was a valid proxy for extended penis length, we mechanically inflated penises of barnacles from an exposed-shore (Prasiola Pt., 48°49'01" N, 125°10'02" W; velocity of breaking waves approx. 5 ms^{-1}, CJN unpublished data) and a protected-shore (Grappler Inlet; 0.05 ms-1, Marchinko and Palmer 2003). To avoid potential density-induced differences in penis form, 25 solitary barnacles – barnacles with plates not touching another barnacle but with an approximately equal number of neighbours within 1-2 cm – were collected in the middle of the *Balanus glandula* zone at each site on December 14, 2007. After freezing for 24 hours at -8°C, barnacles were thawed in seawater and the soma was removed, photographed under a dissecting microscope at 6-8×, blotted dry, and weighed to the nearest 0.1 mg following Arsenault et al. (2001). The soma was then cut between the first and second pair of thoracic legs, inserted onto the tapered end of a seawater-filled plastic capillary tube (1.09 mm outside diameter, 0.38 mm inside diameter, approx. 25 mm long) and carefully glued in place (Krazy Glue, Elmer's Products, Columbus, OH) while keeping the penis tissue moist. The capillary tube was then inserted onto the end of a hypodermic needle (0.5 mm outside diameter, 0.2 mm inside diameter) and fitted onto a 10 ml plastic syringe filled with seawater. The penis and remaining feeding legs were positioned in seawater under a dissecting microscope and photographed to obtain relaxed penis length. The penis was oriented perpendicular to the field of view, and pressure was applied to the syringe to slowly inflate the penis until a) the glue failed, b) the soma tissue or cuticle ruptured, or c) the penis inflated fully. Full inflation was recorded when additional pressure on the syringe failed to extend the penis further and all annulations of the penis cuticle had disappeared. At this point the penis was photographed again. This process was repeated for approx. 20 individuals per site until we had achieved full penis extension for three individuals from each population.

2.2.2 Field survey

Balanus glandula Darwin were collected from eight sites that spanned a wide range of maximum water velocities within Barkley Sound, British Columbia, Canada. Six sites in order of decreasing wave-exposure (Seppings Island, Bordelais Island, Wizard Islets, Kelp Bay, Self Pt., and Ross Islets) are described in Arsenault et al. (2001) and two additional low velocity sites (Bamfield Inlet and Grappler Inlet) are described in Marchinko and Palmer (2003). Barnacles (mean soma mass = 0.0171 g; range = 0.0019 to 0.0491) were collected between Feb. 21-24, 2006, when the majority of individuals in this area are reproductively active (Strathmann 1987) and have fully developed penises (Barnes 1992). Twenty solitary barnacles – barnacles with plates not touching another barnacle but with an approximately equal number of neighbours within 1-2 cm – were collected in the middle of the *B. glandula* zone at each site. Due to errors during dissection, sample sizes used to determine site means differed slightly (from n = 17 to n = 20) among sites and analyses. Due to the difficulty of measuring winter water velocity using standard techniques, we used the known relationship between leg length and maximum velocity of breaking waves (Arsenault et al. 2001) to estimate the maximum water velocity at each site. In addition, we used empirically determined summer velocities at these sites (Arsenault et al. 2001, Marchinko and Palmer 2003) for comparison (Fig. 2-2).

2.2.3 Field transplant experiment

To differentiate between genetic control (differential settlement and/or selective mortality) and environmental control (phenotypic plasticity) of penis form, we transplanted barnacles from two source populations to each of two destination sites. On September 25, 2006, adult *B. glandula* (mean soma mass = 0.0075 g; range = 0.0018 - 0.0449) were collected on mussel shells (*Mytilus* *californianus* Conrad and *M. trossulus* Gould) from two source populations chosen for a substantial difference in wave force between sites (Arsenault et al. 2001) and for a sizeable supply of adult barnacles growing on mussels: a protected shore (Ross Islets (RI); Arsenault et al. 2001) and an exposed shore (Seppings Island (SI); Arsenault et al. 2001) in Barkley Sound, British Columbia, Canada. Mussel shells were cut using a variable speed rotary tool so that one barnacle occupied each mussel shell fragment. Mussel shell fragments were spaced approx 15 mm apart and glued to two 10 by 13 cm Plexiglas plates using marine epoxy putty (Z-spar™ Splash Zone Compound) in a 5 by 8 grid alternating between protected- and exposed-shore source populations. Plates were kept overnight in flowing seawater and then bolted to the rock in the middle of the *B. glandula* zone in two outplant locations chosen for a more than five-fold variation in wave-force: a protected shore (Bamfield Inlet (BI); Marchinko and Palmer 2003) and an exposed shore (Seppings Island (SI); Arsenault et al. 2001). On Feb. 13, 2007 (after 20 weeks), during the local reproductive period, intact transplanted barnacles were collected to measure barnacle penis form. During the outplant period barnacles suffered some mortality at all sites. The final numbers used in the analyses were: Protected (site RI) to Protected (site BI), n = 18; Protected (RI) to Exposed (SI), n = 6; Exposed (SI) to Protected (BI), n = 16; Exposed (SI) to Exposed (SI), n = 13. Furthermore, due to an omission during dissection, soma mass was not measured for one individual in the Protected to Exposed group (RI to BI). Consequently data from this individual were excluded from analyses involving soma mass.

2.2.4 Sample processing

Samples were frozen at -8 °C. and processed within 30 days of collection. Barnacles were thawed in seawater and the soma was removed, blotted dry and weighed to the nearest 0.1 mg following Arsenault et al. (2001). The penis, and the 6th thoracic leg from the left side, were removed, wet mounted in seawater, and photographed under a dissecting microscope at 15-31x. Photographs were measured to the nearest 10 um using ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2008.). For the field-survey barnacles, after being photographed, penises were dried to a constant mass and weighed to the nearest 0.001 mg on a Cahn C-31 analytical microbalance (Thermo Electronic Corporation, Waltham MA).

2.2.5 Statistical analyses

All statistics were calculated using R (R Foundation for Statistical Computing, Vienna, Austria, http://www.R-project.org). Assumptions of equal variance (using Levene's test) and normal distribution (using Shapiro-Wilk test) were met in all cases. To test for differences in extended length and proportional change of artificially inflated penises, we used two-sample *t*-tests on $\log_{10}$-transformed data. For the field survey, least-square means of body size (Fig. 2-2a, b) or penis length (Fig. 2-2c, d) were calculated using Analysis of Covariance (ANCOVA) assuming an equal slope among sites. Regression and ANCOVA analyses were calculated on $\log_{10}$-transformed data. Where slopes varied significantly among groups (Table 2-3) we recalculated least-square means at first, second and third quartiles of penis length assuming unequal slopes (Table 2-6). In all cases the conclusions were not affected (Fig. 2-3). For the field transplant experiment, we computed two 2-way ANCOVAs on $\log_{10}$-transformed data with source population and transplant location as factors and soma mass (Fig. 2-5a) or penis length (Fig. 2-5b) as the covariate.

2.3 Results

2.3.1 Artificial inflation of barnacle penises

Relaxed penis length was a consistent indicator of maximum penis length for both protected-shore and exposed-shore barnacles (Fig. 2-1). Mean proportional inflation was not significantly different between source-populations ($p = 0.772$) and erect penises of protected-shore barnacles remained significantly longer than erect penises of exposed-shore barnacles ($p = 0.039$).

2.3.2 Field survey

We observed differences in both penis size and shape. Corrected for body size (soma mass), penises from the most wave-exposed site were 25% shorter than those from the most protected bay and penis length was strongly negatively correlated with velocity when compared across all eight sites (Fig. 2-2a). Furthermore, penis mass (corrected for body size) was 16% higher in the most exposed site compared to the most protected site and this trait was positively correlated with water velocity across sites (Fig. 2-2b).

In contrast to the modest differences in penis size, we observed substantial differences in penis shape among the same sites. Relative to penis length, penis basal width at the most exposed site was more than 50% larger than penis basal width at the most protected site (Fig. 2-2c, Fig. 2-4). Furthermore, penis mass (standardized for penis length) was more than twice as great in the most wave-exposed site compared to the most protected bay (Fig. 2-2d). In both cases, water velocity explained a high percentage of among-site variation in penis shape: 96% for penis basal width at standard length (Fig. 2-2c), and 95% for penis mass (Fig. 2-2d).

2.3.3 Field transplant experiment

Corrected for body size, barnacles transplanted to the wave-exposed shore produced 25% shorter (analysis of covariance on $\log_{10}$-transformed data, $F_{1,45} = 15.46$, $p < 0.001$, Fig. 2-4a) and 20% wider ($F_{1,46} = 11.57$, $p = 0.001$, Fig. 2-4b) penises than counterparts moved to the protected harbour after a period of 20 weeks. In addition, penis length exhibited a disproportionately greater response to local growth environment in larger barnacles than smaller ones ($F_{1,45} = 4.59$, $p = 0.038$). Finally, regardless of whether barnacles originated from exposed- or protected-shore populations, both penis size (penis length relative to soma mass; $F_{1,45} = 0.39$, $p = 0.534$) and penis shape (penis basal width relative to penis length; $F_{1,46} = 1.90$, $p = 0.175$) converged to similar values at both transplant sites (Fig. 2-5).

2.4 Discussion

2.4.1 Adaptive significance of penis-form variation

The remarkably close fit between wave-force and penis form (Fig. 2-2c, d) suggests that even slight deviations from an optimal shape in a particular environment drastically reduce opportunities to mate. Two lines of argument suggest that the spatial variation in penis form we observed is adaptive.

First, in the absence of hydrodynamic effects, longer penises should increase the number of potential mates because reachable area increases as the square of penis length (Fig. 2-6a). Second, the penis-form variation we observed is consistent with two predictions from engineering theory. Beam theory (Vogel 2003) predicts that stouter, heavier penises should better resist bending caused by drag, so stouter penises should enable more successful copulations on wave-exposed shores (Fig. 2-6b). In addition, hydrodynamic theory predicts that, under high flow conditions, larger penises should be disproportionately stouter than smaller ones because drag, and hence overall bending force, increases to the 2nd power of length (Vogel 2003). Indeed, we observed that penis basal width exhibited significant and similar positive allometry relative to body size and penis length at all sites (Table 2-2), and penises of larger barnacles exhibited a disproportionately greater response when transplanted to different flow conditions (Table 2-7).

Two factors may have resulted in the relatively high within-site variation in penis size (Fig. 2-2a). First, variation in any correlations involving soma mass might arise due to variation in reproductive condition, either directly because penis morphology is known to vary with reproductive condition in many barnacle species, or indirectly because testes and seminal vesicles are part of the soma mass used as a proxy for body size. Second, although we visually controlled for density during collection, density of surrounding individuals may have varied slightly between samples and may play a role in barnacle penis length.

Although the observed variation in penis form seems most likely to be adaptive, one non-adaptive explanation that cannot be ruled out is developmental pleiotropy (Ronemus et al. 1996): mechanisms controlling the development of leg length and penis length may not be independent. Nonetheless, pleiotropy could be an elegant developmental mechanism for ensuring that penis form varied appropriately in response to wave action. Because legs are exposed to flow more frequently, they would be a more reliable sensor of flow conditions, and could “signal” to the penis to change form. In other words, developmental pleiotropy itself could represent an adaptive evolutionary response that couples the developmental control of leg and penis form.

2.4.2 Is penis-form variation plastic?

A close fit between penis form and environment could arise in three ways: a) differential settlement, where genetically different larvae settle in a specific environment appropriate to their genotype, b) selective mortality, where larvae of all genotypes settle independent of environmental conditions but mismatched genotypes suffer higher mortality, or c) phenotypic plasticity, where different phenotypes are directly induced by different growth conditions. We found that barnacles transplanted to quiet water produced longer and thinner penises than those moved to wave-exposed shores and converged on the same phenotype at a given transplant site regardless of source population; therefore, penis size and shape appear to vary among sites primarily due to phenotypic plasticity, and little if at all due to genetic differences (via differential settlement or selective mortality) among populations.

Phenotypic plasticity in penis form may have arisen as an adaptive strategy to cope with spatial and/or temporal variation in flow conditions that developing barnacles experience. Because barnacles have a long pelagic larval duration (Strathmann 1987) and likely travel long distances from their source population prior to settlement, offspring may end up in a vastly different flow environment than that experienced by their parents. Furthermore, independent of where they end up, barnacles may face significant temporal variation in flow. In addition to seasonal patterns in swell-generating storm events (swell increases dramatically in the winter months at our study sites), the settlement, growth, and mortality of other animals may also cause persistent changes in flow between successive years. Therefore, the ability to alter penis form after settlement may be advantageous to cope with aspects of spatial as well as temporal variation in flow conditions.

Although some experimental barnacles died during the transplant experiment (see Methods), our results are unlikely to be influenced by differential mortality. First, a mismatch between penis form and flow conditions is not likely to cause any direct mortality. Second, we found no empty barnacle shells on the transplant plates, implying that all barnacles died due to dislodgement from waves (any other cause of mortality would have left intact shells attached to the transplant plates) not from mismatched penis form. Third, exposed-shore barnacles presumably adapted to high flow conditions still experienced mortality at the exposed site during the experiment. Finally, because barnacle legs and penises must both extend into flow, and barnacle legs are highly plastic in response to flow at these same sites, conditions that favour plasticity in legs are highly likely to favour plasticity in penises as well. Collectively these observations strongly suggest that phenotypic plasticity is responsible for producing the different morphologies observed in different growth environments.

2.4.3 Constraints on penis length

Regardless of how they develop, longer penises are likely constrained by high flow (Fig. 2-2a, Fig. 2-6b). However, at low-flow sites where drag is less likely to limit penis form, several other 'braking' effects may also restrict the development of ever-longer penises in barnacles. First, although oversized male genitals are thought to entail few costs (Eberhard 1985, Chapman et al. 2003), oversized reproductive organs may increase susceptibility to predators by significantly reducing escape velocities (Ramos et al. 2004, Langerhans et al. 2005). Given that even the agile feeding legs of barnacles are eaten by fish (Barnes 1999), barnacles' unwieldy penises are probably even more vulnerable while exposed during copulation. Second, as length increases, the ability to control such a long appendage may become difficult: in one beetle species a similar cost appears to have promoted an elaborate behaviour to retract its outsized intromittent organ without causing irreversible damage (Gack and Peschke 2005). Finally, beyond a certain length, the benefit of greater reach may be outweighed by the cost of producing and housing the large genitalia and ample sperm necessary to fertilize a growing number of distant mates. Collectively, any number of these effects could reduce the advantage of ever-longer penises and may account for the leveling-off of penis length with decreasing water flow that we observed at the most protected sites (Fig. 2-2a).

In sum, our results suggest that penis size and shape in *B. glandula* are strongly influenced by a tradeoff between length and maneuverability that varies with wave-force. Through the capacity to grow wider and heavier penises (for their length) on wave-exposed shores, *B. glandula* may improve their mating success under high flow. This study provides a rare example of conspicuous phenotypic plasticity in animal genitalia and reveals how factors other than the usual drivers of genital diversification – female-choice, sexual conflict, and male-male competition (Eberhard 1985, Arnqvist 1998, Eberhard et al. 1998, Hosken and Stockley 2004) – can influence genital form.

Table 2-1 Extended penis length in animals.

Common name	Scientific name	Penis length relative to body length	Ref.
Burrowing barnacle	*Cryptophialus minutus*	8.0	(Darwin 1854)
Japanese acorn barnacle	*Tetraclita japonica*	3.9	(Murata et al. 2001)
Pacific acorn barnacle	*Balanus glandula*	3.6	*
Rove beetle	*Aleochara tristis*	2.0	(Gack & Peschke 2005)
Argentine lake duck	*Oxyura vittata*	1.0	(McCracken et al. 2001)
Hat snail	*Calyptraea morbida*	1.0	(Chen et al. 2000)
Seed bug	*Lygaeus simulans*	1.0	(Tadler 1999)
Feather mite	*Proterothrix sp.*	1.0	†
Sand flea	*Tunga penetrans*	1.0	†
Slipper limpet	*Crepidula spp.*	0.6	(Brown & Olivares 1996)
Spider	*Tidarren spp.*	0.5	(Ramos et al. 2004)
Ostracod	*Candona suburbana*	0.3	(Cohen & Morin 1990)

(*C. J. N. unpublished data, †pers. comm. H. Proctor.)

Table 2-2 Field Survey scaling relationships.

x	y	Predicted isometric slope	df	r	RMA slope	Probability slopes do not differ from isometry
Log(soma mass)	Log(penis length)	0.333	1,151	0.6957	0.3612	0.188
Log(soma mass)	Log(penis mass)	1	1,149	0.907	1.0072	0.492
Log(soma mass)	Log(penis basal width)	0.33	1,154	0.8572	0.3762	0.008
Log(penis mass)	Log(penis length)	3	1,154	0.7911	3.1496	0.27
Log(penis length)	Log(penis basal width)	1	1,150	0.6933	1.2154	<0.001

(Slopes, and therefore coefficients of allometry, did not vary significantly among sites: $\alpha = 0.01$ after Bonferroni Correction for multiple comparisons, $P > 0.0128$ for equality of slopes for all scaling relationships.)

Table 2-3 Field Survey ANCOVA statistics.

	Log(penis length)				Log(penis mass)			
Source of variation	df	MS	*F*	*p*	df	MS	*F*	*p*
Log(soma mass)	1	0.936	162.10	<0.001	1	13.787	676.53	<0.001
Field site	7	0.025	4.29	<0.001	7	0.018	0.86	0.539
Equality of slopes	7	0.007	1.13	0.245	7	0.015	0.747	0.632
Residual	136	0.006	-	-	135	0.020	-	-

	Log(penis basal width)				Log(penis mass)			
Source of variation	df	MS	*F*	*p*	df	MS	*F*	*p*
Log(penis length)	1	1.341	260.66	<0.0001	1	10.371	385.57	<0.001
Field site	7	0.093	18.13	<0.0001	7	0.325	12.09	<0.001
Equality of slopes	7	0.014	2.67	0.013	7	0.057	2.13	0.045
residual	136	0.005	-	-	131	0.027	-	-

Table 2-4 Field Survey regression statistics calculated using estimated winter water velocities.

	log(penis length at standard soma mass)				
	df	MS	*F*	*p*	adjusted r-squared
water velocity (from leg lengths)	1	0.007	16.476	0.007	0.689
residual	6	0.000			

	log(penis mass at standard soma mass)				
	df	MS	*F*	*p*	adjusted r-squared
water velocity (from leg lengths)	1	0.006	37.857	0.001	0.840
residual	6	0.000			

	log(penis basal width at standard penis length)				
	df	MS	*F*	*p*	adjusted r-squared
water velocity (from leg lengths)	1	0.032	154.050	<0.001	0.956
residual	6	0.000			

	log(penis mass at standard penis length)				
	df	MS	*F*	*p*	adjusted r-squared
water velocity (from leg lengths)	1	0.119	149.050	<0.001	0.955
residual	6	0.001			

Table 2-5 Field Survey regression statistics calculated using summer water velocities.

	log(penis length at standard soma mass)				
	df	MS	*F*	*p*	adjusted r^2
summer water velocity	1	0.007	21.137	0.004	0.742
residual	6	0.002	0.000		

	log(penis mass at standard soma mass)				
	df	MS	*F*	*p*	adjusted r^2
summer water velocity	1	0.006	63.669	<0.001	0.900
residual	6	0.001			

	log(penis basal width at standard penis length)				
	df	MS	*F*	*p*	adjusted r^2
summer water velocity	1	0.028	30.672	0.001	0.809
residual	6	0.001			

	log(penis mass at standard penis length)				
	df	MS	*F*	*p*	adjusted r^2
summer water velocity	1	0.114	69.613	<0.001	0.907
residual	6	0.002			

Table 2-6 Field Survey regression statistics calculated on data standardized to first and third quartiles of penis length for ANCOVAs with interactions.

	log(penis basal width at 1st quartile of penis length)				
	df	MS	*F*	*p*	adjusted r^2
water velocity (from leg lengths)	1	0.030	54.290	<0.001	0.884
residual	6	0.001			

	log(penis basal width at 3rd quartile of penis length)				
	df	MS	*F*	*p*	adjusted r^2
water velocity (from leg lengths)	1	0.045	55.337	<0.001	0.886
residual	6	0.001			

	log(penis mass at 1st quartile of penis length)				
	df	MS	*F*	*p*	adjusted r^2
water velocity (from leg lengths)	1	0.091	57.944	<0.001	0.891
residual	6	0.002			

	log(penis mass at 3rd quartile of penis length)				
	df	MS	*F*	*p*	adjusted r^2
water velocity (from leg lengths)	1	0.181	47.002	<0.001	0.868
residual	6	0.004			

Table 2-7 Field Transplant ANCOVA statistics.

	Log10(penis length)				
	df	MS	*F*	*p*	
Log10(soma mass) (sm)	1	0.463	42.920	0.000	***
Growth environment (env)	1	0.167	15.460	0.000	***
Source population (source)	1	0.004	0.392	0.534	
sm x env	1	0.050	4.591	0.038	*
sm x source	1	0.000	0.001	0.979	
source x env	1	0.001	0.108	0.743	
Error	45	0.011			

	Log10(penis basal width)				
	df	MS	*F*	*p*	
Log10(penis length) (pl)	1	0.171	44.360	0.000	***
Growth environment (env)	1	0.045	11.565	0.001	**
Source population (source)	1	0.007	1.897	0.175	
pl x env	1	0.003	0.707	0.405	
pl x source	1	0.000	0.065	0.800	
source x env	1	0.002	0.577	0.452	
Error	46	0.004			

(*** p <0.001, ** p<0.01, * p<0.05)

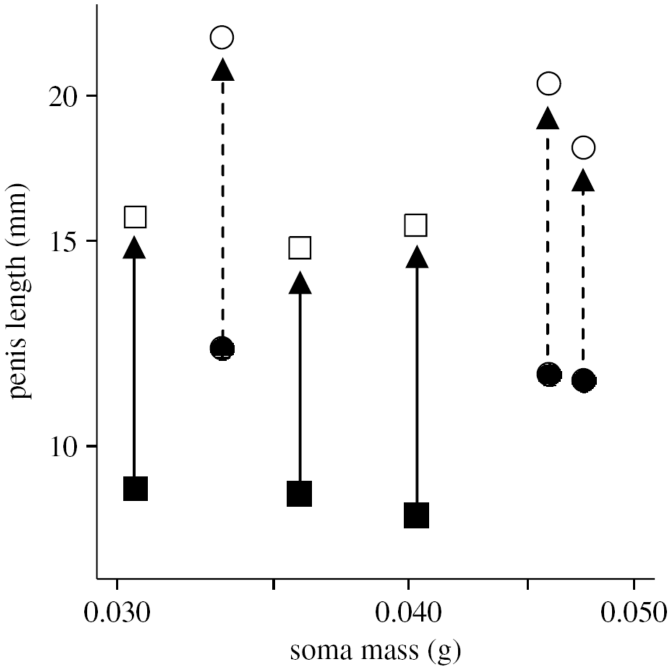

Figure 2-1 Relaxed penis length (closed symbols) and corresponding manually inflated penis length (open symbols) of barnacles from an exposed-shore (Prasiola Point; squares, solid lines) and a protected-shore (Grappler Inlet; circles, dashed lines) in Barkley Sound, British Columbia, Canada. Paired points represent individual barnacles and arrows denote transition from relaxed to inflated penis length (mean proportional inflation ± s.e.m.: protected-shore = 1.82 ± 0.082, exposed-shore = 1.75 ± 0.044, $p = 0.77$).

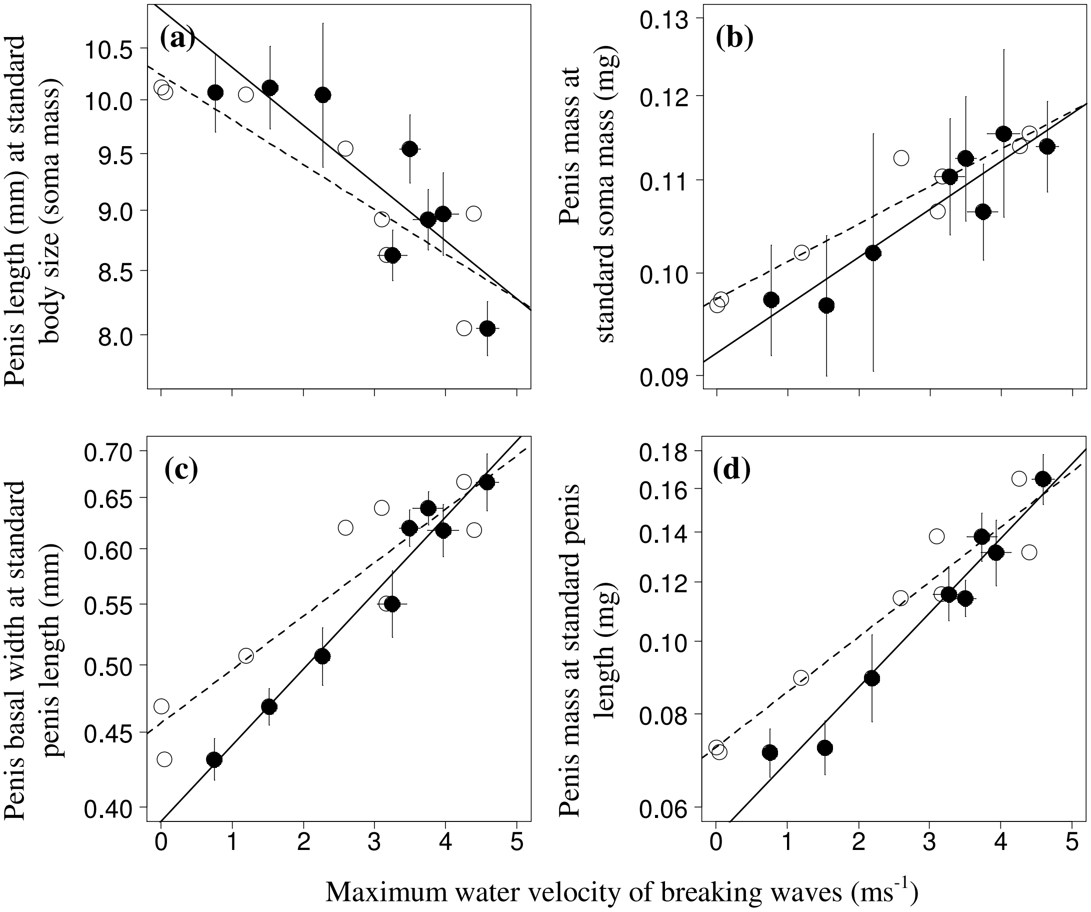

Figure 2-2 Variation in penis form of the intertidal barnacle *Balanus glandula* as a function of wave-exposure among eight sites in Barkley Sound, British Columbia, Canada. (a) Penis length of a standard-sized barnacle (soma wet mass = 0.0172 g, approx. 8 mm basal width; F1,6 = 16.476, p = 0.007). (b) Penis mass of a standard sized-barnacle (soma wet mass = 0.0172 g, approx. 8 mm basal width; F1,6 = 37.857, p < 0.001). (c) Basal width of a standard-length barnacle penis (penis length = 9.21mm; F1,6 = 154.05, p < 0.001, r2 = 0.96). (d) Mass of a standard-length barnacle penis (penis length = 9.21 mm; F1,6 = 149.05, p < 0.001, r2 = 0.95). Closed circles (solid line) represent velocities calculated from the known relationship between leg length and water velocity at each site (see methods). Open circles (dashed line) are empirically determined summer velocities at these sites (Arsenault et al. 2001; Marchinko & Palmer 2003). All points are mean ± s.e.m. (error bars are only shown for closed circles for clarity; in some cases error bars are smaller than the symbol size).

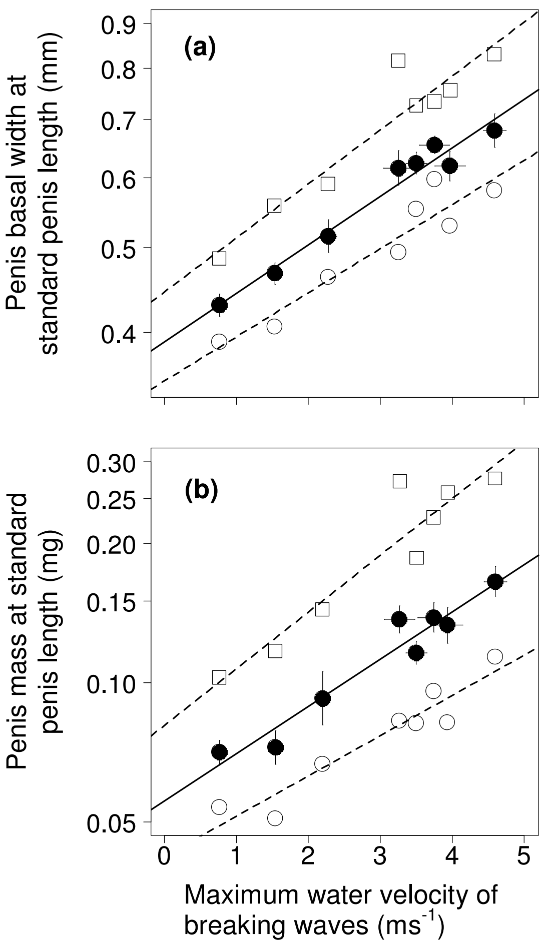

Figure 2-3 Variation in penis shape of the barnacle *Balanus glandula* as a function of wave-exposure, standardized to first quartile (open circles), mean (closed circles), and third quartile (open squares) of penis length using ANCOVA assuming unequal slopes among sites. (a) Penis basal width at standard penis length (corresponds to Fig. 2-2c). (b) Penis mass at standard penis length (corresponds to Fig. 2-2d). All points are mean ± s.e.m. (error bars are only shown for closed circles for clarity and are smaller than the symbols where they appear absent).

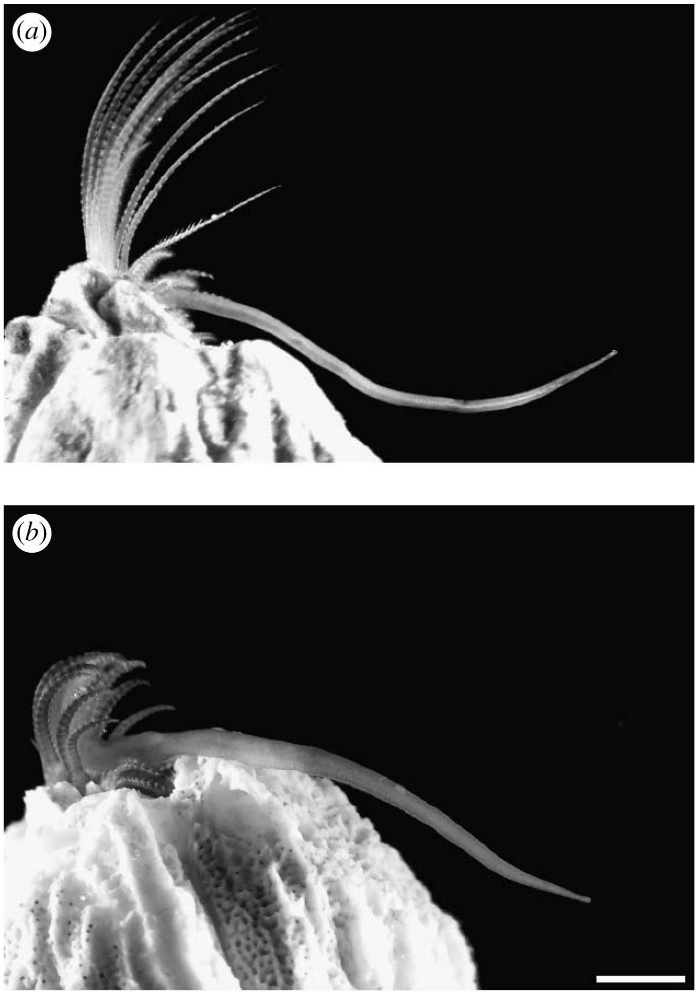

Figure 2-4 Relaxed[2] penis form of similar sized barnacles (*Balanus glandula*) from Barkley Sound, British Columbia, Canada. (a) Protected shore (Grappler Inlet, maximum velocity of breaking waves = 0.75 ms-1; soma wet mass = 0.019 g, basal width = 8.5 mm). (b) Wave-exposed outer coast (Seppings Island, maximum velocity of breaking waves = 4.5 ms-1; soma wet mass = 0.024 g, basal width = 8.9 mm). Scale bar 2 mm.

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[2] Barnacles were frozen for 48 hours and then the penis and legs were gently extracted out the operculum and photographed in seawater under a dissecting microscope.

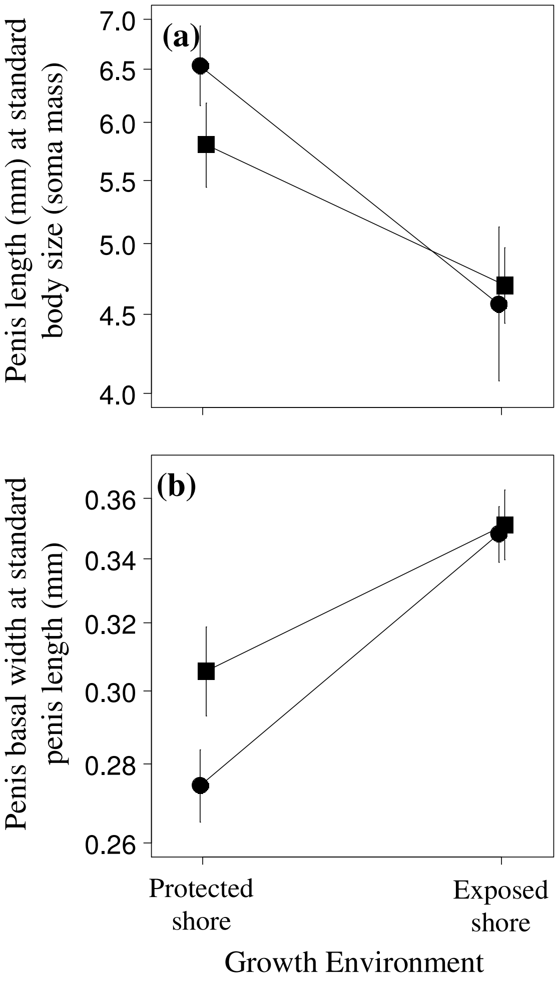

Figure 2-5 Relaxed penis form of the barnacle *Balanus glandula* from a protected shore (Ross Islets; closed circles) and exposed shore (Seppings Island; closed squares) 20 weeks after transplant to a protected shore (Bamfield Inlet) and exposed shore (Seppings Island) in Barkley Sound, British Columbia, Canada. (a) Penis mass of a standard sized-barnacle (soma wet mass = 0.0058 g, basal width approx. 6 mm). (b) Basal width of a standard-length barnacle penis (penis length = 5.59 mm). All points are mean ± s.e.m.

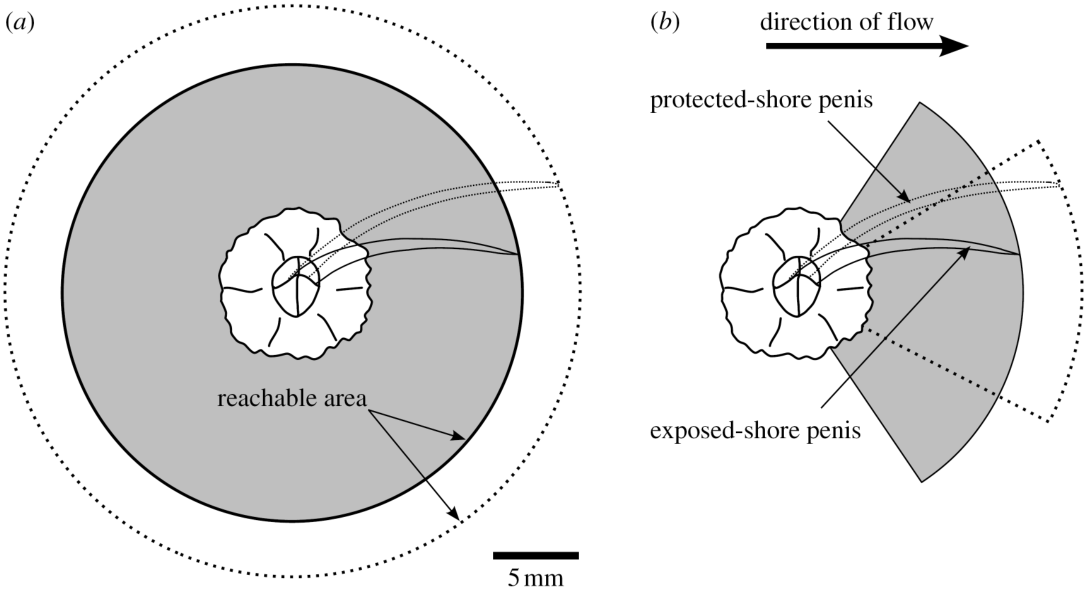

Figure 2-6 Proposed tradeoff between penis form and flow environment. (a) In quiet water, the 25% longer penis of a protected-shore barnacle relative to its exposed-shore counterpart (Fig. 2-2a) may increase the reachable area (the area within which to find mates) by as much as 90%[3] (compare area encompassed by dotted line to shaded area). (b) In high flow, a small increase in the ability of the stouter exposed-shore penis form to resist bending due to drag would enable the penis of an exposed-shore barnacle to extend across more streamlines and yield an effective reachable area (shaded region) greater than that of a similar-sized quiet water barnacle with a penis more prone to downstream deflection due to drag (area bounded by dotted line).

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[3] This calculation in the published paper was incorrect; the actual difference is only 65%.

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CHAPTER III. MODULAR PHENOTYPIC PLASTICITY: DIVERGENT RESPONSES OF BARNACLE PENIS AND FEEDING LEG FORM TO VARIATION IN DENSITY AND WAVE EXPOSURE[4]

3.1 Introduction

The generation of phenotypic variation is a key issue in evolutionary biology because this variation is the raw material upon which natural selection acts to yield phenotypic evolution. But some kinds of variation may arise more easily than others. For example, Darwin (1872) observed that selection on one trait sometimes led to “correlated variation” in other traits not under direct selection (e.g., pigeons bred for small beaks also have small feet). Darwin also noted that correlations sometimes arose even among seemingly disparate traits (e.g., white cats with blue eyes are often deaf). Since then, these and related ideas have received much attention under various headings, including genetic covariance (Atchley 1984), developmental bias (Brakefield 2006), phenotypic integration (Pigliucci and Preston 2004), modularity (West-Eberhard 2003, Klingenberg 2005) and behavioural syndromes (Sih et al. 2004). Although short-term correlations may arise via purely genetic mechanisms (e.g., through linkage or linkage disequilibrium) (Hill 1974), here I define coupled development as a correlation among traits that arises via truly developmental mechanisms (Moran 1992), for example, if traits share components of developmental pathways or compete for shared developmental resources. A good example of coupled development comes from butterflies, where the colours of serially-repeated eyespots on butterfly wings are developmentally coupled through a tissue level response to a signal concentration gradient and thus respond only to selection for coordinated change (Allen et al. 2008). Similarly, the developmental patterning gene *Distal-less* seems to play a key role in the elongation of multiple appendages in arthropods (Panganiban and Rubenstein 2002) including

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beetles (Moczek and Rose 2009), while other patterning genes influence the development of both limbs and genital appendages in mice (Dolle et al. 1993, Podlasek et al. 2002), and even in humans (Del Campo et al. 1999). Conversely, traits may also be developmentally linked through competition for shared developmental resources (Moran 1992), as are the relative size of legs and wings in a butterfly, and the horns and eyes of beetles (Nijhout and Emlen 1998).

Although many such examples of coupled development now exist for developmentally fixed traits, comparatively little is known about developmental coupling among traits that depend on external environmental inputs (but see Gianoli and Palacio-Lopez 2009). Studying coupled development among such morphologically plastic traits is particularly difficult because correlated variation may arise either through coupled development or through parallel plastic responses of independent traits to the same environmental cues. In other words, because phenotypes are a product of both an organism’s genes and its growth environment (West-Eberhard 2003), traits with independent development may nonetheless appear coupled if traits show parallel plastic responses to the same (or correlated) environmental cues. Although difficult, studies of developmental coupling among plastic traits are important because such coupling has the potential to limit the extent of phenotypic variation available to natural selection (Keightley and Hill 1990, Otto 2004) and thus may restrict the evolution of functionally independent plasticity in multiple traits (DeWitt 1998, Auld et al. 2009, Snell-Rood et al. 2010).

I tested whether developmental coupling can restrict the direction of plastic responses by studying the variation of acorn barnacle penis form and leg form in response to alternative, conflicting environmental cues. Acorn barnacles are sessile crustaceans common on rocky shores throughout the world. Most barnacles are hermaphrodites and reproduce by extending unusually long penises to transfer sperm to adjacent individuals (Fig. 3-1). Barnacles feed by filtering particles from the water using highly modified legs (called cirri). Although the feeding legs are homologous to crustacean walking legs, the barnacle penis has no known homolog in other crustaceans (Anderson 1994). Despite this difference in origin, however, the development of barnacle penises and legs may be developmentally linked. The barnacle penis is an unusually long medial structure that arises directly between the 6th (posteriormost) pair of feeding legs (Fig.

3-1). In this sense, penises and legs are directly adjacent body outgrowths – i.e., appendages – that may compete for shared developmental resources (Minelli 2002, 2003). Penises and legs also share some morphological similarities suggestive of similar developmental mechanisms, for example the presence of circumferential cuticular annulations and some shared nerve origins (Darwin 1854, Anderson 1994).

Although specific information on how barnacle legs and penises develop is lacking, what is known is that barnacles show remarkable developmental plasticity in both penis and feeding leg form (Marchinko 2003, Neufeld and Palmer 2008). Barnacles begin life as pelagic larvae that may drift long distances before settlement and metamorphosis (Strathmann 1987). However, as adults they are permanently affixed to the rock and must cope with widespread spatial variation in availability of nearby mates and site-specific flow conditions (Andersen et al. 2007). As a result, widely-separated populations show little detectable genetic differentiation (Brown et al. 2001, Sotka et al. 2004), and instead display remarkable levels of morphological plasticity in leg and penis form facilitated by regular molting of the cuticle throughout adulthood (Anderson 1994). For example, when transplanted to a wave-exposed shore, the common Pacific barnacle *Balanus glandula* Darwin grows penises and feeding legs that are substantially shorter and stouter than their protected-shore counterparts (Marchinko 2003, Neufeld and Palmer 2008). This demonstrably adaptive response for feeding legs (Marchinko 2007) is presumably adaptive for penises as well (Neufeld and Palmer 2008), an inference supported by the superior performance of wider penises on wave exposed shores in an Atlantic barnacle (Hoch 2009). Adult density may also influence penis and leg form. In an Atlantic barnacle, individuals from high-density aggregations have shorter penises than individuals with fewer neighbours (Hoch 2008); in another species, high density is associated with slightly longer legs (Lopez et al. 2007). However, such responses of individual appendages (i.e., penises or legs) to individual cues (i.e., water velocity or conspecific density) are uninformative as to how responses to one cue may limit adaptive responses to another.

To determine whether barnacles are capable of simultaneous adaptive responses to multiple cues, I tested how penis and leg form varied in response to two conflicting environmental cues – water velocity and conspecific density – in *Balanus glandula*. First, to assess the independence of leg- and penis-form variation (see Fig. 3-2) across different density and flow conditions, I compared leg and penis form variation of both isolated and grouped barnacles at multiple low-flow and high-flow sites. Second, using the same dataset, I asked whether the direction of concurrent responses of legs and penises to variation in conspecific density and water velocity were consistent with documented adaptive responses from past studies on barnacles involving single cues.

If development restricts adaptive plastic responses of penises and legs in barnacles, i) penis and leg form variation should be correlated across environments (Fig. 3-2a, b), and ii) for a given barnacle size, *longer* legs should always accompany *longer* penises across environments (or *shorter* penises if they show an antagonistic developmental trade-off). Furthermore, if development restricts the shape of penises or legs, appendages should maintain a consistent shape regardless of overall appendage size. For example, for a positive correlation, *longer* appendages would always also be *wider* (or always *narrower* for a negative correlation).

In contrast, if the direction of plastic responses in legs and penises is not restricted by shared development, lowered conspecific density should induce opposite responses in barnacle penises and legs: low density reduces the number of nearby mates which should favor longer penises (Hoch 2008), yet fewer neighbours increases the local water velocity through a reduction in the boundary layer (Abelson et al. 1993) which should favour shorter legs (Lopez et al. 2007). On the other hand, increased water velocity on wave-exposed shores should select for *parallel* responses in penises and legs: shorter and stouter penises *and* legs are favored to better resist bending due to drag (Arsenault et al. 2001, Neufeld and Palmer 2008). Unrestricted development would also allow the shape of individual appendages to vary independently (e.g., legs could get longer or shorter in different environments without becoming proportionally narrower or wider).

3.2 Materials and Methods

3.2.1 Field sampling

Adult barnacles (*Balanus glandula* Darwin; mean soma mass = 0.0203 g; range = 0.0103 - 0.0567) were collected between March 31 and April 4, 2008 at two wave-exposed and two wave-sheltered sites representing the extreme ends of a water velocity gradient in Barkley Sound, BC, Canada. All sites were west-facing with 5 to 15 degree slopes. Sheltered sites (S1 = Bamfield Inlet, S2 = Grappler Inlet, max. velocity approx. 0.05 m/s) are described in Marchinko and Palmer (2003); exposed sites (E1 = Prasiola Point, E2 = Seppings Island, max. velocity approx. 5 m/s) are described in Neufeld and Palmer (2008). Twenty-five barnacles were collected at each of two densities in the middle of the *B. glandula* zone at each site. The lateral wall plates of “low density” barnacles did not touch another barnacle but the centre of the operculum of at least one adjacent barnacle had to be within 2-3 cm of the centre of the operculum of the focal barnacle. The lateral wall plates of “high density” barnacles touched other barnacles on all sides and at least four adjacent barnacles had to have opercula within 1.5 cm. This method selected barnacles at both ends of the density range, but ensured that at least one potential mate was within reach of all barnacles sampled.

3.2.2 Sample processing

Samples were frozen at -20°C for at least 24 hours and measured following Neufeld and Palmer (2008). First, barnacles were thawed in seawater. Then, to reduce damage to the penis prior to measurement, it was carefully removed at the base (proximal to the relatively-rigid pedicel, taking care not to stretch or deform the penis during removal) and wet-mounted before the soma was blotted dry and weighed to the nearest 0.1 mg. Finally, the left, posterior-most thoracic leg was removed and wet-mounted and both the penis and legs were photographed under a dissecting microscope at 15-30× magnification using a six megapixel digital camera. Photographs were measured to the nearest 10 μm using Image-J (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2010). Penis basal diameter was measured at the proximal-most annulation (closest to the pedicel). Penis length was measured as a complex curve along the midline from this point to the tip of the penis (excluding setae). Leg basal diameter was measured at the basal-most segment of the ramus. Leg length was measured as a curve along the outer edge of the endopodite from the base to the tip of the ramus. To assess measurement error, I began by re-measuring a sub-sample of photos from 24 randomly selected individuals from across site and density combinations. This revealed repeatabilities (Whitlock and Schluter 2009) of 0.97-0.99 depending on the trait. In other words, 97-99% of variance in a trait was due to true differences, and only 1-3% due to error in measuring photographs. I did not assess the error due to small conformational changes in appendages due to wet-mounting in this study, but a number of observations suggest these differences will also be small. First, the relatively rigid cuticle (Neufeld, unpublished data) that covers both the penis and feeding legs should maintain a constant cross-sectional shape during handling. Furthermore, small differences in the curvature of the extended feeding legs should not influence measured leg length, much like bending a flexible stick should not influence its measured length. Penis length may be the most prone to error because the cuticle of the penis consists of many small folds (called annulations) that allow the penis to extend and contract. However, much care was taken to minimize conformational changes during handling; the penis was ablated below the unarticulated pedicel and handled immersed in seawater. Any dissections where the penis was stretched during removal were discarded and repeated with another sample.

3.2.3 Statistical analyses

All analyses were performed on $\log_{10}$-transformed data using R (R Development Core Team 2009). To correct for the effect of body size, measurements were standardized to a common soma mass of 0.0177 g using ANCOVA (after testing for a common slope among all treatment combinations, Table 3-1). Scatter plots and linear regressions on size-standardized means (on grouped data for each exposure-density combination) tested for correlations among trait values across environments (Fig. 3-3). ANCOVAs on unadjusted trait relationships are also reported for completeness (Table 3-2; Figure 3-4); however unadjusted data cannot detect how traits vary relative to one another where body size varies substantially so are not discussed in detail. Split-plot ANOVAs on size-standardized means at each treatment and site combination tested the role of exposure and density on leg and penis form (Table 3-3). All models conformed to assumptions of equal variances and normality of residuals.

3.3 Results

3.3.1 Appendage size co-variation

For a given barnacle size, the relative length of penises and feeding legs were independent across the range of density and exposure environments sampled (Fig. 3-3a) but, on average, barnacles with broader feeding legs also had broader penises (Fig. 3-3b) across the same range of environments.

3.3.2 Appendage shape co-variation

For a given barnacle size, barnacles with broad penises did not consistently have relatively longer or shorter penises (Fig 3c) across the range of environments sampled; however, across environments, barnacles with broad feeding legs also had shorter legs (Fig. 3-3d).

3.3.3 Direction of concurrent responses

Variation in conspecific density yielded opposite responses of barnacle penis length and leg length (Fig. 3-1, Fig. 3-5a, b, Table 3-1, 3-2). On average, penises were 18% shorter at high density (Fig. 3-5a, $F_{1,3} = 23.672$, $p = 0.017$), while legs were approx. 6% longer at high density (Fig. 3-5b, $F_{1,3} = 39.716$, $p = 0.008$). Density effects were independent of wave-exposure for both penis length (exposure-density interaction, $F_{1,2} = 1.902$, $p = 0.302$) and leg length (exposure-density interaction, $F_{1,2} = 0.598$, $p = 0.520$). In contrast, density had no effect on basal diameter of barnacle penises (Fig. 3-5c, $F_{1,3} = 0.449$, $p = 0.551$) and no consistent effect on leg basal diameter (Fig. 3-5d, $F_{1,2} = 7.865$, $p = 0.107$), but I found a significant interaction between density and wave-exposure on leg basal diameter ($F_{1,2} = 121.406$, $p = 0.008$).

Wave-exposure had no consistent effect on penis length ($F_{1,2} = 0.070$, $p = 0.817$) but legs were nearly 50% shorter on exposed shores ($F_{1,2} = 114.57$, $p = 0.009$). In addition, both penises (Fig. 3-5c, $F_{1,2} = 5133.3$, $p < 0.001$) and legs (Fig. 3-2d, $F_{1,2} = 75.697$, $p = 0.013$) were approx. 20% wider at the base on wave-exposed shores compared to wave-protected sites.

3.4.1 Discussion

Despite being closely adjacent, elongate appendages that extend several body lengths from the trunk (Fig. 3-1), barnacle legs and penises varied independently in response to multiple environmental cues (Fig. 3-3), and this variation in leg and penis form was consistent with documented adaptive responses from past studies of individual cues (Fig. 3-5). Most dramatically, high conspecific density yielded shorter penises but longer legs on all wave-exposed and all wave-protected shores (Fig. 3-5). In addition, at high flow, feeding legs were half as long as their low-flow counterparts yet penis lengths did not differ between low- and high-flow sites. Penis length and basal diameter also showed no association across environments (Fig. 3-3c) and varied in an adaptive way in response to multiple conflicting cues: penis basal diameter varied almost exclusively due to variation in wave exposure while penis length varied almost exclusively with variation in density (Fig. 3-5).

A decoupling of appendage length and basal diameter is consistent with past studies on barnacles. The Atlantic barnacle *Semibalanus balanoides* grows wider penises on wave-exposed shores compared to quiet harbours, but penis length does not vary with water velocity (Hoch 2009). Conversely, only density (and not exposure) influences penis length in *S. balanoides* (Hoch 2008). In four common Pacific barnacles (representing two orders and three families, including the species in the current study *B. glandula*) length and basal diameter of legs show contrasting responses to flow (Marchinko and Palmer 2003). Furthermore, a reciprocal transplant of *B. glandula* showed that wave exposure has opposite effects on penis basal diameter and penis length (Neufeld and Palmer 2008). I found no effect of wave exposure on penis length in the current study. However, the high among-site variation in penis length in this system (Neufeld and Palmer 2008) may have simply obscured any flow-induced variation in penis length in the current study, which surveyed fewer sites.

In contrast to the largely-independent variation in appendage length and penis shape, variation in wave exposure yielded a) nearly parallel changes in basal diameter of penises and legs (Fig. 3-3b), and b) antagonistic variation in leg length and basal diameter. So, developmental coupling among some traits cannot be ruled out. However, the decrease in feeding leg length and increase in feeding leg basal diameter at high-flow sites is adaptive in *B. glandula* (Marchinko 2007), as is the increase in basal diameter of penises in another species (Hoch 2009) – an increase in diameter should reduce bending due to drag, and the shorter appendages likely create less drag in the first place (Vogel 2003). Therefore, if present, such coupling could itself be an adaptive strategy to eliminate excess developmental baggage in traits where selection favors parallel variation in form (Cheverud 1996, Neufeld and Palmer 2008). It is of course worth noting that although developmental coupling does not appear to restrict the direction or extent of plastic responses in the legs and penises of barnacles, this does not imply that the developmental programs of these two appendages are entirely independent. Information on the mechanics of development of both legs and penises could shed light on the specific developmental processes that produced the observed variation in form; this is a promising avenue for future research.

Collectively, these observations suggest barnacles can sense multiple – sometimes conflicting – environmental cues and independently modify penis and leg form to suit local conditions. However, how a barnacle senses flow and neighborhood density remains unknown. One species has a set of remarkably refined mechanosensory proprioceptors at the base of the 6th pair of feeding legs, likely capable of sensing joint position, movement rate, and muscle tension (Clark and Dorsett 1978). These proprioceptors may simply allow barnacles to sense the position of their legs, or they might monitor stress or strain on the extended feeding net. If they do detect stress or strain, proprioceptors could provide a reliable trigger for the development of a flow-appropriate leg form. The cues that trigger a change in penis length are even less clear, but a few seem plausible. Chemicals released by a barnacle's neighbours may signal density. Alternatively, barnacles may simply respond to physical contact with a neighbour's shell. However, barnacles may actually be able to sense the spatial environment itself. Past observations suggest penis extension and movement (termed 'groping') accounts for as much as 10 percent of active time in *B. glandula* (Pentcheff 1995) and may be a reliable source of information about the number of reachable mates.

Because the current study used naturally occurring variation as a proxy for a developmental response, it is possible that some of the observed patterns in leg and penis form are due to viability selection (i.e., differential mortality) instead of developmental plasticity. However, this explanation seems unlikely. First, *B. glandula* is highly plastic and is known to dramatically change leg and penis form (Marchinko 2003, Neufeld and Palmer 2008). Second, it is hard to imagine how the shape of a barnacle's penis could increase the chance of mortality at all, and mortality due to slightly mismatched legs at a single site also seems improbable. For example, the approx. 6% difference in leg length between high and low density barnacles at any one site, although significant in the context of this paper, should be much too small to cause an increase in mortality, although barnacles with slightly mismatched legs may experience a slight decrease in growth while the optimum phenotype is produced.

Despite the apparent absence of developmental trade-offs among legs and penises in barnacles, barnacle penises represent a substantial energetic investment because of their huge size relative to the body (Fig. 3-1). Therefore, if certain developmental resources are limiting, modular plasticity in barnacle appendages may hinder the development of other traits through similar trade-offs. For example, on wave-exposed shores with low conspecific density, barnacles must grow wider penises to resist the force of breaking waves while simultaneously developing longer penises to reach more-distant mates. Therefore, in microenvironments where high water velocity occurs simultaneously with low density, the growth of longer, wider (Fig. 3-5), more-massive (Neufeld and Palmer 2008) penises may come at a cost of reduced overall growth or fecundity in these environments.

A few recent examples show how developmental coupling between plastic traits may restrict adaptation. In a study of two perennial plants, the amount of phenotypic integration (defined as the number of significant correlations with other traits) was negatively correlated with the amount of plasticity in any given trait (Gianoli and Palacio-Lopez 2009). In other words, the most plastic traits show the fewest correlations with other traits. Other studies suggest variation in temperature and photoperiod can affect plants' growth responses to light (Weinig 2000, reviewed in Valladares et al. 2007). In some animals, the development of an adaptive defense against one predator may limit the adaptive response to another. For example, a snail may attempt to fend off shell-entry predators by allocating shell material to the shell opening, but in doing so, the snail may reduce its ability to defend against shell crushing predators which attack other parts of the shell (DeWitt et al. 2000).

In summary, I found no evidence that developmental coupling restricted the direction of plastic responses in adjacent appendages of barnacles (*B. glandula*). This study represents one of the rare tests of developmental coupling between multiple (demonstrably adaptive) plastic traits, and suggests that barnacle legs and penises possess the capacity for independent (modular) adaptive plasticity. However, more such tests are required to assess whether developmental coupling limits the evolution of adaptive plasticity in other systems (Moran 1992, DeWitt et al. 1998, Auld et al. 2009).

Table 3-1 Results of ANCOVAs used in size-standardization of barnacle penis and leg measurements in figures 3 and 5. When testing equality of slopes the error degrees of freedom were 188. Significant p values (< 0.05) are highlighted in bold.

	Log(Penis length)				Log(Leg length)			
Source of variation	df	MS	F	p	df	MS	F	p
Log(soma mass)	1	0.485	118.59	< **0.001**	1	0.765	564.29	< **0.001**
Treatment combination	7	0.053	12.98	< **0.001**	7	0.831	612.92	< **0.001**
Error	195	0.75147	0.004		195	0.0014		
Equality of slopes	7	0.007	1.67	0.118	7	0.001	0.98	0.445

	Log(Penis basal width)				Log(Leg basal width)			
Source of variation	df	MS	F	p	df	MS	F	p
Log(soma mass)	1	1.189	405.09	< **0.001**	1	0.598	515.08	< **0.001**
Treatment combination	7	0.062	21.20	< **0.001**	7	0.030	25.70	< **0.001**
Error	195	0.003			195	0.001		
Equality of slopes	7	0.003	1.11	0.356	7	0.002	1.93	0.066

Table 3-2 Results of ANCOVAs on unadjusted trait relationships among exposure and density combinations (Fig. 3-4). Significant p values (< 0.05) are highlighted in bold.

	Log(Penis length)			
Source of variation	df	MS	F	p
Log(Leg length)	1	0.041	7.007	**0.009**
Exposure	1	0.197	34.056	**< 0.001**
Density	1	0.262	45.354	**< 0.001**
Error	200	0.006		

	Log(Penis basal diameter)			
Source of variation	df	MS	F	p
Log(Leg basal diameter)	1	1.326	312.845	**< 0.001**
Exposure	1	0.020	4.614	0.033
Density	1	0.003	0.788	0.376
Error	200	0.004		

	Log(Penis length)			
Source of variation	Df	MS	F	p
Log(Penis basal diameter) (PD)	1	0.447	102.885	**< 0.001**
Exposure (E)	1	0.102	23.562	**< 0.001**
Density (D)	1	0.210	48.224	**< 0.001**
PD × E	1	0.002	0.362	0.548
PD × D	1	0.038	8.747	**0.003**
E × D	1	0.001	0.177	0.674
Residuals	197	0.004		

	Log(Leg length)			
Source of variation	Df	MS	F	p
Log(Leg basal diameter) (LD)	1	0.099	59.185	**< 0.001**
Exposure (E)	1	6.339	3802.243	**< 0.001**
Density (D)	1	0.048	28.985	**< 0.001**
LD × E	1	0.000	0.170	0.680
LD × D	1	0.003	1.578	0.211
E × D	1	0.021	12.825	**< 0.001**
LD × E × D	1	0.008	4.922	**0.028**
Residuals	196	0.002		

Table 3-3 Results from split-plot ANOVAs used to test the role of exposure and density on barnacle penis and leg form (Fig. 3-5). Where not significant, interactions were removed from final models. Significant p values (< 0.05) are highlighted in bold.

	Log(Penis length)				Log(Leg length)			
Source of variation	df	MS	F	p	df	MS	F	p
Exposure	1	0.016	0.07	0.817	1	22.254	114.57	**0.009**
Error (Site)	2	0.229			2	0.194		
Density	1	4.865	23.672	**0.017**	1	0.085	39.716	**0.008**
Error (Site:Density)	3	0.206			3	0.002		

	Log(Penis basal width)				Log(Leg basal width)			
Source of variation	df	MS	F	p	df	MS	F	p
Exposure	1	0.018	5133.300	**< 0.001**	1	0.002	75.697	**0.013**
Error (Site)	2	0.000			2	0.000		
Density	1	0.000	0.449	0.551	1	0.000	7.865	0.107
Density:Exposure	-	-	-	-	1	0.000	121.406	**0.008**
Error (Site:Density)	3	0.000			2	0.000		

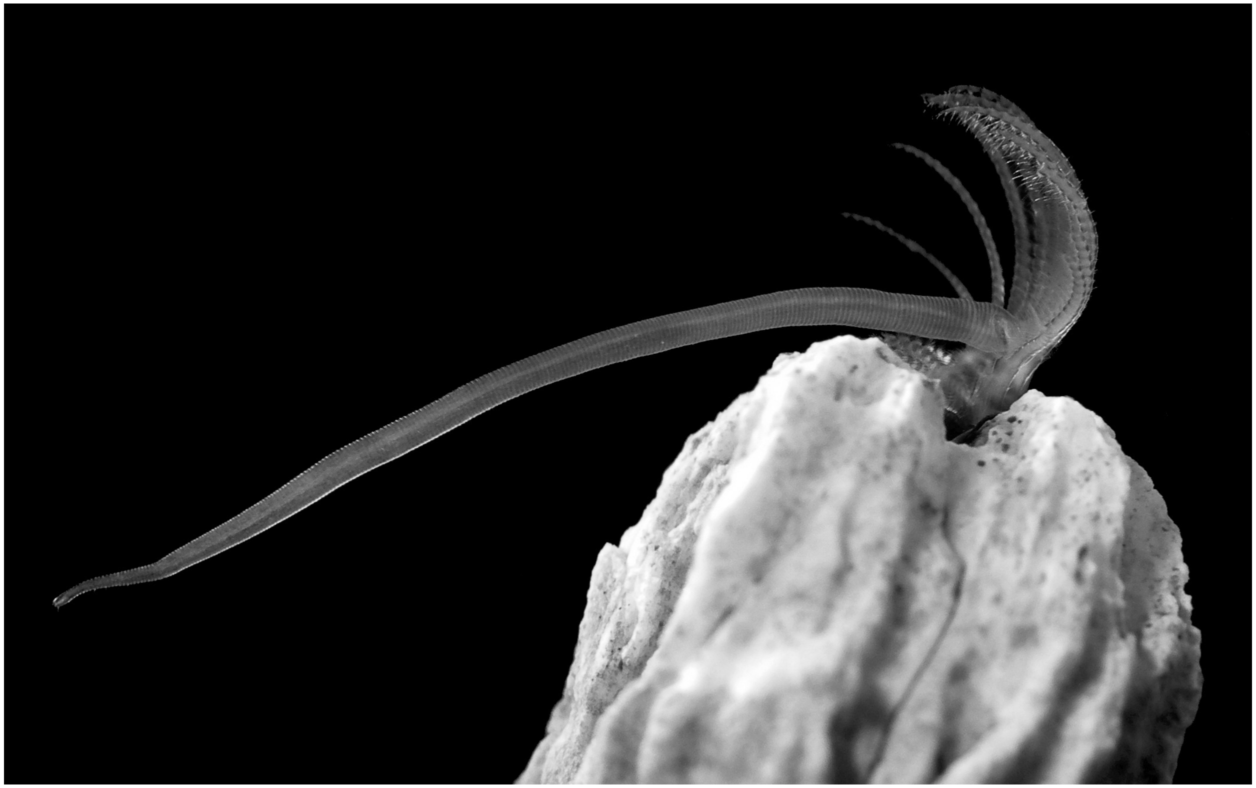

Figure 3-1 Partially extended penis and extended feeding legs of the Pacific intertidal barnacle *Balanus glandula* from a moderately exposed shore (Ross Islets, maximum force of breaking waves approx. 1 m s^{-1}, Neufeld and Palmer 2008). [5]

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[5] The barnacle was frozen for 48 hours and then the penis and legs were gently extracted out the operculum and photographed in seawater under a dissecting microscope. Soma wet mass = 0.0153 g; approx. 7 mm basal diameter.

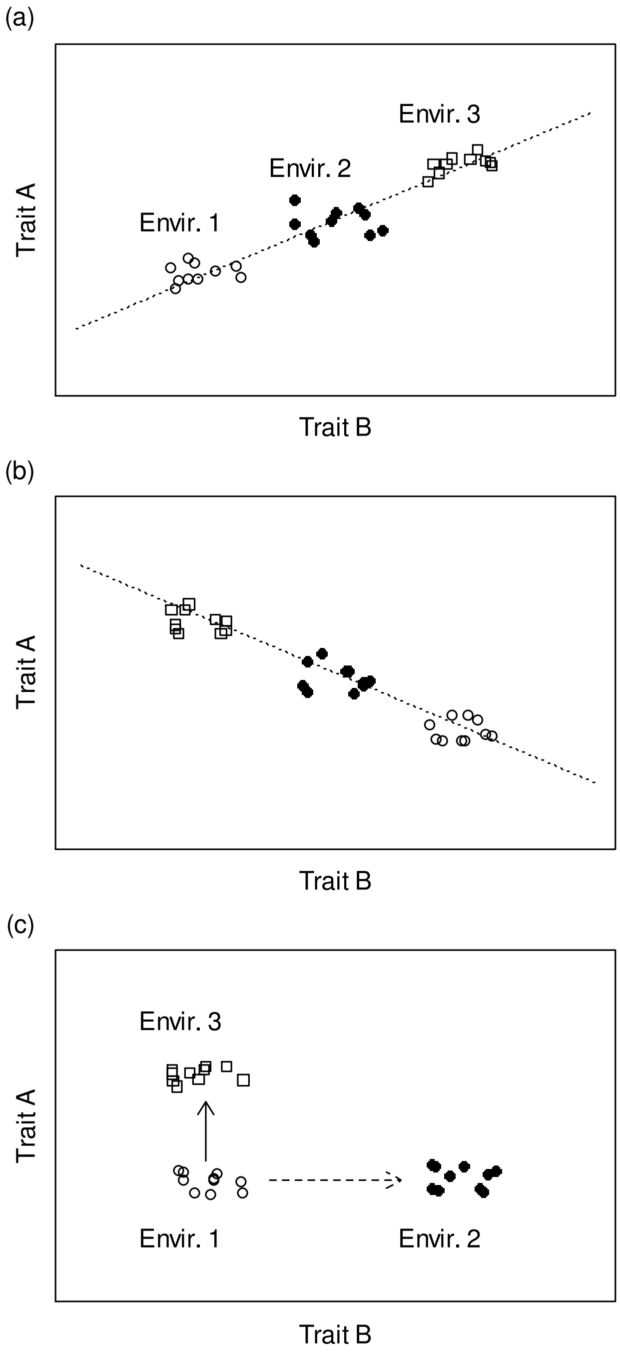

Figure 3-2 Hypothetical patterns of co-variation between two plastic traits in genetically homogeneous organisms raised in three different environments (each point corresponds to an individual organism). a, b) Coupled development: variation in trait A depends on variation in trait B (dotted line) across all environments, but the co-variation may be either parallel (a) or antagonistic (b). c) Independent development: traits A and B respond independently to different environmental cues (e.g., when compared to the phenotype expressed in environment 1, organisms in environment 2 alter trait B without changing trait A (dashed arrow), while in environment 3 they alter trait A but not trait B (solid arrow).

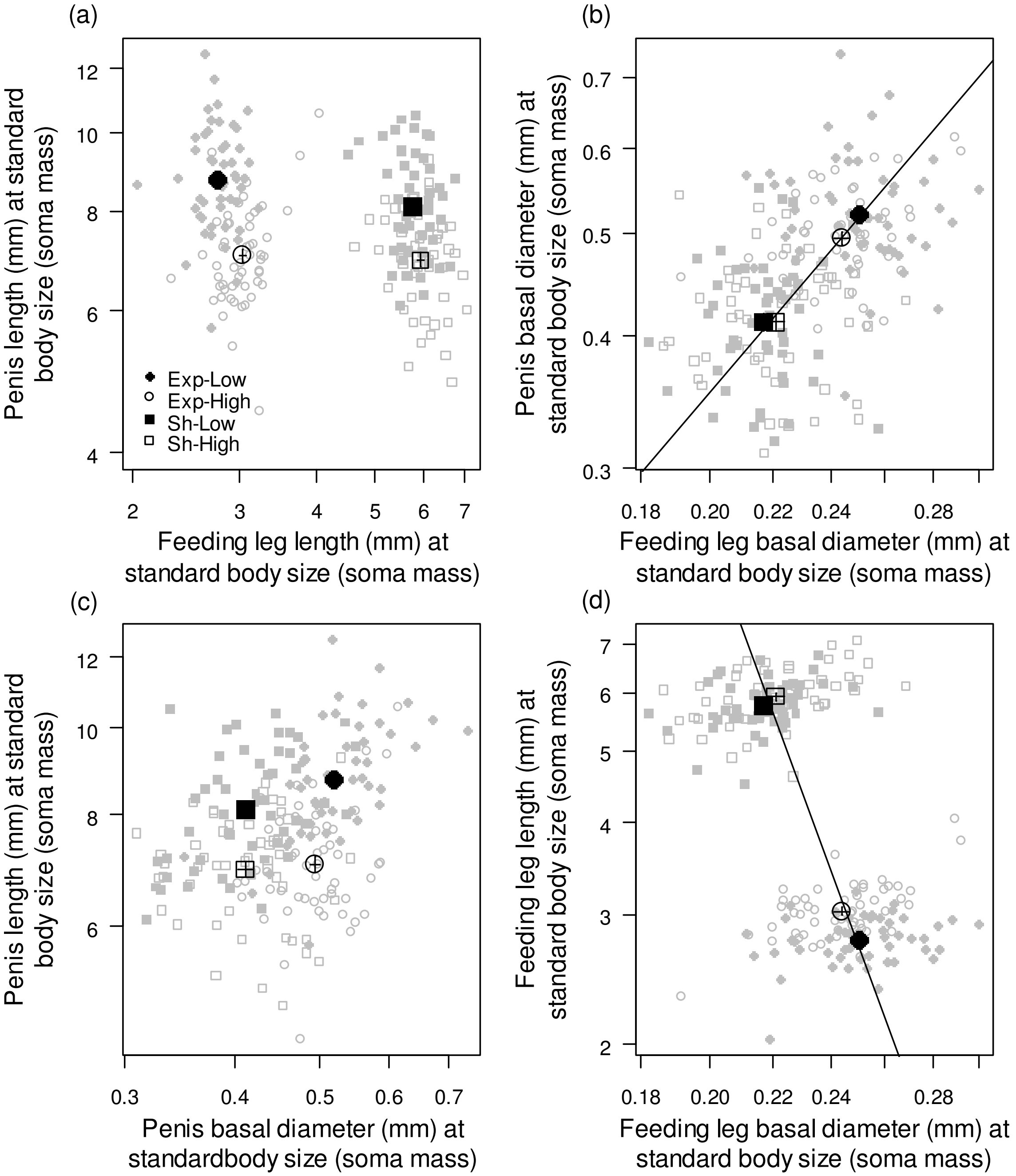

Figure 3-3 Co-variation between penis and feeding leg form as a function of wave-exposure and adult barnacle density in Barkley Sound, BC, Canada (data for the replicate sites at each exposure level are pooled). All measurements were standardized to an average-sized barnacle (soma wet mass = 0.0177 g, approx. 8 mm basal diameter) using ANCOVA (Table 3-1). Large symbols represent means for each unique exposure and density combination ± s.e.m. and small symbols are individual data points. Where significant, lines represent best-fit linear regressions on means. a) Penis length vs. Feeding leg length (Linear regression, F1, 2 = 0.222, p = 0.684), b) Penis basal diameter vs. Feeding leg basal diameter (F1, 2 = 193.2, p = **0.005**), c) Penis length vs. Penis basal diameter (F1, 2 = 0.3041, p = 0.637), d) Feeding leg length vs. Feeding leg basal diameter (F1, 2 = 94.66, p = **0.011**).

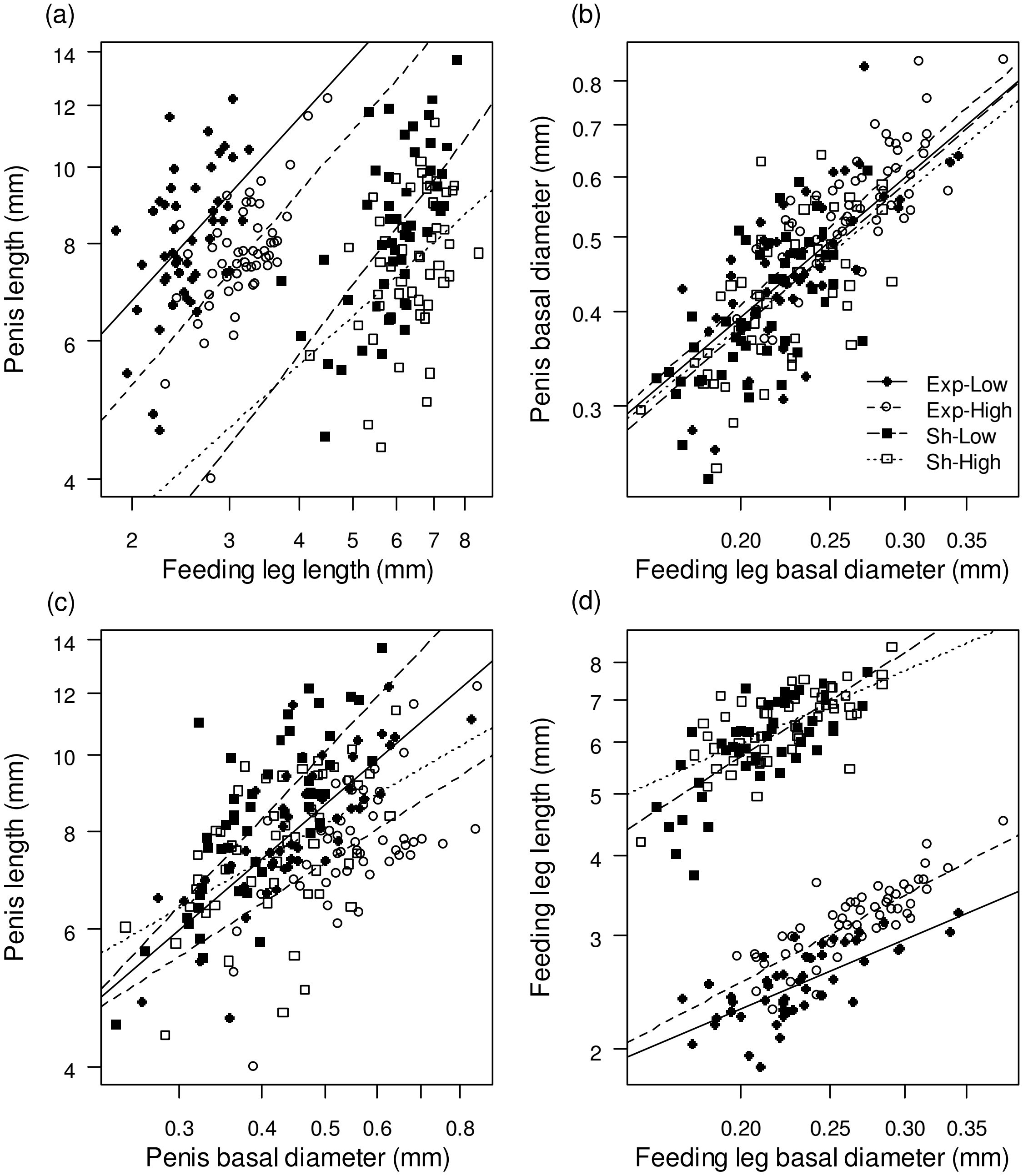

Figure 3-4 Relationship between unadjusted barnacle penis and feeding leg form as a function of wave-exposure and density in Barkley Sound, BC, Canada. Symbols represent unique exposure and density combinations pooled across sites. a) barnacle penis length was positively correlated with feeding leg length but also influenced by exposure and density (Table 3-2), b) penis diameter was positively correlated with leg basal diameter, (Table 3-2) and was only marginally influenced by variation in exposure ($R^2 = 0.009$) and not at all by density, c) penis length was positively correlated with penis basal diameter and this relationship was influenced by exposure and density, though the interaction between penis basal diameter and density was also significant, d) leg length was positively correlated with leg basal diameter, and this relationship also depended on numerous interactions among basal diameter, density, and exposure (Table 3-2).

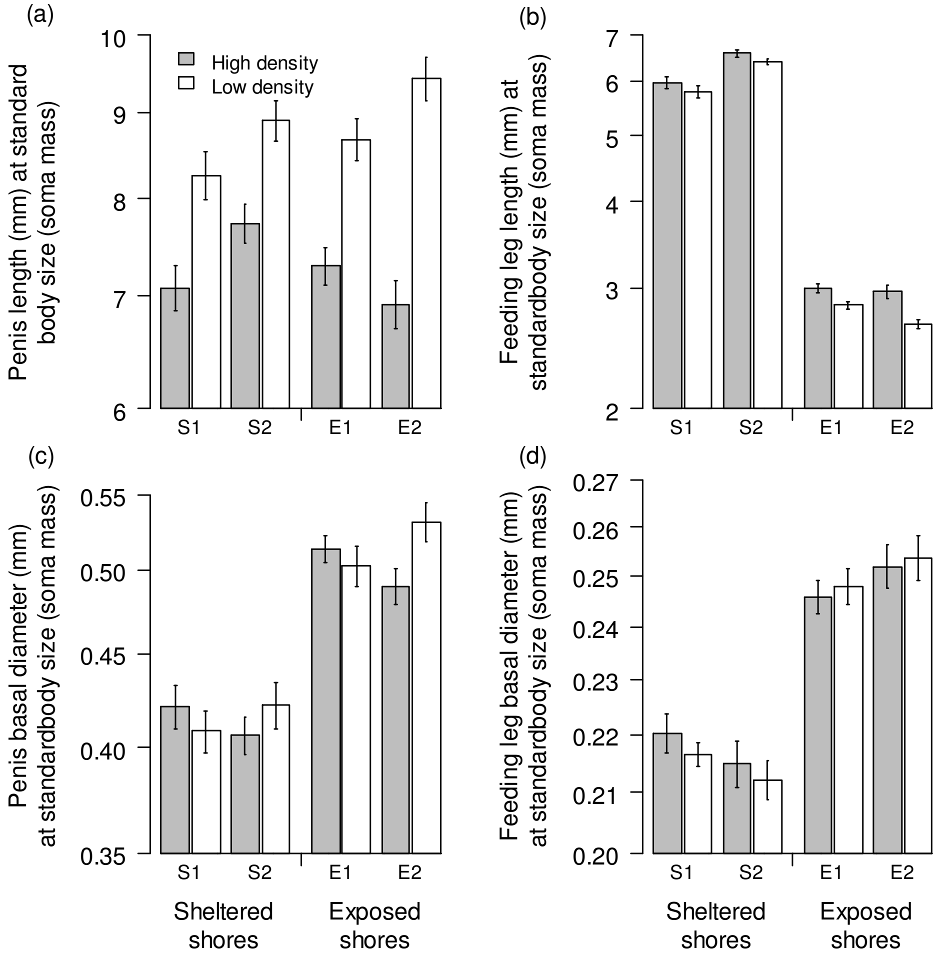

Figure 3-5 Penis and feeding leg form of barnacles (*Balanus glandula*) as a function of wave-exposure and density among four field sites in Barkley Sound, BC, Canada (Table 3-3). All measurements were standardized to an average-sized barnacle (soma wet mass = 0.0177 g, approx. 8 mm basal diameter) using ANCOVA (Table 3-1). a) Relaxed penis length, b) feeding leg length, c) penis basal diameter, and d) leg basal diameter. All bars show mean ± s.e.m.

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CHAPTER IV. BARNACLE APPENDAGE PLASTICITY: ASYMMETRICAL RESPONSE TIME LAGS, DEVELOPMENTAL MECHANICS AND SEASONAL VARIATION[6]

4.1 Introduction

To cope with variation in their environment, many organisms change their body form to match current conditions. Countless examples of such morphological plasticity now exist across a wide range of taxa and experimental systems (Pigliucci 2001): many plants change leaf, stem, and root form to better tolerate abiotic conditions and reduce competition (Callaway et al. 2003); plants and animals change body form to defend against would-be predators (reviewed in Tollrain and Harvell 1999); and both plants (Agrawal et al. 1999) and animals (Marshall 2008) can even alter the form of their offspring in response to environmental change. For morphological plasticity to be effective, however, responses must be fast enough to produce a body form matched to current conditions (Moran 1992, Tufto 2000). If responses are too slow, the environment for which a phenotype was developed may already have changed; if responses are too quick, the costs associated with producing a new phenotype may outweigh the benefits of the new form (Padilla and Adolph 1996, Gabriel et al. 2005).

Two theoretical models suggest that such response time-lags have a major impact on the evolution of reversible plasticity in variable environments. In a relatively simple model incorporating two fixed environmental states, plasticity is favoured over multiple fixed phenotypes when responses are quick, but plasticity ultimately becomes disadvantageous as responses get slower (Padilla and Adolph 1996). Similarly, in a more complex model incorporating a continuous variable environment, the benefits of plastic responses quickly decrease as responses become slower (Gabriel et al. 2005). Despite this well-recognized theoretical importance, surprisingly little is known about response time lags in natural systems (Vanalstyne 1988, Harvell and Padilla 1990, Gabriel et al. 2005), nor about the developmental mechanics that ultimately produce

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changes in form. However, development ultimately determines the speed of plastic responses, so knowing how – and how quickly – organisms can change body form is important in predicting when morphological plasticity should evolve (Gabriel et al. 2005).

I measured response time-lags and developmental mechanics of appendage form plasticity in the common Pacific acorn barnacle *Balanus glandula* Darwin following reciprocal transplants between wave-exposed and protected shores. Barnacles are famously plastic in many aspects of shell and body form; some species can alter shell form when exposed to cues from a potential predator (Lively 1986a, b), and many can dramatically modify shell form in response to many other environmental cues (Bourget and Crisp 1975, Crisp and Bourget 1985). Feeding leg and penis form of barnacles is also developmentally plastic (Marchinko 2003, Neufeld and Palmer 2008, Hoch 2009). In *B. glandula,* individuals on wave-exposed shores grow feeding legs and penises that are substantially shorter and stouter than their protected-shore counterparts (Marchinko 2003, Neufeld and Palmer 2008). Variation in conspecific density is also associated with independent and likely adaptive variation in leg and penis form (Neufeld 2011).

Despite this well-documented developmental plasticity in many aspects of barnacles appendage form in response to multiple environmental cues, a number of key questions remain unanswered. First, exposed-shore barnacles transplanted to a subtidal site in a quiet harbour grow longer feeding legs quickly (within 18 days; Marchinko 2003). However, response times under natural conditions in the intertidal are unknown. On the high shore where *B. glandula* is most common, immersion time (and thus access to food) is greatly reduced, potentially imposing an important energetic limit on response times. Furthermore, response time lags for the reciprocal transplant (from low to high flow) have not been investigated. Response time lags may be asymmetrical for two reasons, either because historic patterns of selection are more pronounced in one direction than the other, or due to asymmetrical developmental limits.

Second, very little is known about the developmental mechanisms that produce these changes in leg form. Barnacle feeding legs are composed of a short two-segment protopodite that then divides into two rami, each of which is made up of many jointed segments that allow the feeding legs to curl into the shell when not in use. Although varied terminology has been used to describe the individual units that make up the jointed paired rami of barnacles (Darwin 1854, Boxshall 2004), here I will follow Boxshall (2004) and use the term “segment” to refer to an individual element of the jointed ramus (I discuss this terminology in greater detail in the Discussion). Given this jointed construction, barnacles may change feeding leg length in two ways: by altering a) the number of ramus segments, and/or b) the length of each segment. Finally, barnacles experience variation in water velocity both in space (because larvae spend approx. 30 days in the water column before settlement), and in time (i.e., after settlement, due to seasonal winter storms and through changes in conspecific density due to settlement and mortality of nearby individuals). Past studies have compared barnacle leg and penis form among environments at a single time interval (Arsenault et al. 2001, Marchinko and Palmer 2003, Neufeld and Palmer 2008), or have relied on the results of short-term transplant experiments (Marchinko 2003, Neufeld and Palmer 2008, Hoch 2009). Therefore, whether barnacles respond to temporal variation at individual sites remains unclear.

In light of these gaps in knowledge, the goals of this study were to determine: (1) the time course of the plastic response following reciprocal transplants between low and high water velocity under natural conditions in the intertidal, (2) whether leg length changes are facilitated through changes in the number of ramus segments, and/or the length of each segment, and (3) whether populations of barnacles modify leg and penis form at multiple sites over time.

4.2 Methods

4.2.1 Transplant experiment

I transplanted barnacles from two source populations to each of four destination sites in Barkley Sound, British Columbia, Canada. On September 12, 2006, adult *Balanus glandula* Darwin (mean soma mass = 0.0064 g; range = 0.0004 - 0.0351) were collected on mussel shells (*Mytilus californianus* Conrad and *M. trossulus* Gould) from two source populations chosen for a substantial difference in wave force between sites and for a sizeable supply of adult barnacles growing on mussels: a moderately-protected shore (Ross Islets (RI) (Arsenault et al. 2001)) and an exposed shore (Seppings Island (SI) (Arsenault et al. 2001)) in Barkley Sound. Mussel shells were cut using a rotary tool so that one barnacle occupied each mussel shell fragment. 20 barnacles on mussel shell fragments were randomly selected to determine initial leg lengths from each source population. The remainder of mussel shell fragments were spaced approx. 13 mm apart and glued to twelve 10 by 13 cm Plexiglas plates using marine epoxy putty (Z-spar™ Splash Zone Compound) in a 10 by 7 grid alternating between protected- and exposed-shore source populations (yielding 35 barnacles from each source on each of three plates per site). Plates were kept overnight in flowing seawater and then three plates were bolted to the rock in the middle of the *B. glandula* zone at each of four outplant locations chosen for a more than five-fold variation in wave-force: a protected shore (Bamfield Inlet (BMSC); Marchinko and Palmer 2003), a moderately-protected shore (Ross Islets (Ross); Arsenault et al. 2001), and two exposed shores (Seppings Island (Seppings); Arsenault et al. 2001, Prasiola Point (Prasiola); Neufeld and Palmer 2008). At two-week intervals, two rows of barnacles were selected from each of three plates at each site and frozen prior to processing. Due to some mortality at each site, bi-weekly samples from each plate contained 3-7 barnacles (mean = 6) from each source population.

4.2.2 Seasonal variation survey

To determine whether barnacles respond to seasonal variation in wave force, I measured leg and penis form in three populations of adult barnacles (quiet water, moderately-exposed, and exposed-shore) every 2-3 months over approximately two years. I collected barnacles (mean soma mass = 0.0142 g; range = 0.0007 - 0.0683) from natural populations near three of the four transplant destination sites in Barkley Sound, British Columbia, Canada: BMSC, Ross, and Prasiola (all described above). Sites were chosen to span a range of wave-exposures and for ease of access during stormy winter months. At each site and sampling interval, I collected 10-15 barnacles from the middle of the *B. glandula* zone. To minimize the effect of conspecific density on leg length (Neufeld 2011), sampled barnacles touched other barnacles on all sides and at least four adjacent barnacles had opercula within 1.5 cm. Offshore wave height data was obtained from an offshore buoy (National Data Buoy Centre, station C46206 – La Perouse Bank). A past study on the California coast found a significant positive correlation between measured offshore wave height and onshore wave force in the majority of sites, suggesting such data are at least a reasonable proxy for onshore data where direct measurements are unavailable (Helmuth and Denny 2003).

4.2.3 Sample processing and measurement

Samples were frozen at -20 °C and processed as time permitted. Barnacles were thawed in seawater and the soma was removed, blotted dry and weighed to the nearest 0.1 mg following Arsenault et al. (2001). The 6th thoracic leg, from the left side, was wet mounted in seawater and photographed under a dissecting microscope at 15× with a 6mp digital camera. Photographs were measured to the nearest 10 μm using ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2010.). Feeding leg length and setae length were measured following Marchinko and Palmer (2003). Penis form was measured following Neufeld and Palmer (2008). Segment length was calculated as total ramus length divided by the number of segments (i.e., the mean segment length for each ramus).

4.2.4 Statistical analyses

All statistics were calculated using R 2.10.1 (R Development Core Team 2009) on $\log_{10}$-transformed data. However, for ease of interpretation, plots display back-transformed values plotted on a log scale wherever possible. For the transplant experiment, individual leg length measurements from the entire experiment were first adjusted to a common body size (0.0052g, soma mass) using ANCOVA (Table 4-1). Mean size-adjusted leg length was then calculated for each plate at each time interval and these plate means were used as independent replicates in all subsequent analyses. Speed of response was defined as the interval from the start of the experiment to the first interval that showed a clear departure from initial (explicitly defined as the first instance where confidence intervals of initial length and current leg length in the opposite wave-force regime did not overlap). Segment traits at day 57 were also adjusted to a common body size (0.005 g, soma mass) using ANCOVA (Table 4-2); then, to determine if segment number and segment length were plastic, I used a 2-way ANOVA with origin site, transplant site, and their interaction (Table 4-3).

For the seasonal variation experiment, traits were adjusted to a common body size (0.013 g, soma mass), or penis length (4.56 mm) using ANCOVA (Table 4-4). Then, to determine if natural populations of barnacles show temporal variation in leg and penis form I used a General Additive Model (GAM) on mean size-adjusted values at each site and sampling interval (Wood 2006). Data from the BMSC site is missing after July 2007 because of total mortality at this site, likely due to thermal stress during the previous month from an unusually hot day that coincided with a mid-day low tide (Neufeld, pers. obs.). Therefore, because not all sites were sampled at each interval, I applied a single smooth across all sites (Wood 2006), and included site as a covariate without interaction to account for consistent penis and leg form variation among sites (Neufeld and Palmer 2008). This approach allowed me to test whether, on average across all sites, barnacles showed any predictable temporal trends in aspects of leg and penis form (exemplified by a significant smoothed term in the GAM), but did not allow me to test whether the temporal trends differed among sites (Wood 2006).

4.3 Results

4.3.1 Speed and asymmetry of response under natural conditions

Feeding-leg length of quiet-water barnacles transplanted to an exposed shore took nearly twice as long to diverge from initial values (57 days, Fig. 4-1a) than did feeding-leg length of barnacles transplanted from an exposed shore to a protected bay (28 days, Fig. 4-1b), when corrected for body size. Furthermore, feeding-leg length of exposed-shore- and quiet-water barnacles converged more quickly in quiet-water transplant sites (Fig. 4-2a, c; Fig. 4-3) than when transplanted to exposed-shore sites (Fig. 4-2b, d; Fig. 4-3).

4.3.2 Developmental mechanics of leg length change

Leg-segment number and leg-segment length of transplanted barnacles were influenced by both transplant location and origin location. Barnacles *transplanted* to a wave-exposed shore produced feeding legs with 15% fewer segments ($F_{1,9} = 39.9$, $p < 0.001$; Fig. 4-4a) and 25% shorter segments ($F_{1,9} = 213.6$, $p < 0.001$; Fig. 4-4b) than counterparts moved to a protected harbour after 57 days, when corrected for body size. Barnacles *originating* from a wave-exposed shore produced feeding legs with 25% more segments ($F_{1,9} = 85.7$, $p < 0.001$; Fig. 4-4a) but only 9% longer segments ($F_{1,9} = 16.99$, $p = 0.003$; Fig. 4-4b) than barnacles originating from a protected harbour, and these effects were independent of transplant location (interaction: $F_{1,9} = 2.587$, $p = 0.146$; $F_{1,9} = 0.179$, $p = 0.683$).

4.3.3 Temporal variation of barnacle appendage form

Barnacle leg and penis form varied approximately seasonally throughout the sampling period. Leg length (GAM smooth term, $F_{6.6,\,34.4} = 4.195$, $p = 0.0015$; total deviance explained by the model (TDE) = 97.3%), and leg setae length ($F_{5.4,\,17.6} = 3.663$, $p = 0.014$; TDE = 96.1%) varied over time. Legs (Fig. 4-5a) and leg setae (data not shown) were longest in the summer.

Penis length ($F_{5.9,\,17.1} = 4.634$, $p = 0.0045$; TDE = 74.6%), and penis basal diameter ($F_{6,\,17} = 8.038$, $p < 0.001$; TDE = 82.9%) also varied over time; penises were longest (Fig. 4-5b) and widest (Fig. 4-5c) in the winter. Furthermore, basal diameter of penises adjusted to a common penis length also varied seasonally ($F_{4,\,22} = 5.275$, $p = 0.003$; TDE = 85%), being widest (for their length) in the winter (Fig. 4-5d).

4.4 Discussion

4.4.1 Response time-lags under natural conditions

Barnacles changed leg length almost twice as fast when moved from an exposed shore to a protected shore (28 days) than when moved in the opposite direction (57 days). This time lag asymmetry is in the opposite direction to a previous prediction for barnacles (Marchinko 2003), and conflicts with numerous predictions of time lag asymmetries in other systems. Past studies have suggested that time lags should be shorter in the direction deemed most stressful or risky. For example, two intertidal snails (*Nucella lapillus* (Linnaeus); Etter 1988, and *Littorina obtusata* (Linnaeus); Trussell 1997) and an intertidal sponge (Palumbi 1984) all develop wave-tolerant forms more quickly when moved from a protected shore to an exposed shore. In all three cases, the authors propose that the high risk of dislodgment and/or death of a mismatched body form on exposed shores has selected for shorter response time lags to minimize this cost. A related prediction has been supported in at least one unrelated system as well; red knots (a common shorebird) grow larger more muscular gizzards to a greater extent and slightly more quickly when switching to hard prey (a larger gizzard is required to digest such prey) than the speed and extent of change when switching back to more digestible soft prey (Starck 1999, Dekinga et al. 2001).

Why do barnacles appear to exhibit the opposite time lag asymmetry than that which would be predicted from past studies? Perhaps for barnacles, protected shores are the more stressful environment (i.e., patterns of stress or risk are opposite to those for snails and sponges). However, this explanation seems unlikely. The long legs of protected-shore *B. glandula* deform and ultimately become ineffective at food capture at much lower sustained water velocities than do the short legs of wave-exposed individuals (Marchinko 2007). Therefore, long-legged barnacles should have great difficulty feeding on exposed shores, where water velocities often reach more than 50× the threshold for effective feeding of quiet-water forms (Miller 2007). In contrast, although exposed-shore barnacles should capture somewhat less food than protected-shore barnacles in quiet water – because their feeding fans are smaller and their extending setae are spaced farther apart (Marchinko and Palmer 2003) – barnacles should still be able to feed regularly in this calm environment because they can extend their feeding nets continuously without risk of damage or deformation due to breaking waves.

Instead, I propose that the asymmetrical response time lags in the current study involve an interplay between leg length change, molting rate, and food acquisition. Barnacle legs are covered in a layer of cuticle, so barnacles must molt to change leg length. Molting rate, in turn, is tightly and positively correlated with food acquisition in many species. In *B. glandula*, molting declines nearly two-fold at times of year when barnacles acquire less food (as measured by fecal pellet production; Wu and Levings 1978). A similar pattern exists for other barnacles; when food is reduced by half in the laboratory, *Semibalanus balanoides* produce nearly 40% fewer molts and 40% fewer fecal pellets (Barnes and Barnes 1982), while *Balanus amphitrite* and *Balanus eburneus* nearly stop molting when starved in the laboratory (El-komi and Kajihara 1991). Therefore, because quiet water barnacles transplanted to wave-exposed shores should have very few opportunities to feed, their capacity for leg length change should also be greatly restricted. This is the most likely explanation for the substantially slower plastic response of barnacles transplanted under natural conditions in the current study.

The same energetic argument proposed above can also explain the nearly two-fold longer time lag in the current study, when compared to an earlier study (Marchinko 2003) where barnacles were transplanted to low flow and continuously immersed under a floating dock. In the high intertidal, barnacles are frequently out of the water (Harley and Helmuth 2003) and must wait until the tide returns before they can feed, while barnacles held under the dock have continuous access to food. Therefore, response time lags of barnacles on the high shore may be similarly limited by the ability to acquire enough food to initiate molting. More generally, barnacles should be limited in the speed or extent of plastic responses in any instances where food capture is restricted, whether this limit is due to reduced immersion time, asymmetrical form-environment mismatch, or any other cause.

Two experiments could shed light on the nature of such an energetic constraint on response times in barnacles. First, response times measured under a range of food regimes in the laboratory could directly investigate the link between food availability, molting rate, and leg-length change. This experiment could be easily performed using a series of recirculation flow chambers and an artificial food source. Presuming such an energetic limit exists, this experiment would also shed light on whether slower response times are associated with a slower overall molting rate, or merely less change per molt. Second, direct measurement of feeding efficiency of different feeding fans in different environments would confirm the extent of form-environment mismatch of exposed-shore and protected-shore feeding legs in both high and low flow. Together, these studies would elegantly parse out the mechanism behind what appears to be an important energetic constraint on developmental plasticity in barnacles.

Other documented examples of energetic constraints on plasticity are rare. However, at least one example confirms similar limits can negatively impact response times in other systems. In *Arabidopsis* (Brooks et al. 2010), seedlings adapted more quickly to a change in vertical orientation when supplied with more energy: younger seedlings (with more stored food reserves) reacted quicker than old ones, large seedlings reacted more quickly than small ones, and seedlings reacted more quickly when grown on a higher energy diet than on a more nutrient-limited one. Despite the rarity of other documented examples, such energetic limitations should be common anywhere plastic morphological responses influence resource acquisition (e.g., responses to light in plants), or where energetically costly processes (e.g. molting in arthropods) are necessary to produce a change in form.

The process of appendage regeneration in arthropods may offer some additional insight into the relationship between energetics and response time lags (albeit in the slightly different context of replacing a lost structure rather than the modification of an existing one). In arthropods, appendage regeneration can either accelerate or delay molting (reviewed in Maginnis 2006), suggesting the energetic requirements of molting, and the costs of losing an appendage, may vary among species and environments. Limb regeneration requires at least one molt, yet molting can be energetically costly (Maginnis 2006). In the absence of energetic costs, molting should occur quickly to alleviate the many possible costs of a missing appendage. However, where molting is costly, delayed molting may allow the acquisition of additional resources to offset this cost. Therefore, instances where molting is delayed could occur due to a lack of the necessary energetic resources, or because costs of a missing appendage are low. Conversely, accelerated molting may result either from low costs of molting, or because costs of a missing appendage are high. Interestingly, a link between resource availability and the overall cost of molting is supported by one additional observation; energetic trade-offs between regeneration and other functions such as growth or reproduction appear most often in field studies (where resources may be limited), yet often disappear when animals are fed ad libitum in the lab (Maginnis 2006).

An energetic argument may also explain the asymmetrical response time lags in sponges described earlier. Sponges from exposed shores have substantially smaller canals to circulate water (and thus food) around the organism than do protected-shore forms, resulting in more than two-fold higher energetic costs of pumping water through the animal in high-flow morphs (Palumbi 1984, 1986). The details of how sponges change body form is unknown; however, assuming some energetic investment is required, the higher cost of circulating water in exposed-shore sponges and its corresponding limit on food acquisition could explain the slower response of sponges transplanted from exposed- to protected shores (Palumbi 1984).

One final observation regarding the time lag asymmetry is worth mentioning. Given the severe limitations to food capture for protected-shore barnacles moved to an exposed shore, how could barnacles capture any food under these extreme conditions? The answer is twofold. First, many barnacles can feed by pumping water through their shells while keeping the feeding legs partly or fully retracted (Crisp and Southward 1961), allowing them to feed without the risk of extending their feeding legs into breaking waves. Second, in at least one species (*Chthamalus fissus*), individuals display remarkably fast feeding movements and are able to extend their feeding legs into the water column between wave impacts when flow is momentarily reduced (Miller 2007; Trager et al. 1992). Assuming this behaviour is common in other species, the ability to sense flow and dodge the largest waves should allow long-legged barnacles to extend their feeding nets during the brief periods of low flow on wave-exposed shores.

4.4.2 Developmental mechanics of leg length change

Leg-segment number and mean segment-length were influenced both by transplant location and source population. This provides clear evidence that both leg-segment number and segment length are developmentally plastic, and that barnacles use a combination of two mechanisms to lengthen or shorten their legs to cope with changes in water velocity.

Developmentally plastic responses of leg-segment length do not seem surprising, but plastic changes in leg-segment number raise a number of interesting developmental questions. Typically, leg segment number is fixed in arthropod limbs (Boxshall 2004), and segment identity is determined by molecular cues (Ibeas and Bray 2003, Angelini and Kaufman 2005). However, it is not clear whether barnacle feeding legs are composed of true segments. Boxshall (2004) defines true segments as “characterized by the presence of intrinsic muscles that originate, insert, or attach within each segment”, in contrast to “annuli” which “lack intrinsic muscle origins, intermediate attachments or insertions…”. In barnacle legs, the proximal section consists of a protopodite divided into two true segments, each of which clearly contains intrinsic musculature (Anderson 1994). Distal to the protopodite, the multiarticulated rami each contain only a single retractor muscle extending out to the tip of the ramus. However, because this retractor muscle appears to form at least some intermediate attachment in each ramus article (Neufeld, unpublished data; Boxshall 2004), the articulations of the ramus are not merely annuli. Instead, Boxshall (2004) calls this condition “secondarily multisegmented”, and describes a similar condition in at least one other group (the antenna of conchostracan crustaceans). In the *Drosophila* leg, true segments (e.g. the tarsus) develop before dividing further into separate annuli (e.g. tarsomeres), and these two processes often involve the expression of different developmental genetic pathways (Kojima 2004). In contrast, the Notch pathway is required for the development of all joints, regardless of whether these fall on true segments, or on further segmental subdivisions or annuli (de Celis et al. 1998, Kojima 2004). Therefore, knowing what genes are involved in barnacle leg segmentation may shed some light on how this secondary segmentation of the barnacle feeding legs relates to the development of segmented appendages in other arthropods, such as *Drosophila*, where patterns of gene expression are better known (Angelini and Kaufman 2005). Furthermore, how barnacles alter the number of “segments” from one molt to the next is another fascinating question worthy of further study, because the number of “segments” of the old cuticle would seem to constrain the potential number of “segments” of new leg developing inside that cuticle prior to molting. Interestingly, when a single ramus has been damaged in the field, regenerating segments appear beneath the cuticle of the distal-most complete segment of the damaged leg (Neufeld personal observation). However, whether a similar process is involved in the plastic response to wave force is unknown.

In additional to the variation arising via developmental plasticity, the significant effect of source population (i.e., origin location) suggests leg-form variation is influenced by a genetic or historic component. In other words, although barnacles always produced legs with more and longer segments in low flow, barnacles from exposed shores always have fewer (and slightly shorter segments) than barnacles from quiet harbours, regardless of transplant location. This origin-site effect could be due to three causes: i) selective settlement, where barnacles with fewer and shorter segments preferentially settle in low flow areas, ii) selective mortality, where barnacles settle at random, but those with fewer and shorter segments preferentially survive in low flow, or iii) historic effects, where early experience determines the range of plastic responses available to each individual later in life. Unfortunately, the current study cannot distinguish among these three causes. Regardless of the cause, however, this origin site effect suggests developmental limits to segment number – and to a lesser extent, segment length – may account for the lack of convergence in leg length between source populations at any of the transplant sites (Fig. 4-2).

4.4.3 Seasonal variation in leg and penis form

Most aspects of barnacle leg and penis form varied in a manner consistent with adaptation to seasonal variation in water velocity over the 1.5 to 2 years of sampling (Fig. 4-5). Feeding legs and leg setae were longest in the summer when wave-height was low, and shortest in the winter when wave-force increased due to winter storm events. Penis diameter and diameter at standard length were also greatest in the winter. Shorter, stouter legs and leg setae, and wider penises are all adaptive in high flow (Marchinko 2007; Neufeld and Palmer 2008; Hoch 2009) because they should all reduce bending due to drag (Vogel 2003). Therefore, this study provides the first evidence that barnacles use phenotypic plasticity to adapt to variation in flow at a single site during their lifetimes.

However, not all traits varied in a manner consistent with adaptation to seasonal wave force. Penis length was greatest in the winter when wave force is largest (Fig. 4-5), even though longer penises are expected to increase bending due to drag (Vogel 2003), suggesting other factors besides seasonal variation in wave force are acting on penis form plasticity. Many barnacles grow longer penises during the winter mating season, and reduce the size of their penises (or lose them entirely in the most extreme cases) when not in use (Barnes 1992). Although this seasonal reduction in penis length appears to be quite modest for *B. glandula*, the seasonal change in penis form should allow barnacles to maintain a long penis only when it is needed for mating, while reducing the energetic cost of producing and maintaining such a large structure when not in use.

4.4.4 Conclusions

Although development is often mentioned as the ultimate limit to the speed of plastic responses, energetic constraints on response times are rarely studied. Here I show that developmental response time lags are longer under conditions where energy acquisition should be lower, a pattern consistent with a developmental limit restricting response time lags in barnacles. Results also shed light on the developmental processes barnacles use to change leg form, showing barnacles alter both segment length and the number of leg segments to change overall leg length. Finally, this study provides the first evidence that barnacles change leg and penis form in a manner consistent with adaptation to seasonal variation in wave force.

Table 4-1 Results of ANCOVA used in size-standardization of barnacle leg length in the transplant experiment (Figs. 4-1 to 4-3). When testing equality of slopes the error degrees of freedom were 620. Significant p values (< 0.05) are highlighted in bold.

	log (leg length)			
	df	MS	F	p
log (soma mass)	1	5.1318	1786.961	**< 0.0001**
unique origin-transplant-day combination	41	0.143	49.794	**< 0.0001**
error	661	0.0029	-	-
equality of slopes	41	0.0037	1.3139	0.09498

Table 4-2 Results of ANCOVA used in size-standardization of barnacle leg segment number and mean segment length in transplant experiment (see Fig. 4-4). When testing equality of slopes the error degrees of freedom were 126. Significant p values (< 0.05) are highlighted in bold.

	log (segment number)				log (mean segment length)			
	df	MS	F	p	df	MS	F	p
log (soma mass)	1	0.1088	107.4100	**< 0.0001**	1	0.5497	172.3060	**< 0.0001**
unique site-day combination	7	0.0630	62.2500	**< 0.0001**	7	0.0441	38.3820	**< 0.0001**
error	133	0.0010	-	-	133	0.0012	-	-
equality of slopes	7	0.0014	1.4224	0.2019	7	0.0004	0.3471	0.9305

Table 4-3 Results of ANOVA testing effects of source population and transplant location on segment number and segment length (Fig. 4-4). When testing the interaction the error degrees of freedom were 8. Significant p values (< 0.05) are highlighted in bold.

	log (segment number at standard body size)				log (mean segment length at standard body size)			
	df	MS	F	p	df	MS	F	p
source	1	0.0288	85.7040	**< 0.0001**	1	0.0029	16.9950	**0.0026**
transplant	1	0.0134	39.9000	**0.0001**	1	0.0367	213.6430	**< 0.0001**
residuals	9	0.0003	-	-	9	0.0002	-	-
source x transplant	1	0.0007	2.5872	0.1464	1	0.0000	0.1788	0.6835

Table 4-4 Results of ANCOVA used in size-standardization of traits in seasonal variation survey (see Fig. 4-5). When testing the interaction the error degrees of freedom were 543. Significant p values (< 0.05) are highlighted in bold.

	df	MS	F	p
	log (feeding leg length)			
log (soma mass)	1	0.1218	57.2054	**< 0.0001**
site	2	2.4797	1164.8866	**< 0.0001**
residuals	545	0.0021	-	-
interaction	2	0.0060	2.8464	0.0589
	log (feeding leg setae length)			
log (soma mass)	1	0.0397	11.3612	**0.0008**
site	2	1.0109	289.2299	**< 0.0001**
residuals	359	0.0035	-	-
interaction	2	0.0029	0.8415	0.4319
	log (penis length)			
log (soma mass)	1	2.7152	170.7228	**< 0.0001**
site	2	0.1263	7.9440	**0.0004**
residuals	280	0.0159	-	-
interaction	2	0.0154	0.9695	0.3806
	log (penis diameter)			
log (soma mass)	1	4.5479	496.7774	**< 0.0001**
site	2	0.1354	14.7884	**< 0.0001**
residuals	282	0.0092	-	-
interaction	2	0.0041	0.4495	0.6384
	log (penis diameter)			
log(penis length)	1	3.7915	360.3018	**< 0.0001**
site	2	0.6255	59.4392	**< 0.0001**
residuals	291	0.0105	-	-
interaction	2	0.0153	1.4567	0.2347

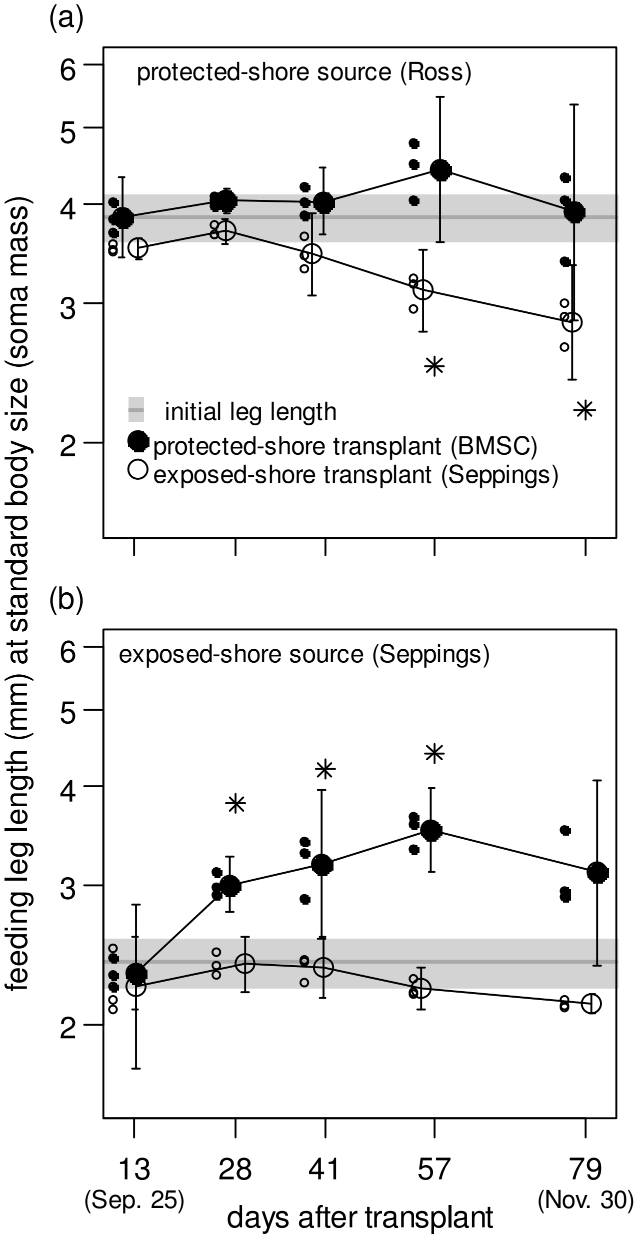

Figure 4-1 Feeding-leg length of barnacles (*B. glandula*) at standard body size (soma mass, 0.0052g) from two source populations after transplant to a protected-shore (BMSC) and an exposed-shore (Seppings) in Barkley Sound, British Columbia, Canada. Plate means (small points) were used to calculate grand mean (large points) ± 95% confidence intervals (CI). Initial leg length denoted by horizontal grey line (± 95% CI, grey shading). Stars denote intervals where CIs of initial source populations do not overlap CIs from barnacles transplanted to the opposite wave-force regime (i.e., intervals showing significant departures from initial values).

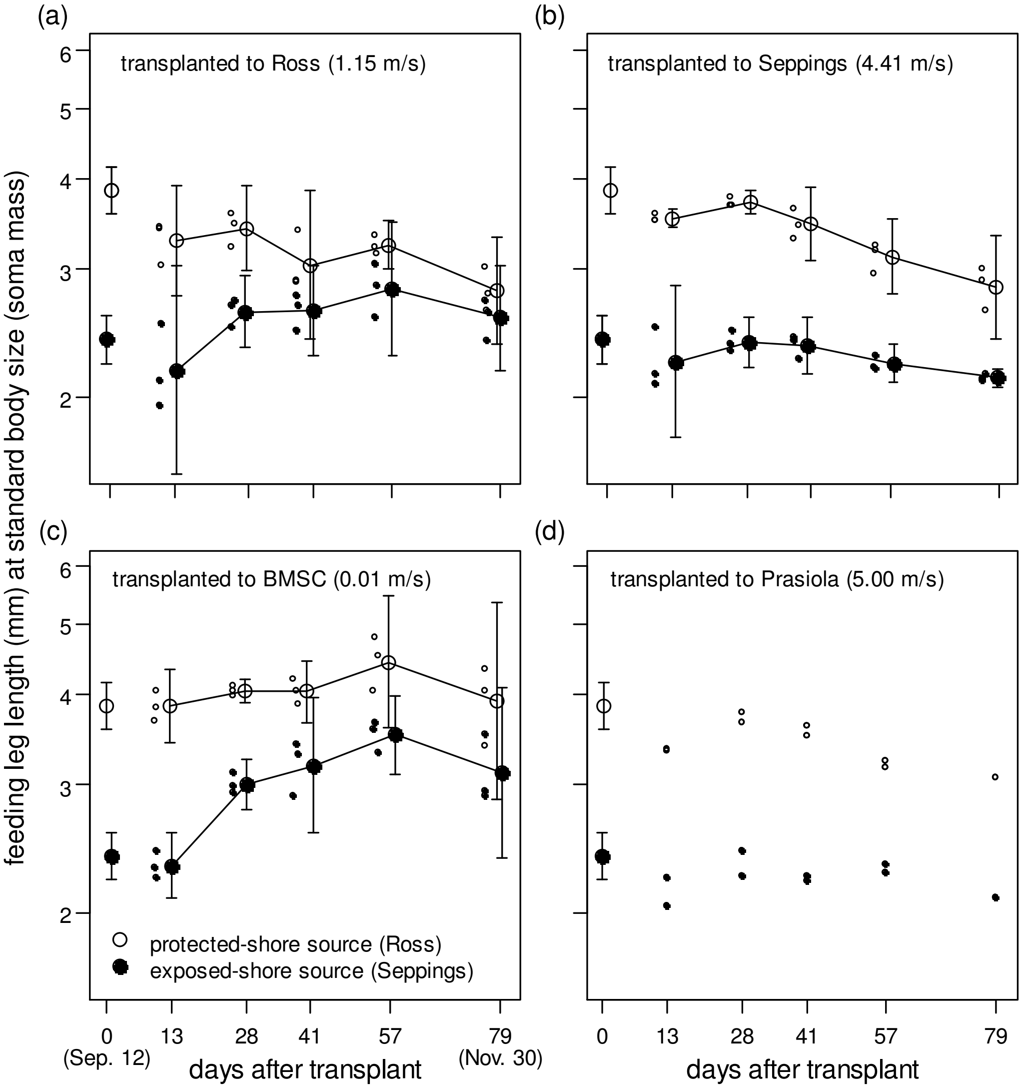

Figure 4-2 Feeding leg length of barnacles (*B. glandula*) at standard body size (soma mass, 0.0052g) from two source populations after transplant to four sites (a-d) along a range of wave-exposure in Barkley Sound, British Columbia, Canada (water velocities in parentheses correspond to Neufeld and Palmer 2008). Plate means (small points) were used to calculate grand mean (large points) ± 95% confidence intervals (CI). Due to loss of one plate at Prasiola Point during a storm, grand means and CIs are not displayed for this site.

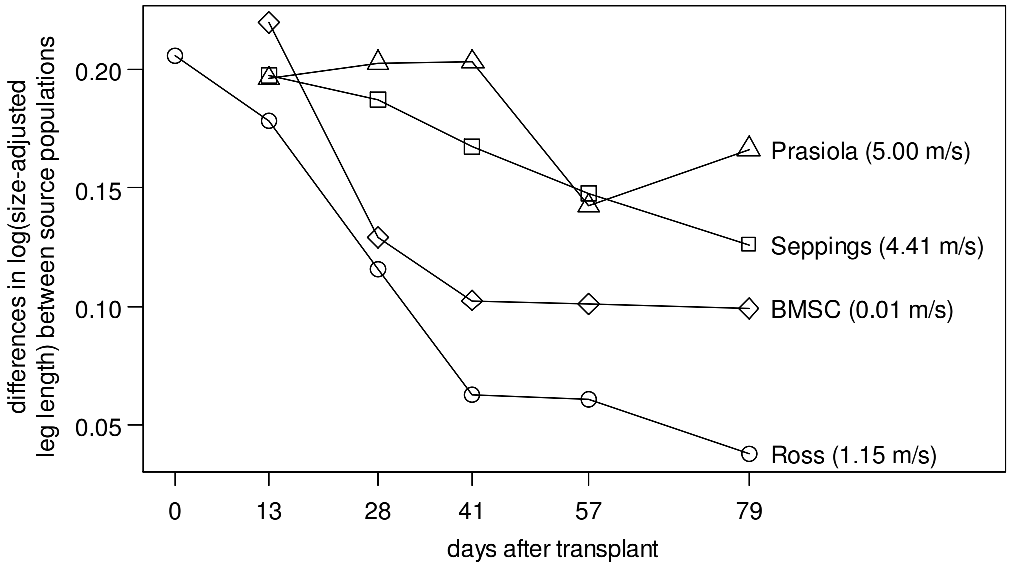

Figure 4-3 Differences in log-size-adjusted leg length between source populations at four transplant sites in Barkley Sound, British Columbia, Canada (water velocities in parentheses correspond to Neufeld and Palmer 2008). Values correspond to difference between source populations at each site and time interval in Figure 4-2. A decrease over time suggests source populations are converging at transplant sites; a consistent value over time suggests a lasting source population effect.

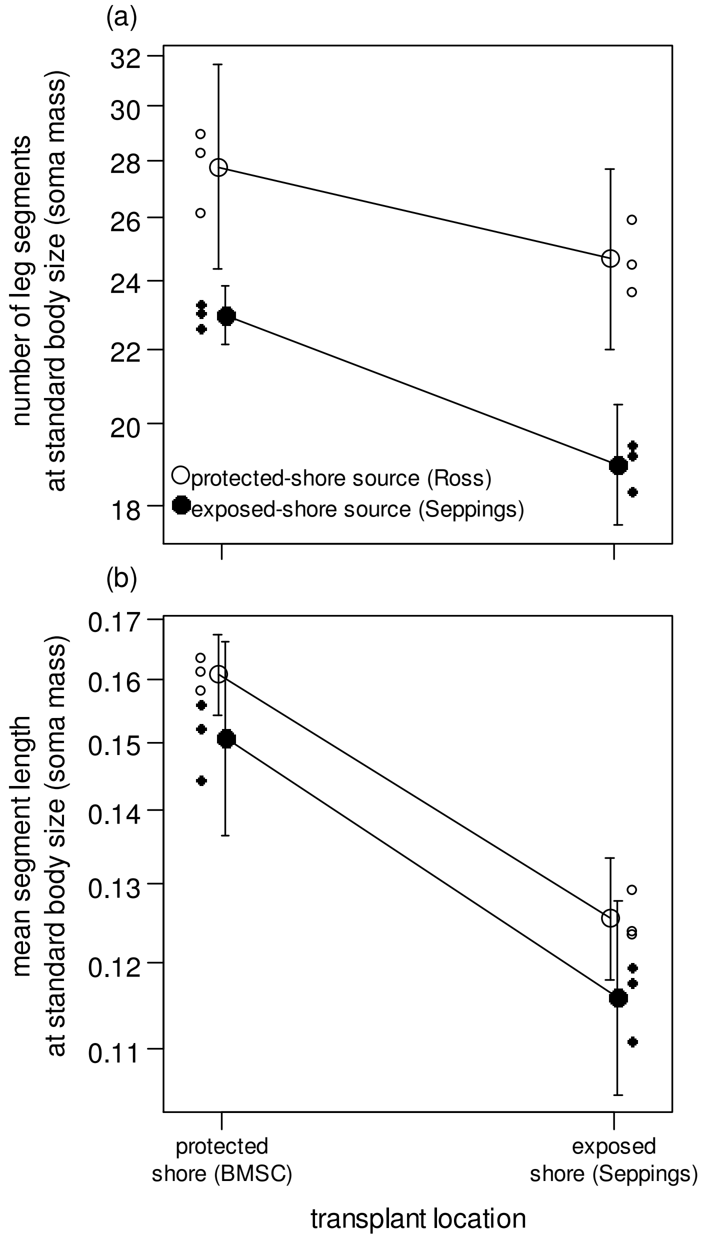

Figure 4-4 Leg-segment number of *Balanus glandula* at day 57 of transplant experiment: a) segment number at standard body size (soma mass, 0.0051 g) and b) mean segment length at standard body size (soma mass, 0.0051 g). Small points are plate means used to calculate grand mean (large points) ± 95% confidence intervals.

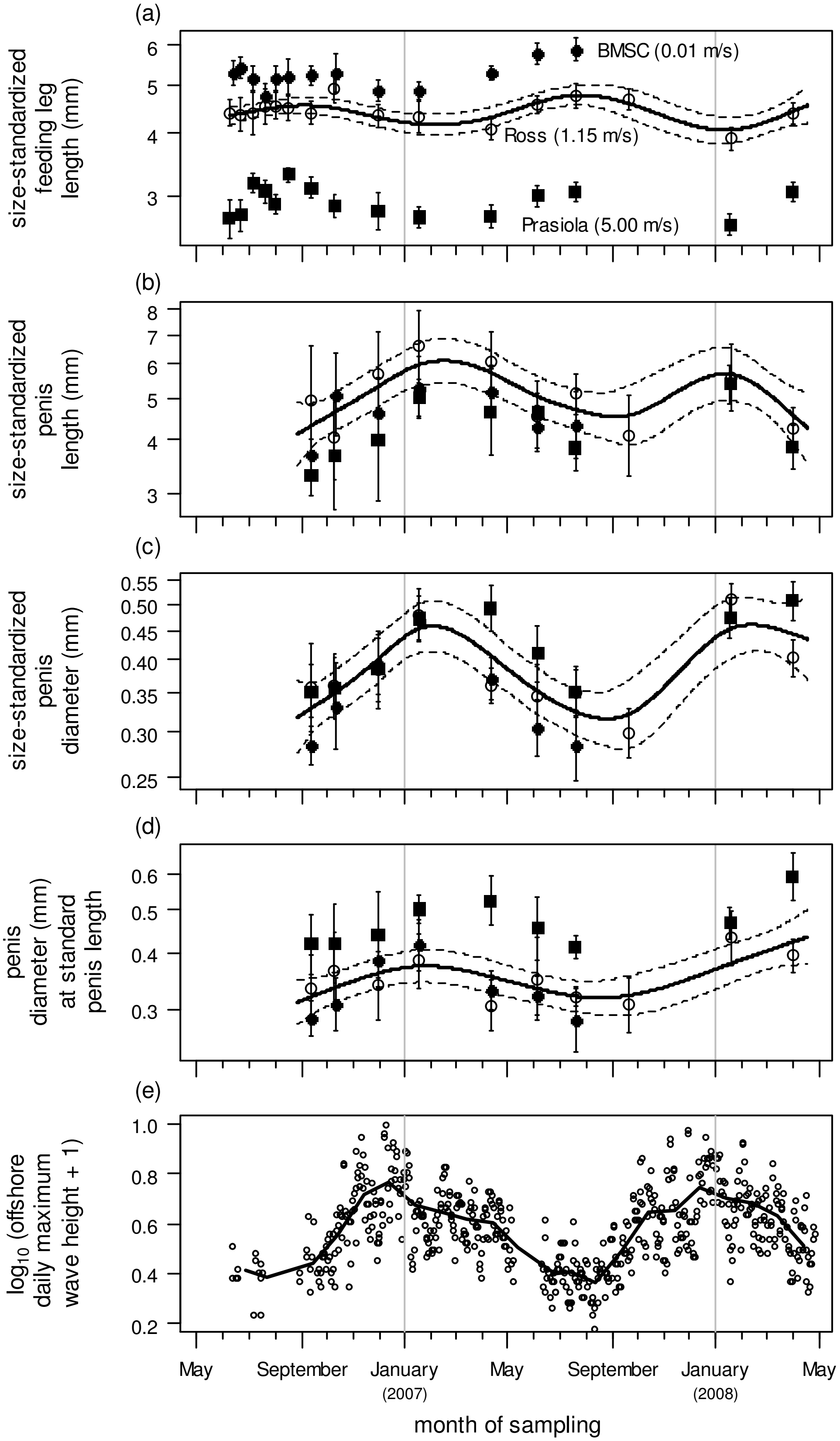
(a)
size-standardized feeding leg length (mm)
6
5
4
3
BMSC (0.01 m/s)
Ross (1.15 m/s)
Prasiola (5.00 m/s)
(b)
size-standardized penis length (mm)
8
7
6
5
4
3
(c)
size-standardized penis diameter (mm)
0.55
0.50
0.45
0.40
0.35
0.30
0.25
(d)
penis diameter (mm) at standard penis length
0.6
0.5
0.4
0.3
(e)
$\log_{10}$ (offshore daily maximum wave height + 1)
1.0
0.8
0.6
0.4
0.2
May
September
January
(2007)
May
September
January
(2008)
May
month of sampling

Figure 4-5

Figure 4-5 Temporal variation in feeding leg and penis form in three natural populations of the Pacific acorn barnacle *Balanus glandula* in Barkley Sound, BC, Canada (water velocities in parentheses correspond to Neufeld and Palmer 2008). n = 7-15 (mean = 13) per site/sampling interval combination. (a) to (d): points are size-standardized means ± 95% confidence interval (CI); solid line is best-fit from a General Additive Model on means with population as a covariate (dashed lines, ± 95% CI); because only the intercept varies among smoothed model fits for each population, model fit is only plotted for Ross for clarity. (e): points are maximum daily offshore wave height at La Perouse Bank off the coast of Vancouver Island (National Data Buoy Center, station C46206); line follows monthly mean of maximum daily wave height. Traits adjusted to common soma mass (0.0125 g, approximately 4 mm opercular diameter; a-c) or penis length (4.56 mm; d) using ANCOVA (Table 4-4).

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CHAPTER V. MECHANICAL DESIGN OF BARNACLE FEEDING LEGS AND PENISES: CUTICLE THICKNESS, MUSCLE CROSS-SECTIONAL AREA, SARCOMERE LENGTH, AND EXPERIMENTAL PENIS INFLATION[7]

5.1 Introduction

Rocky shores are one of the most extreme and variable hydrodynamic environments on earth. On wave-exposed shores, the maximum velocity of breaking waves can exceed 25 m s^{-1} while in nearby protected bays, water velocity may be two orders of magnitude slower, never exceeding 10 cm s^{-1} (Denny 1988, Denny et al. 2003). Adding to this extreme spatial variability, water velocity at any one place can change dramatically as waves break and recede and it may also vary significantly throughout the year due to the frequency and severity of storm events (Denny 1988). Surprisingly, despite this widespread variation in water velocity over space and time, a diverse assemblage of plants and animals thrives under these extreme conditions.

Intertidal barnacles are one particularly successful group that attains large population sizes on rocky shores worldwide. These small sessile crustaceans are filter feeders that extend modified legs, called cirri, to capture particles from the surrounding water. However, filter-feeding in this environment poses a challenge because many intertidal barnacle species live under a wide range of water velocities (Anderson 1994). On exposed shores, feeding legs must be able to withstand the force of breaking waves, while in quiet water, barnacles must maintain adequate water flow to capture enough food and facilitate gas exchange (Anderson 1994). Furthermore, feeding legs are not the only appendages that barnacles must extend into these extreme and variable flows. To reproduce, barnacles use impressively long – up to 8 times their body length (Neufeld and Palmer 2008) – and dexterous penises to seek out and fertilize nearby mates (most

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barnacles are simultaneous hermaphrodites, so any neighbour may serve as a mate; Anderson 1994).

To cope with these challenges, many barnacles have evolved the capacity to change the size and shape of their feeding legs and penises to suit water flow conditions (Arsenault et al. 2001, Marchinko 2003, Marchinko and Palmer 2003, Hoch 2009). For example, in the Pacific acorn barnacle *Balanus glandula* Darwin, individuals transplanted to high flow grow feeding legs that are approximately 50% shorter and 25% wider (Marchinko 2003) and penises that are up to 25% shorter and 50% wider for their length (Neufeld and Palmer 2008) relative to individuals grown in calm water. In addition to these dramatic responses to changes in water velocity, barnacle leg and penis form also varies with the density of nearby conspecifics. With few neighbours, barnacles have shorter legs (Lopez et al. 2007, Neufeld 2011), likely to cope with the reduced boundary layer, and longer penises (Neufeld 2011), likely to reach more-distant neighbours. Furthermore, at least one species responds to seasonal variation in wave force, growing shorter, stouter legs and wider penises during stormier winter months (Chapter IV, Neufeld in review).

Although appendage variation in barnacles has received growing attention in the last 10 years, all past studies of barnacle penis and leg form involve measurements on overall form (of leg length and width, for example). However, comparatively little is known about how individual components of these structures (like cuticle and muscle, for example) vary as overall form varies. Knowing whether tissue-level responses parallel functional changes in overall appendage form will help us understand the complexity and extent of form variation in acorn barnacle appendages, and may also shed light on tradeoffs that could limit patterns of variation available to selection within and between environments.

The barnacle feeding fan is a complex structure. In most acorn barnacles, three pairs of biramous thoracic legs, or cirri, align to form a sieve-like structure (Anderson 1994). Barnacles extend the feeding fan via an increase in hydrostatic pressure generated within a closed circulatory system, and retract it with a bundle of striated muscle fibres that runs from the base to the tip of each ramus (Anderson 1994). The penis is also extended by increased hydrostatic pressure, while its movements are controlled by striated longitudinal muscles that run the length of the penis along the outer wall (Klepal et al. 1972). These same muscles are used during retraction.

We therefore examined two tissue-level components of legs and penises that might affect their ability to withstand high velocities on exposed shores: muscle and cuticle. Because the total stress generated by a muscle is proportional to its cross-sectional area (Vogel 2003), we predicted that exposed-shore barnacles should have muscles with greater total cross-sectional area to control the feeding legs under higher drag forces. Furthermore, striated muscle fibres are composed of repeating units known as sarcomeres, each made up of thick myosin and thin F-actin filaments that contract by sliding past each other (Wainwright et al. 1982). According to this sliding-filament model of muscle contraction, longer sarcomeres should produce greater stress because more actin-myosin cross-bridges are acting in parallel. Indeed, Taylor (2000) reported an isometric scaling of resting sarcomere length with stress production across several animal groups including crustaceans. Also, longer sarcomeres (>6 μm) are more often associated with slow-twitch fibres possessing higher endurance while shorter sarcomeres (<4 μm) are more often found in fast-twitch muscle fibres (Jahromi and Atwood 1971, Costello and Govind 1983). Therefore, one could make two contrasting predictions: i) feeding-leg retractor muscles of exposed shore barnacles may have longer sarcomeres to function better under the greater stresses experienced in high flow, or ii) exposed shore retractor muscles may have shorter sarcomeres to allow feeding legs to retract more quickly when water velocities rise above the threshold for effective feeding (Miller 2007).

The cuticular exoskeleton is another important functional element of both legs and penises. It constrains the bounds of the internal fluid during hydrostatic extension of the penis and legs. Differences in cuticle thickness would also affect appendage stiffness in flow. Therefore, we predicted that barnacles from an exposed shore should have thicker cuticle in both legs and penises. Furthermore, past measurements of penis form have been made on relaxed penises (Hoch 2008, Neufeld and Palmer 2008, Hoch 2009, Neufeld 2011), and little is known about how much the penis is capable of stretching (but see Neufeld and Palmer 2008), or how the penis functions effectively when extended. Therefore, we assessed whether patterns of variation observed in relaxed penises held true for extended penises as well. Finally, designing a structure that extends via hydrostatic pressure poses a significant material challenge. In a pressurized cylinder with a wall of uniform material properties, the circumferential stress is twice the longitudinal stress (Wainwright et al. 1982). In barnacles, therefore, in the absence of difference in structural or material properties of the penis wall, a unit increase in pressure would result in a twofold increase in circumference for every unit increase in length. Therefore, we also studied how, and how well, barnacle penises are able to resist circumferential expansion during extension.

To answer these questions we compared cuticle and muscle development in legs and penises of clumped and solitary barnacles from one wave-protected and one wave-exposed site.

5.2 Materials and Methods

5.2.1 Cuticle thickness and muscle cross-sectional area histology and measurements

On Feb. 24, and Feb 27, 2009, *Balanus glandula* Darwin (mean soma mass = 0.0321 g; range = 0.0152 - 0.068) were collected from two sites representing the extremes of a wave-exposure gradient in Barkley Sound, near Bamfield British Columbia, Canada: the low-velocity site (Bamfield Inlet) and the high velocity site (Seppings Island) are described by Neufeld and Palmer (2008). The penis and 6th left thoracic leg were removed at the base and preserved in Bouin’s solution for 24-28 hours prior to being rinsed and moved to 70% ethanol before embedding. Samples were processed using a

LEICA Tissue Processor 1020 using a standard program for paraffin embedding. First, samples were rinsed in 70% ethanol for 1 hour, then moved to 90% ethanol for one hour, and then to two separate baths of 100% ethanol for 1.5 hours each. Then samples were moved to a 1:1 ethanol toluene mix for 1.25 hours, then to two separate baths of 50% toluene for 30 minutes each. Finally, samples were moved to two separate baths of paraffin for 2 hours each, and then embedded in paraffin blocks prior to sectioning. 5 µm cross-sections were taken at the base of the penis and legs for muscle cross-section measurements. For the penis, the paraffin block was then mounted at 90° and a longitudinal section was taken at the widest part near the base of the penis. For the legs, sections were taken through the base of both rami after they divide from the protopodite. All sections were mounted on glass slides using Mayer's fixative, and stained with hematoxylin and eosin. For staining, slides were first rinsed in two baths of toluene for a minimum of five minutes each, followed by two minutes each in: i) 100% ethanol, ii) 100% ethanol, iii) 90% ethanol, iv) 70% ethanol, v) 50% ethanol, and vi) distilled water. Then, slides were immersed in hematoxylin for 2 minutes, rinsed in distilled water, rinsed in slow-flowing tap water for fifteen minutes, and 70% ethanol for two minutes. Then slides were immersed in eosin for 30 seconds, two successive baths of 100% ethanol for two minutes each, and two successive baths of toluene for two minutes each. Cover slips were mounted with DPX and kept at 37°C overnight prior to photographing through a compound microscope at 100-400x with a 6MP digital camera.

Photographs were measured to the nearest 10 µm using ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2011). For penis muscle area, the area inside the centre-most muscle bundles was subtracted from the area inside the outer-most muscle bundles to get an approximate cross-section. Leg muscle area was calculated as the total muscle cross-sectional area (defined as the area between the outermost and innermost muscle bundles) of both rami divided by two. Leg cuticle thickness was measured at the outer (convex) edge of a single ramus, chosen for the most well-defined cuticle margins. For penis cuticle measurements, data are means of three measurements taken at the convex outer edges of the three most intact and in-focus regions nearest the base of the penis. All photographs were measured without knowledge of collection location or density. To assess measurement error, we re-measured traits from photographs in 10 randomly selected individuals. Repeatabilities (Whitlock and Schluter 2009) varied from 0.93-0.99 depending on the trait. In other words, 93-99% of variance in a trait was due to true differences, and only 1-7% due to error in measuring photographs.

5.2.2 Sarcomere measurements

On Oct 29, 2009, *B. glandula* (mean soma mass = 0.0191 g; range = 0.0021 - 0.0524) were collected live with attached substrate from Barkley Sound, near Bamfield, British Columbia, Canada. Wave-protected specimens were from Grappler Inlet and wave exposed individuals were from Wizard Island. Barnacles were transported live to Edmonton, Alberta and held in recirculating seawater aquaria at the University of Alberta aquatics department. Mature individuals with aperture lengths between 3 mm and 5 mm were removed from their substrate and placed into 7% $MgCl_2$ in seawater overnight (15-20 hrs) to relax muscles and anesthetize the animals. Individuals were then removed from their shells and severed between the 3rd and 4th pairs of cirri with a scalpel under a dissecting microscope, being careful not to manipulate the cirral fan in order to prevent conformational changes to the sarcomeres. Tissues were then fixed in 5% paraformaldehyde overnight and rinsed twice in 100 mM phosphate buffered saline (PBS). Again using a scalpel and dissecting microscope, the 4th and 6th cirral pairs were severed below the protopodite and the outer edge of the cuticle was split twice at the base, middle, and tip of each ramus to allow for actin label penetration. The cirri were then placed in 0.6 ml microcentrifuge tubes and perforated with 0.1% Triton X-100 (PBTX) for 10 min before adding 50 µL of F-actin label (1 mL actin label: 10 µL Bodipy Fluorescein Phallacidin; 40 µL 10% bovine serum albumin (BSA); 950 µL PBTX). Tissues were soaked in the F-actin label overnight, rinsed twice for ten minutes in PBS, counterstained with 1 mL Hoescht 33342 in PBTX for one minute and then rinsed three more times in PBS. The cirri were whole mounted on glass slides in Mowiol® mounting medium with cover slips sealed with clear nail polish. Slides were stored in a refrigerator at 4°C and kept dark to prevent the active fluorochrome from fading prior to viewing. Images were taken of the base, middle, and tip of each ramus using FITC and DAPI UV filters on a Zeiss Axioskop 2 plus fluorescent scope with a QImaging QICAM CCD 10-bit digital camera operated with Northern Eclipse software. Sarcomere lengths were measured using ImageJ as the distance between two successive I bands. Five sarcomeres were measured from each image and the mean was used in all calculations. Soma mass was back-calculated from measured leg length using OLS regressions in Marchinko and Palmer (2003). Although this approach likely introduced some error, the tight correlation between body size and leg length at most sites suggests this approach is reasonable. Furthermore, the small effect of body size on sarcomere lengths (3% in this study) suggests any errors in soma mass calculations should not appreciably influence results.

5.2.3 Statistical analyses and calculations

Analyses were conducted using R 2.10.1 (R Development Core Team 2010). To test the role of conspecific density and exposure a) on muscle cross-sectional area and cuticle thickness of legs and penises, and b) on relaxed and inflated penis form, we used ANCOVA on $\log_{10}$-transformed data using body size (soma mass) as a covariate. To test for differences in mean sarcomere length with exposure, feeding leg region, and leg number (i.e., leg 6 or leg 4), we used ANCOVA on $\log_{10}$-transformed data with body size (soma mass) as a covariate. Where appropriate, interaction terms were sequentially removed following Hendrix et al. (1982). Because the final model contained a significant leg number × body size interaction, terms involving leg number were also tested at one standard-deviation above and below mean body size to determine if conclusions about sarcomere length between leg 4 and leg 6 changed appreciably at different body sizes. Models conformed to assumptions of equal variance and residuals were approximately normally distributed.

5.2.4 Penis inflation

To investigate the overall form and material properties of barnacle penises, barnacles (*B. glandula*; mean soma mass = 0.0398 g; range = 0.0106 - 0.0720) were collected from Bamfield Inlet and Seppings Island (sites described above) at both low and high conspecific density. Approximately 15 barnacles were collected from each site and density combination in February 2009, and February 2010 and frozen for > 72 hours before use. Prior to inflation, the soma was removed, and the 6th thoracic leg from the left side and the penis were ablated, wet-mounted, and photographed using a 6 MP digital camera. The soma was then blotted dry and weighed to the nearest 0.01 mg.

For inflation, each penis was ablated below the peduncle and inserted onto a thin capillary tube (PE 10) and carefully glued in place (Krazy Glue, Elmer's Products, Columbus, OH) while keeping the penis tissue moist and ensuring no air was allowed to enter into the system. The capillary tube was attached to a 30 gauge hypodermic needle connected to a 10 ml serological pipette. The pipette was held using a micromanipulator to facilitate precise positioning of the setup during mounting and inflation. The other end of the pipette was attached to 10 m of flexible rubber tubing (4 mm internal diameter), which in turn was connected to a 100 ml open-top bottle. The entire system was filled with seawater at ambient room temperature throughout the experiments. The bottle was capable of being raised to a height of 4.25 m above the tip of the needle using a pulley system, allowing pressure at the tip of the needle to be varied incrementally from ambient pressure up to 43 kPa (approximately 6 PSI). The penis was positioned in seawater under a dissecting microscope and oriented perpendicular to the field of view. As pressure in the system was increased at approximately 2.5 kPa intervals, the penis was photographed through the microscope using a 6 MP digital camera (after extension stabilized at each pressure interval) until: a) maximum pressure was reached, or b) the penis ruptured. Photographs were calibrated with a stage micrometer and measured to the nearest 10 µm using ImageJ following Neufeld and Palmer (2008).

The capacity to stretch was calculated as the change in length divided by the initial length and was computed for both longitudinal and circumferential changes during inflation. This is equivalent to calculating engineering strain in a homogenous material. However, we use the word “stretch” because the barnacle penis is not a uniform material but instead is made up of many layers of material with presumably different material properties (cuticle vs. muscle for example), making the use of the word “strain” misleading when discussing the overall capacity to stretch of this complex structure. However, we did compute material stiffness of the penis by calculating Young’s modulus on stress-strain curves using pressures above 20 kPa (data not shown) using the mean cuticle thickness for each exposure treatment from Fig. 5-2A. This allowed us to compare stiffness of the penis wall when stretched longitudinally vs. circumferentially.

Although inflation was attempted for all individuals from both years, due to the extreme difficulty of working at this fine scale, penises were sometimes damaged during dissection and mounting, so sample sizes vary and data from both years was combined in all analyses. Furthermore, despite being frozen for > 72 hours, some penises began moving autonomously during dissection. These were excluded from all analyses, except for data shown in Figure 8. Re-measurement of 10 randomly selected photographs revealed repeatabilities (Whitlock and Schluter 2009) of 0.95 for penis length, and 0.97 for penis basal diameter.

5.3 Results

5.3.1 Cuticle and muscle

Comparison of muscle and cuticle among barnacles from different flow regimes and population densities revealed that barnacles on an exposed shore had cuticle that was approximately 50% thicker in penises (Fig. 5-1; Fig. 5-2A), and approximately 25% thicker in legs (Fig. 5-1; Fig. 5-2B), when compared to cuticle of penises and legs of equal-sized barnacles from a nearby protected harbour (Table 5-1). Penis and leg muscle cross-sectional area was approximately 100% greater in barnacles from an exposed shore, compared to equal-sized barnacles from a nearby protected harbour (Fig. 5-2C, D; Table 5-1). However, cuticle thickness and muscle cross-sectional area in legs and penises was not associated with variation in conspecific density (Table 5-1).

Sarcomeres at the base of the feeding legs were more than twice as long as those at the tip (and were of intermediate length in the middle) for both leg 6 and leg 4, and leg region explained more than 85% of total variation in sarcomere length (Fig. 5-3; Table 5-2). Sarcomere length was also associated with variation in wave exposure; barnacles from an exposed shore had approximately 20% shorter sarcomeres, compared to equal-sized barnacles from a nearby protected bay (Fig. 5-3). This effect was consistent for both leg 6 and leg 4, although the magnitude of the effect differed slightly among leg regions (Table 5-2). Sarcomere length also depended slightly on the two-way interaction between wave-exposure and region and the three-way interaction among leg number, region, and wave-exposure, though these effects were very small, explaining only 0.3% and 0.5% of total variation in sarcomere length, respectively. The final model (Table 5-2) also included a small but significant interaction between body size and leg number; subsequent analyses (Hendrix et al. 1982) shows that the small significant effect of leg number is only present at smaller body sizes (Fig. 5-4; Table 5-3).

5.3.2 Artificial penis inflation

Mechanical inflation confirmed barnacles from a protected harbour had penises that were approximately 25% longer than penises of barnacles from a nearby exposed shore; this pattern was present both before (Fig. 5-5A) and after (Fig. 5-5B) artificial penis inflation. Barnacles with few reachable neighbours had approximately 20% longer penises compared to barnacles with many reachable neighbours both before (Fig. 5-5A) and after (Fig. 5-5B) inflation and this pattern was similar at both sites (Table 5-4). However, we observed no consistent differences in the capacity to stretch during inflation among penises of barnacles from different water velocities or conspecific densities, despite more than three-fold variation in the capacity for both longitudinal and circumferential stretch among individual barnacle penises (Fig. 5-6). Barnacle penises stretched approximately five times more in length than in circumference at maximum inflation (Figs. 5-6, 5-7). Pressure-stretch curves appear to decrease in slope at higher pressures (Fig. 5-7), though we observed no consistent differences in inflation curves among barnacles from different water velocities and conspecific densities (Fig. 5-7). Material stiffness of the penis wall (mean Young's modulus ± standard error) was nearly tenfold higher when stretched circumferentially (40.57 MPa ± 3.0) than when stretched longitudinally (4.36 MPa ± 0.43). Body size was not related to maximum longitudinal or circumferential stretch ($p > 0.61$). Also, penises of exposed-shore barnacles showed no clear evidence of being able to sustain higher pressure before rupturing, despite having 50% thicker cuticle (Fig. 5-1A) relative to similar-sized protected-shore individuals. Of the 14 protected-shore penises and 10 exposed-shore penises that ruptured before maximum experimental pressure was reached, only five penises each of exposed-shore and protected-shore barnacles ruptured beyond the base and thus can be attributed to a natural failing of the cuticle rather than to artifacts of the attachment procedure. Of these natural failure events, there was no significant difference in pressure to rupture between exposed-shore (mean = 37 kPa) and protected-shore (mean = 32 kPa) barnacle penises ($t = 1.3804$, $p = 0.222$). Penises with living muscle tissue omitted from earlier analyses showed less longitudinal extension at low pressures (approximately 0-20 kPa), compared to penises of barnacles with inactive muscle tissue, but converged at approximately equal inflation at higher pressures (approximately 20-25 kPa; Fig. 5-8A). We did not observe any obvious differences in circumferential inflation curves between living and dead tissue (Fig. 5-8B).

5.3.3 Tissue organization

In the penis, at least two distinct layers of longitudinal muscles were visible. Those muscle bands that lie close to the epidermis were nearly perpendicular in cross-section, and relatively straight in longitudinal section, while the inner muscle bands appeared more oval in penis cross-sections and appeared folded in the longitudinal sections of the relaxed penis (Fig. 5-9A, B). Furthermore, the annulations of the penis cuticle were clearly composed of accordion-like folds of relatively uniform thickness, and were underlain by a layer of epidermal cells that also appeared folded in longitudinal sections through the relaxed penis (Fig. 5-9B). Longitudinal sections also revealed that the outer and folded inner longitudinal muscles of the penis were clearly attached to many cuticular annulations, though we could not determine from our sections whether muscles attached at every annulation. However, longitudinal sections through the feeding legs showed that the retractor muscle clearly attached to the cuticle at the base of each articulation (Fig. 5-9D). We also observed an obvious decrease in cuticle thickness moving from the external (nearest the anus) convex side to the inner (nearest the mouth) concave side of each ramus on all specimens (Fig. 5-1).

5.4 Discussion

5.4.1 Mechanical design of barnacle legs and penises

We identified a number of new traits in barnacle legs and penises that vary in line with predictions from engineering theory and that are consistent with adaptation to the increased hydrodynamic stress encountered on wave-exposed shores. Specifically, to cope with the extreme forces (Denny 2006) imposed by breaking waves on exposed shores, barnacle feeding fans and penises must meet two important criteria: i) legs and penises must resist deformation and structural failure under load, and ii) they must possess muscles strong enough to control these structures in the extreme and variable flows. Below we address each of these in turn.

5.4.2 Thicker cuticle of legs and penises should help resist deformation

In the feeding legs, the 25% thicker cuticle of wave-exposed individuals should significantly increase resistance to compression and to local buckling under load. When the feeding fan is oriented into flow, drag exerted on the feeding legs puts the inside surface in tension, while the outer surface is put in compression. Therefore, the thicker cuticle on the outer surface of the feeding legs should distribute compression forces over a greater cross-sectional area and increase the second moment of area, thereby allowing the legs to withstand higher drag without buckling. More generally, the allocation of thicker cuticle on the posterior side of the feeding legs in both wave-exposed and wave-protected individuals (Fig. 5-1) should increase strength while economizing on material on the inside surface of the legs where the cuticle should be under tension when the feeding fan is extended in flow.

In the penis, the thicker cuticle of wave-exposed individuals should also reduce the risk of structural failure, but for a slightly different reason. Unlike the feeding legs, which must resist deformation largely in one direction, the extended penis must cope with forces in all directions. Thicker cuticle should help barnacle penises resist the larger forces on wave-exposed shores for two reasons, both related to local buckling. When a bending force is exerted on a cylinder, the material on one side is put in tension, while the other side is put in compression. This has two important implications for barnacle penises. First, at any given pressure, thicker cuticle should increase the force required to initiate buckling under compression that will ultimately result in a sharp bending and lack of proper penis function (Kelly 2007), as sometimes seen in copulating dogs (Grandage 1972). Second, and perhaps more importantly, because wall stress is inversely related to wall thickness (Wainwright et al. 1982), thicker cuticle should be able to withstand greater pressures before breaking. Although we found only a small and non-significant difference in mean pressure-to-rupture between exposed-shore and protected-shore barnacle penises, this is as likely an artifact of the experimental procedure as it is a true result. Specifically, we excluded all rupture events where cuticle failed at the base, because we could not separate failures due to damage during mounting from true failure of the cuticle. Because wall stress increases with radius (Wainwright et al. 1982), and because penises are widest at the base, we'd expect most penises of living barnacles to fail at the base as well. Therefore, the inflation methodology makes it difficult to disentangle experimental artifact from the true pattern in nature. However, the idea that exposed-shore barnacle penises can and do operate at higher internal pressures is supported by one additional observation. When we consider all failure events (including those where the penis ruptured at the point of attachment), protected-shore barnacle penises generally failed at lower pressures (as exemplified by the pressure where the lines end in Figure 5-6). If exposed-shore barnacle penises are indeed able to withstand and function at higher internal pressures, this increased pressure should act to resist the inward force at a local bend and thereby further decrease the risk of local buckling and failure. The thicker cuticle of exposed-shore barnacle penises may have one additional benefit as well. The shells of barnacles are quite sharp, as discovered by anyone who has fallen in the rocky intertidal and experienced “barnacle bites”. Therefore, in the higher flow experienced on wave-exposed shores, the thicker cuticle may reduce the risk of damage to penises during mating.

Collectively, when the results reported here are considered alongside past work documenting an approximately 20% increase in basal diameter of legs and penises on exposed shores (Marchinko and Palmer 2003, Neufeld and Palmer 2008), the likely increase in bending stiffness of legs and penises from exposed shores becomes even more significant. Specifically, the second-moment of area (which is proportional to bending stiffness) scales with the radius to the fourth power, and also increases as new material is added at greater distances from the neutral axis (Vogel 2003). For example, for a cylinder, a 20% increase in diameter would result in a 31% increase in flexural stiffness, while a 25% increase in wall thickness (assuming new material is added on the outside) would increase flexural stiffness by 30%. However, taken together, a 25% thicker wall and a 20% greater diameter would increase flexural stiffness by a remarkable 70%. Although legs and penises are clearly structurally more complex than a simple cylinder, this simplistic example none the less suggests that changes in structure of legs and penises seen on exposed shores should significantly increase flexural stiffness. This is seen in the ability of exposed-shore barnacles to extend their legs in higher flows in the laboratory (Marchinko 2007), and in the increased fertilization success on exposed shores of barnacles with wider penises (Hoch 2009). Furthermore, when combined with predicted decrease in drag of shorter feeding legs (Marchinko and Palmer 2003), and penises (Neufeld and Palmer 2008), barnacles on exposed shores appear remarkably well adapted for life in these conditions.

5.4.3 Functional significance of variation in muscle cross-sectional area and sarcomere length

In addition to resisting deformation, to feed and mate on wave-exposed shores, barnacle legs and penises must have muscles strong enough to effectively control these appendages while resisting the higher forces imposed on them in these high-energy environments. The increases we observed in cross sectional area of muscles in the legs and penises of barnacles from the wave-exposed shore should allow them to do just this. Specifically, because the force generated by a muscle is proportional to cross-sectional area, a doubling of cross-sectional area should equate to a two-fold increase in total force production.

We also tested the prediction that sarcomeres should be longer on exposed shores to control the feeding legs under increased drag (recall that longer sarcomeres are associated with greater force production; Costello and Govind 1983, Taylor 2000). Contrary to our predictions, however, we observed 20% *shorter* sarcomeres in wave-exposed individuals, relative to equal-sized wave-protected counterparts. One possible explanation for this unexpected result involves the interaction between feeding behaviour and another trait often correlated with sarcomere length: muscle endurance. Muscles with longer sarcomeres are generally composed of more slow-twitch muscle fibres with high endurance, while muscles with shorter sarcomeres often contain more fast-twitch aerobic muscle tissue better for short bursts of activity (Jahromi and Atwood 1971, Costello and Govind 1983, Vogel 2003). Because wave-protected individuals feed with a fast and relatively constant cirral beat of approximately 20-40 beats per minute, while wave-exposed forms typically feed by holding the cirral net steady and taking advantage of passive flow generated by breaking waves, wave-protected individuals should benefit from a greater concentration of slow-twitch, high endurance muscle fibres to facilitate the higher sustained rate of cirral beating.

Furthermore, when the variation in sarcomere length is taken together with the parallel increase in muscle cross-sectional area, the combination of more muscle with shorter sarcomeres on exposed shores may in fact be an elegant solution to the need for both quick and strong muscles in these areas. Specifically, the increased speed commonly associated with fast-twitch muscles may allow for quick and carefully-timed cirral movements required to dodge the largest waves on exposed shores (Miller 2007), while the increase in muscle cross-sectional area should maximize total force production to counteract the greater forces experienced by the feeding fan in breaking waves.

We also found approximately 50% shorter sarcomeres at the tip of the feeding legs compared to the base. Although this variation was unexpected, two plausible adaptive explanations could account for this striking pattern, acting either alone or in concert. If shorter sarcomeres are indeed faster (Jahromi and Atwood 1971, Costello and Govind 1983), increased retraction speed at the tip of the cirri could prevent captured food from flowing out past the top of the fan before transfer to the maxillipeds on the forward sweep of the cirral beat, and could also be adaptive against predators. Barnacle cirri are frequently grazed upon by fish and other predators (Harvey et al. 2003), so faster retraction speeds at the tip of the feeding legs could help avoid this ‘grazing’ behaviour by fish. Furthermore, presuming muscles with longer sarcomeres are stronger (Costello and Govind 1983, Taylor 2000), greater force generation at the base of the feeding legs may be adaptive to counteract the greater cumulative drag force experienced in this leg region (the drag force exerted at the base of the feeding legs should be greatest because the entire leg is exerting force at the base, while the tip experiences drag only from the distal region). Given these clear functional predictions of variation in sarcomere length, one promising avenue for future research would be to use high-speed video to quantify leg retraction speeds along the length of the feeding legs from exposed-shore and protected-shore barnacles.

5.4.4 Penis inflation mechanics

Changes in length and basal diameter of relaxed and extended penis form were consistent with each other and with results of past studies. This confirms that conclusions from two previous studies of relaxed penis form of *B. glandula* (Neufeld and Palmer 2008, Neufeld 2011) should apply to extended penises as well. However, despite documenting for the first time clear variation in extended penis form among sites and densities in *B. glandula*, we did not see any difference in the ability to stretch of penises from different wave-exposure environments or densities. This suggests variation in structure rather than variation in material properties (of the cuticle for example) account for variation in penis form among wave exposures and population densities.

Barnacle penises stretched a remarkable five times more in length than in circumference, likely a result of the well-defined cuticular annulations that run the length of the penis (Fig. 5-9B). Much like the folds of an accordion, these annulations should allow the penis to stretch along its length while resisting circumferential expansion. The observed increase in stiffness at higher pressures (exemplified by a decrease in extension for a given increase in pressure; Fig. 5-7) is consistent with a dominant role of cuticular folds in facilitating longitudinal extension. This is also supported by the nearly ten-fold higher stiffness of penis tissue during inflation when stretched in circumference than when stretched in length. Penises in other animals have adaptations that appear to serve a similar function. For example, the penises of armadillos (Kelly 1999b) and turtles (Kelly 1999a, 2004) are inflatable structures both surrounded by an axial-orthogonal array of collagen fibers. When the penis is relaxed, the fibres are folded, and when the penis extends, these fibres straighten out (Kelly 1997, 2004), resulting in a sharp increase in stiffness at maximum strain (Kelly 1999b). In barnacles, the folded cuticle likely serves a similar function (Fig. 5-9).

We also identified an unusual organization of muscle bands that should allow the penis to remain moveable and flexible at different extensions. Our histological sections clearly showed multiple muscle bands attached to the cuticle, and some more folded in the relaxed penis than others. Such muscle bands of different lengths would allow different bands to be used for penis control as the penis stretches, without individual muscle bands needing to operate across a wide range of extensions. This organization of muscles was not noted in a previous study of barnacle penis histology (Klepal et al. 1972), so it is unclear how many barnacles possess such a muscle organization. This arrangement of muscles would therefore help *B. glandula* control penis movements over a wide range of extensions. However, one additional observation suggests barnacles may have another solution as well. The adductor muscles of at least one barnacle in the same genus (*B. nubilus*) can shorten to one sixth of resting length (Hoyle and Smyth 1963). If the muscles of the penis can shorten to a similar degree, this could serve as an additional mechanism for controlling the penis at multiple extensions. Both these adaptations should also aid in penis retraction.

The variation in circumferential and longitudinal strain from base to tip may also serve an adaptive function. Because strain in a cylinder is proportional to its radius, strain should decrease approximately linearly as the penis narrows along its length. This may have two benefits. First, the increase in flexural stiffness at the base should resist deformation as outlined earlier. Second, the reduced longitudinal strain near the tip should not require as strong muscles to control the penis – most of the more elaborate searching movements performed before copulation (Klepal 1990) are performed by the tip of the penis, with the base remaining relatively inactive when the penis is extended (pers. obs.).

The barnacle penis clearly faces an unusual set of structural requirements. Not only must it be retractable to be stored safely and compactly when not in use, it also must remain strong, flexible, and moveable while extending across a wide range of distances from the animal. Above, we have identified numerous features that may help barnacles cope with these unusual functional constraints. However, a number of future studies would be fruitful. For example, although differences in penis inflation curves between living and dead muscle offer some clue about the range of pressures at which the penis functions, comparison of inflation curves of penises with chemically relaxed and fully contracted muscle using the procedure outlined in Hoyle and Smith (1963) could quantify maximum muscle strength among different population densities and habitats, and would also confirm the range of pressures that barnacle penis muscles are capable of resisting. Furthermore, to complement laboratory studies, direct measurement of *in vivo* pressures of barnacles from exposed and protected shores could directly document the pressures required to extend penises in the field.

5.4.5 Conclusions

On an exposed shore, barnacles had feeding legs and penises with higher total muscle cross-sectional area and thicker cuticle, and feeding leg muscles with shorter sarcomeres, relative to similar-sized barnacles from a protected bay. We also found an unexpected and striking two-fold decrease in sarcomere length between the base and tip of the feeding legs, and propose a hypothesis that this difference may result from a tradeoff between speed and strength in muscles. We show that previous patterns observed in relaxed penises hold true for extended ones as well. Inflated penises stretch approximately five-fold more in length than in circumference, though we found no clear differences in extensibility among penises of barnacles from sites that differed in water velocity. Finally, we documented an unusual muscle arrangement in the barnacle penis – multiple muscle bands that appear to vary in length – and suggest that this arrangement should help the penis function at a range of pressures and extensions. Together these results round out previous research on barnacle appendage form variation and plasticity, and suggest a clear adaptive functional explanation for the remarkable and complex variation in barnacle leg and penis form among sites that differ dramatically in water velocity.

Table 5-1 Results of final ANCOVAs on cuticle thickness and muscle cross-sectional area after all non-significant interactions were removed (results are shown in Figs. 5-1, 5-2).

	df	ms	F	p
$\log_{10}$(penis cuticle thickness at base)				
$\log_{10}$(body size)	1	0.239	9.61	**0.0034**
exposure	1	0.459	18.42	**<0.0001**
density	1	0.004	0.17	0.6840
residuals	43	0.025	-	-
$\log_{10}$(feeding leg cuticle thickness at base)				
$\log_{10}$(body size)	1	0.206	14.13	**0.0005**
exposure	1	0.174	11.98	**0.0013**
density	1	0.007	0.47	0.4983
residuals	41	0.014	-	-
$\log_{10}$(penis muscle x.s. area at base)				
$\log_{10}$(body size)	1	0.461	30.7	**<0.0001**
exposure	1	0.679	45.26	**<0.0001**
density	1	0.022	1.45	0.2355
residuals	42	0.015	-	-
$\log_{10}$(leg muscle x.s. area at base)				
$\log_{10}$(body size)	1	0.596	22.73	**<0.0001**
exposure	1	1.332	50.79	**<0.0001**
density	1	0.007	0.27	0.6061
residuals	41	0.026	-	-

Table 5-2 Results of final ANCOVA on sarcomere length after all non-significant interactions were removed (results are shown in Fig. 5-3).

	$\log_{10}$(sarcomere length)				
	df	ms	F	p	R^2
$\log_{10}$(body size) (S)	1	0.12	80.52	**< 0.001**	0.031
exposure (E)	1	0.12	80.71	**< 0.001**	0.031
leg (L)	1	0.03	19.41	**< 0.001**	0.008
region (R)	2	1.68	1139.18	**< 0.001**	0.862
E ×L	1	0	0.43	0.514	0.000
E ×R	2	0.01	5.52	**0.005**	0.005
L ×R	2	0.02	13.7	**< 0.001**	0.010
S × L	1	0.01	7.11	**0.008**	0.003
E × L × R	2	0.01	3.7	**0.028**	0.003
residuals	130	0.001	-	-	-

Table 5-3 Adjusted ANCOVA on sarcomere length, at one standard deviation below the mean body size (soma mass), at the mean, and at one standard deviation above the mean (results are shown in Fig. 5-4).

log$_{10}$(sarcomere length)					
location	source	df	ms	F	p
- 1 SD	leg (L)	1	0.13	288.12	**< 0.001**
mean	leg (L)	1	0.03	19.41	**< 0.001**
+ 1 SD	leg (L)	1	0	0.38	0.538

Table 5-4 Results of final ANCOVAs on relaxed and inflated length after all non-significant interactions were removed (results are shown in Fig. 5-5).

	df	ms	F	p
$\log_{10}$(relaxed penis length)				
$\log_{10}$(body size)	1	0.004	1.72	0.0196
exposure	1	0.034	14.20	**0.0005**
density	1	0.031	13.16	**0.0007**
residuals	47	0.111		
$\log_{10}$(extended penis length)				
$\log_{10}$(body size)	1	0.011	3.93	0.0534
exposure	1	0.082	30.11	**<0.0001**
density	1	0.045	16.49	**0.0002**
residuals	47	0.128		

Figure 5-1 Cross-sections of feeding legs (paired rami from leg six) and longitudinal sections of the penis near base (inset) from approximately equal-sized barnacles (soma mass = 0.024-0.026 g; opercular diameter approx. 4.6 mm; *Balanus glandula*) from an exposed shore (A) and a protected shore (B) in Barkley Sound, British Columbia, Canada. Labels in A: relative cuticle thickness measured at the outer convex (dorsal) surface of the feeding legs (noted by c.t.), and retractor muscle (r.m.). Insets (in A, B) show relative thickness of external cuticular annulations (c.a.). Scale bar 0.1 mm; scale equal in all images.

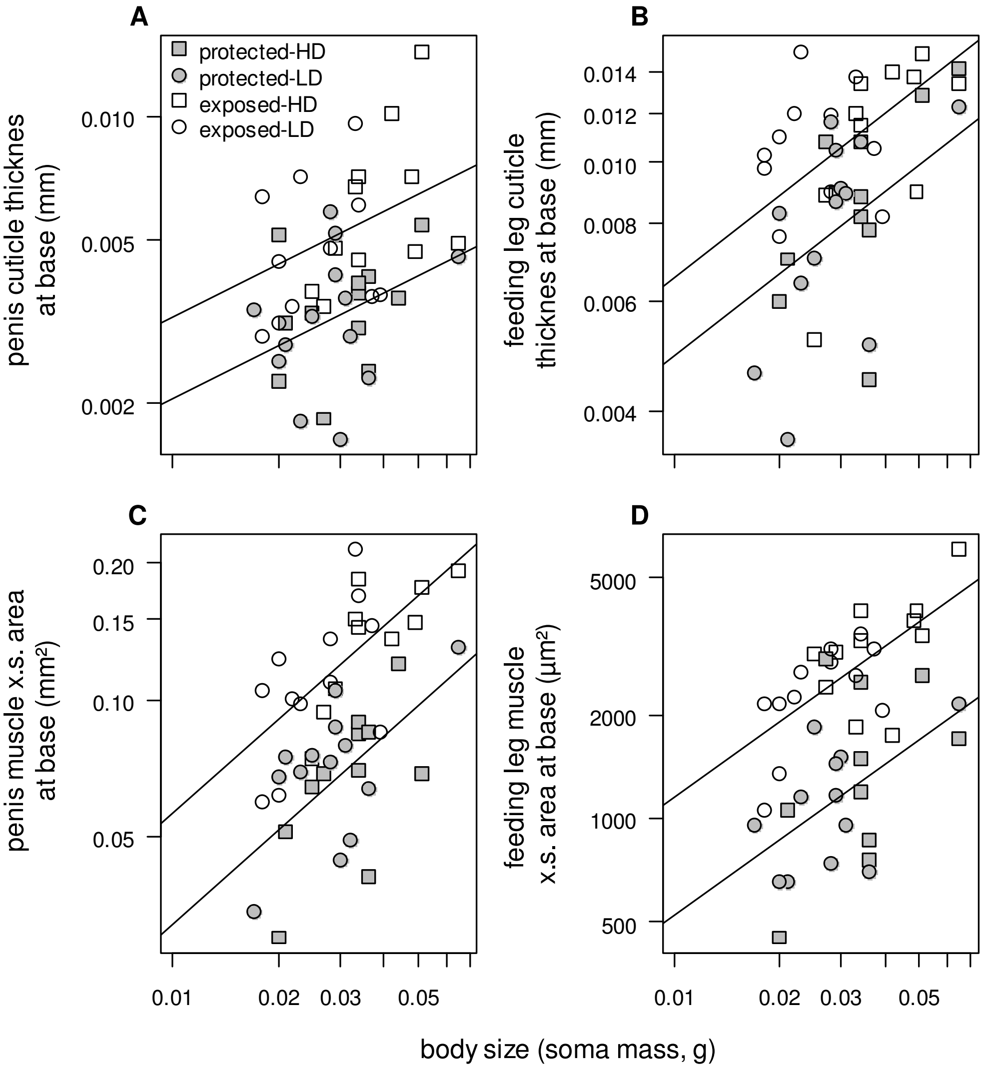

Figure 5-2 Cuticle thickness (A, B) and total muscle cross-sectional area (C, D) of penises (A, C) and feeding legs (mean for a single ramus of leg 6) (B, D) of the Pacific acorn barnacle *Balanus glandula* as a function of body size (soma mass) from an exposed shore (open symbols) and a protected shore (closed symbols) in Barkley Sound, British Columbia, Canada. Squares represent barnacles with many neighbours; closed symbols represent barnacles with few neighbours. Lines represent best fit regressions from final models (with few and many neighbours pooled at each site, Table 5-1).

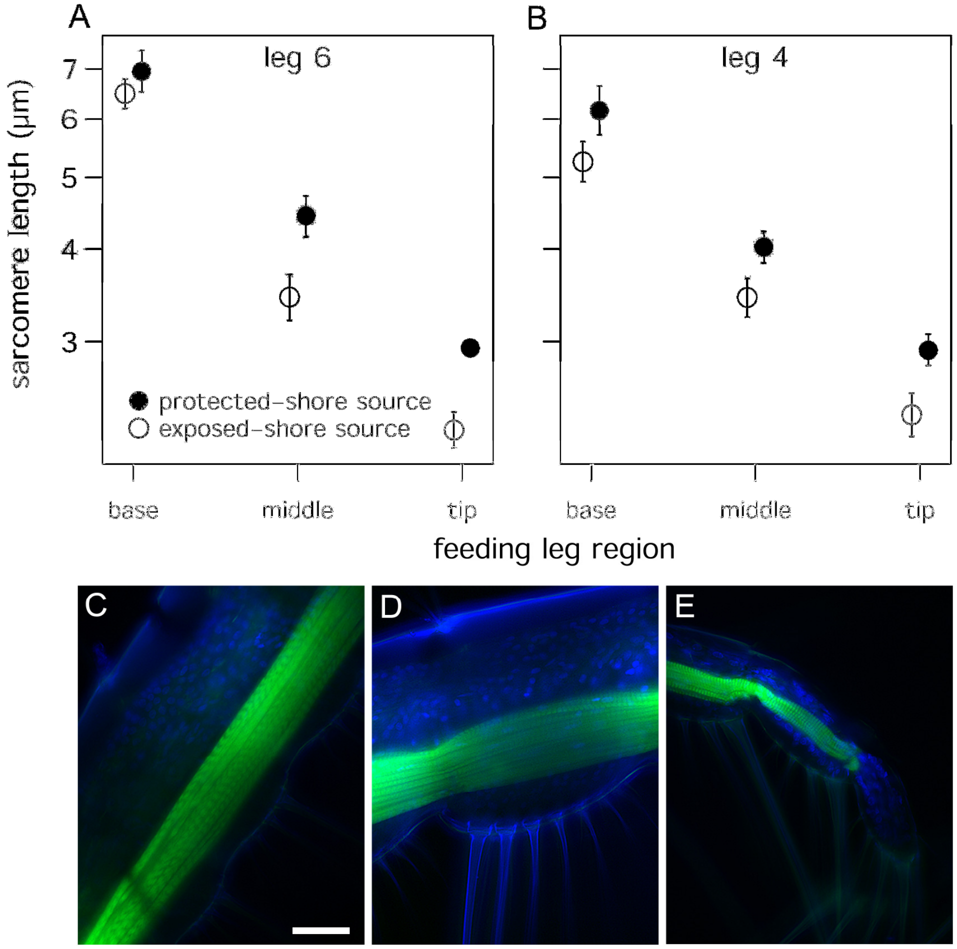

Figure 5-3 Sarcomere lengths of feeding-leg retractor muscles of the acorn barnacle *Balanus glandula*. A, B) Mean body-size adjusted sarcomere length from leg 6 (A) and leg 4 (B) (representing the medial- and lateral-most legs of the cirral fan, respectively) compared across leg regions from an exposed-shore (open symbols) and a protected shore (closed symbols). All measurements were standardized to an average-sized barnacle (soma wet mass = 0.0125 g, approximately 4 mm opercular diameter) using ANCOVA (Table 5-2). C-E) Representative images from fluorescent microscopy taken at the base (C) middle (D) and tip (E) of a barnacle feeding leg (leg 6). The labeled F-actin of the retractor muscle is visible in green. Scale is consistent across all images. Scale bar, 50 µm.

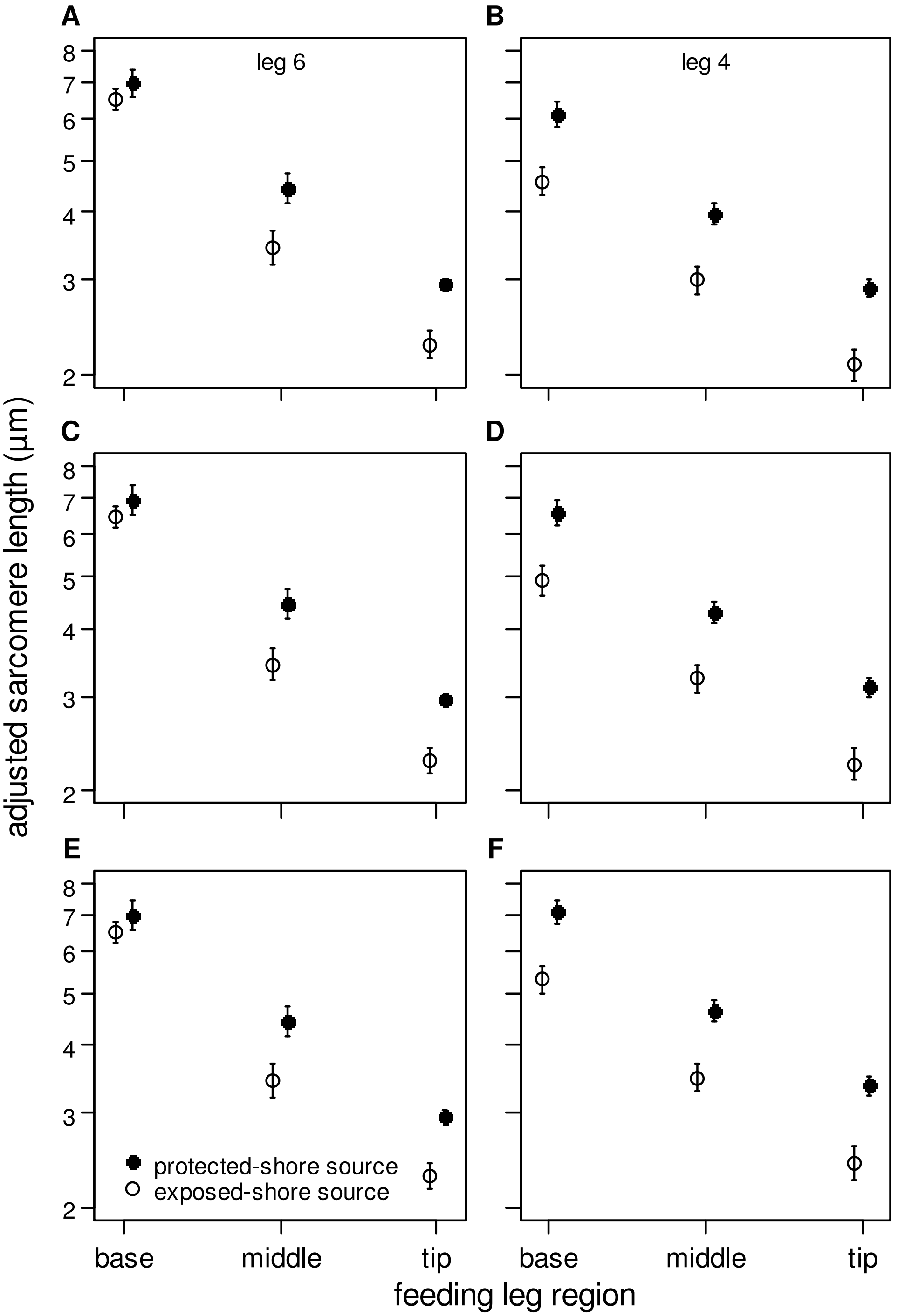

Figure 5-4 Feeding-leg sarcomere length of *Balanus glandula* adjusted to: (A, B) one standard deviation below the mean body size (soma mass), (C, D) at mean body size, and (E, F) one standard deviation above the mean body size.

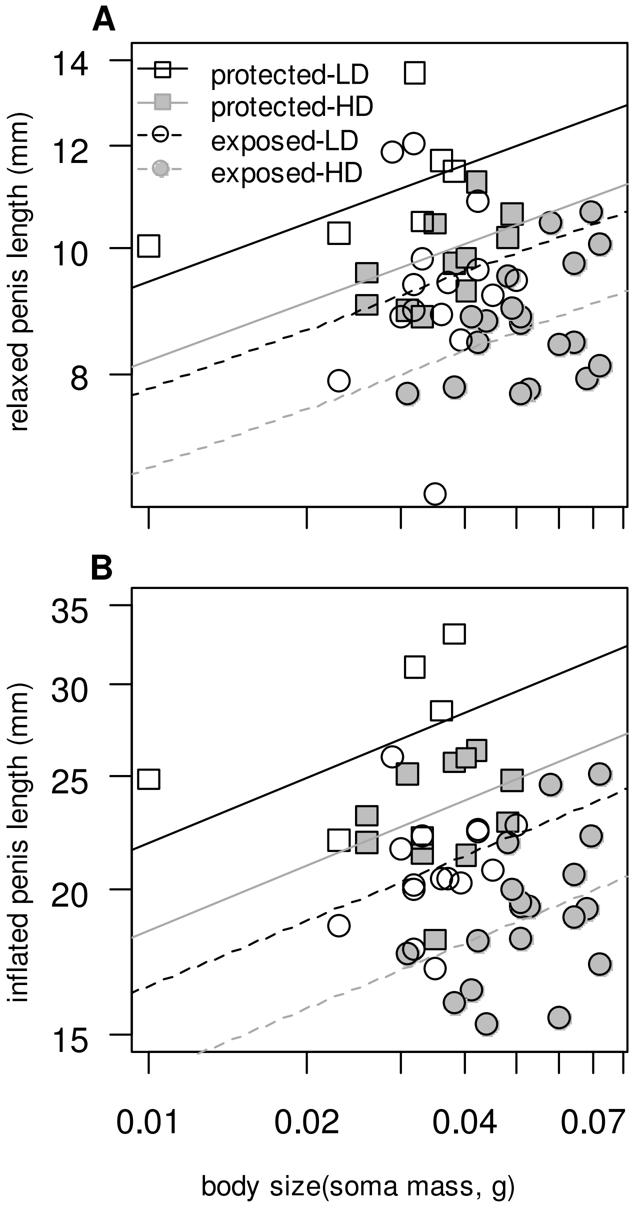

Figure 5-5 Relaxed (A) and inflated (B) penis length before and after artificial inflation for acorn barnacles (*Balanus glandula*) from low- and high conspecific density at a wave-exposed shore and a protected shore in Barkley Sound, British Columbia, Canada. Lines represent best-fit regressions from final models after removing all non-significant interactions (Table 5-4). Squares and solid lines are protected-shore barnacles, circles and dotted lines are exposed-shore barnacles; open symbols and black lines are barnacles with few neighbours, grey symbols and lines are barnacles with many neighbours.

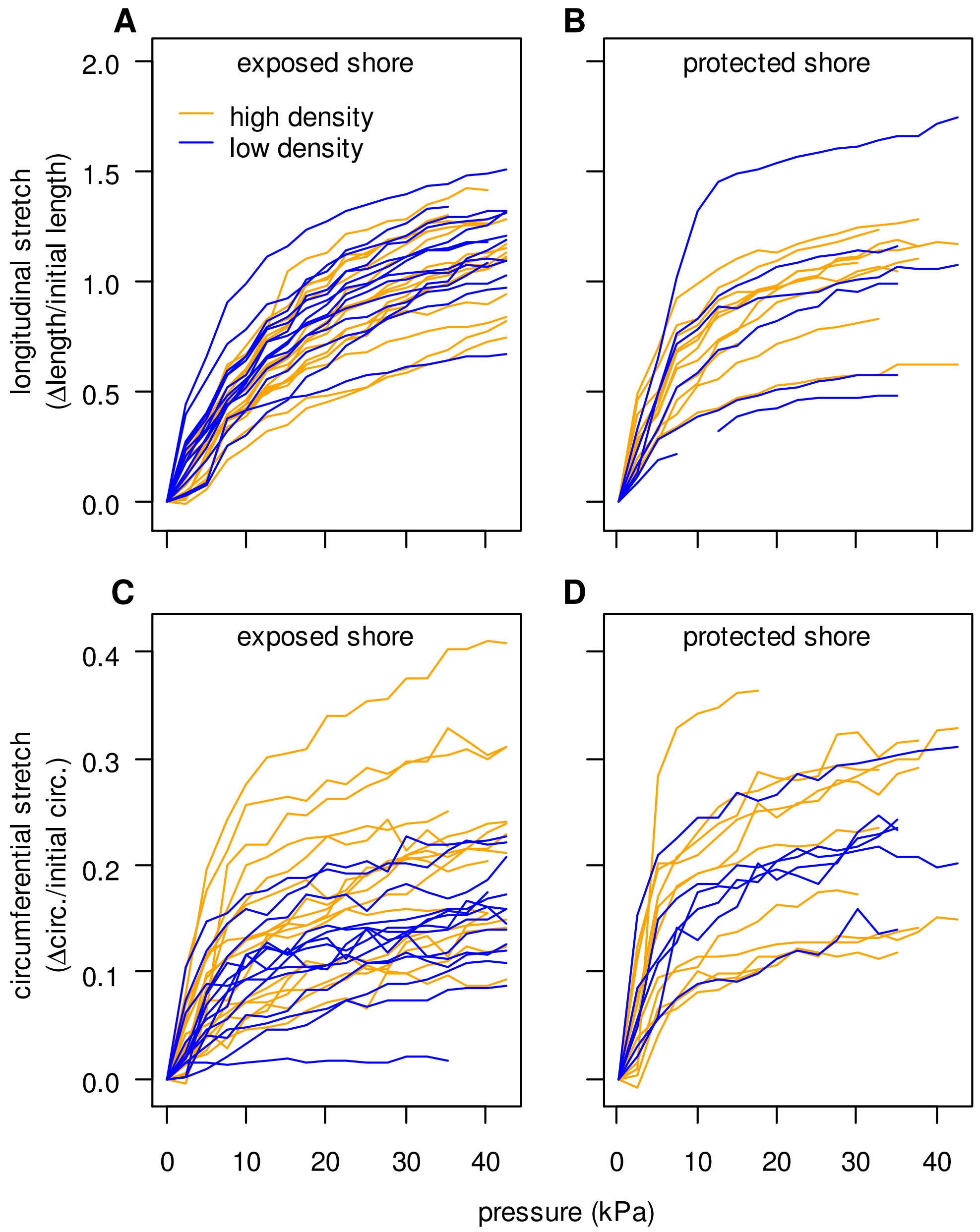

Figure 5-6 Standardized barnacle penis form during artificial inflation. A, B) Longitudinal stretch, and C, D) circumferential stretch as a function of pressure. Acorn barnacles (*Balanus glandula*) from both high and low conspecific densities were collected from an exposed shore (A, C) and a protected shore (B, D) in Barkley Sound, British Columbia, Canada. Lines represent curves for individual barnacles.

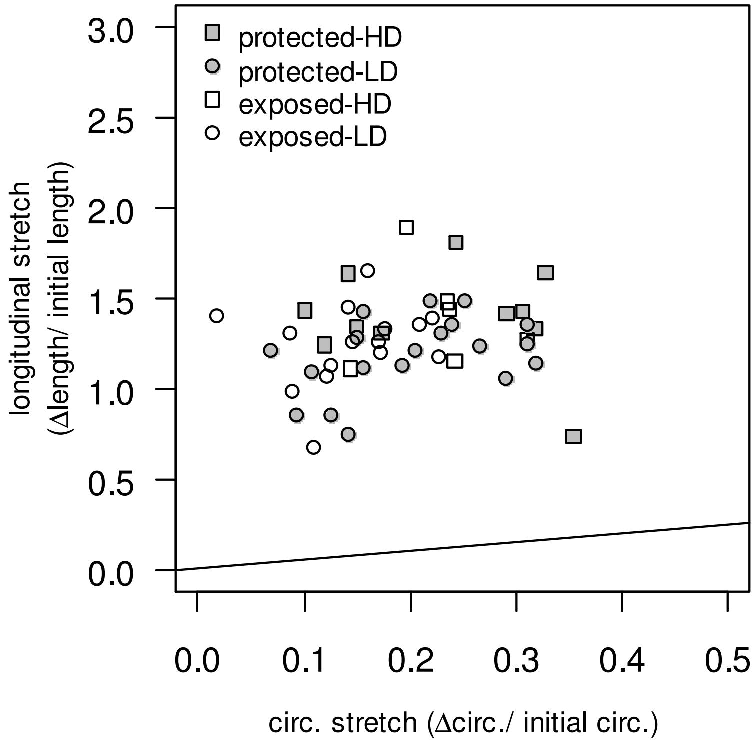

Figure 5-7 Standardized longitudinal extension (stretch) as a function of circumferential stretch during artificial inflation at maximum pressure (43 kPa) for acorn barnacles (*Balanus glandula*). Symbols represent penises of barnacles from both high (squares) and low (circles) conspecific densities from an exposed shore (open) and a protected shore (grey) in Barkley Sound, British Columbia, Canada. Each point represents an individual barnacle. The line represents the prediction for a pressurized cylinder, the walls of which are composed of an isotropic material.

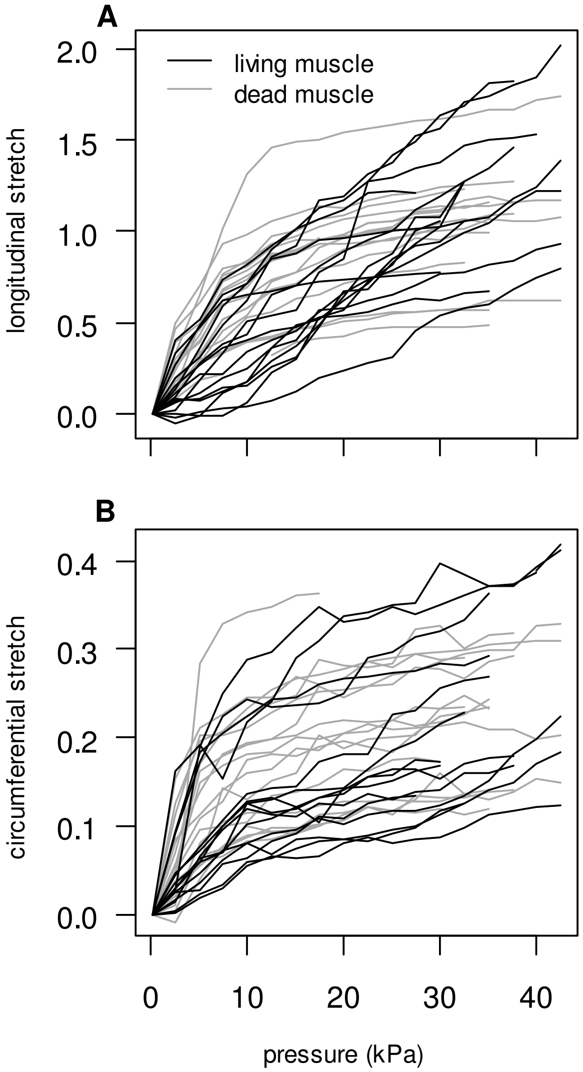

Figure 5-8 Standardized penis wall extension (stretch) during artificial inflation of barnacle penises (*Balanus glandula*). A) longitudinal stretch and B) circumferential stretch as a function of pressure. Black lines are additional barnacles excluded from earlier analyses because penis tissue was still living during inflations (see methods).

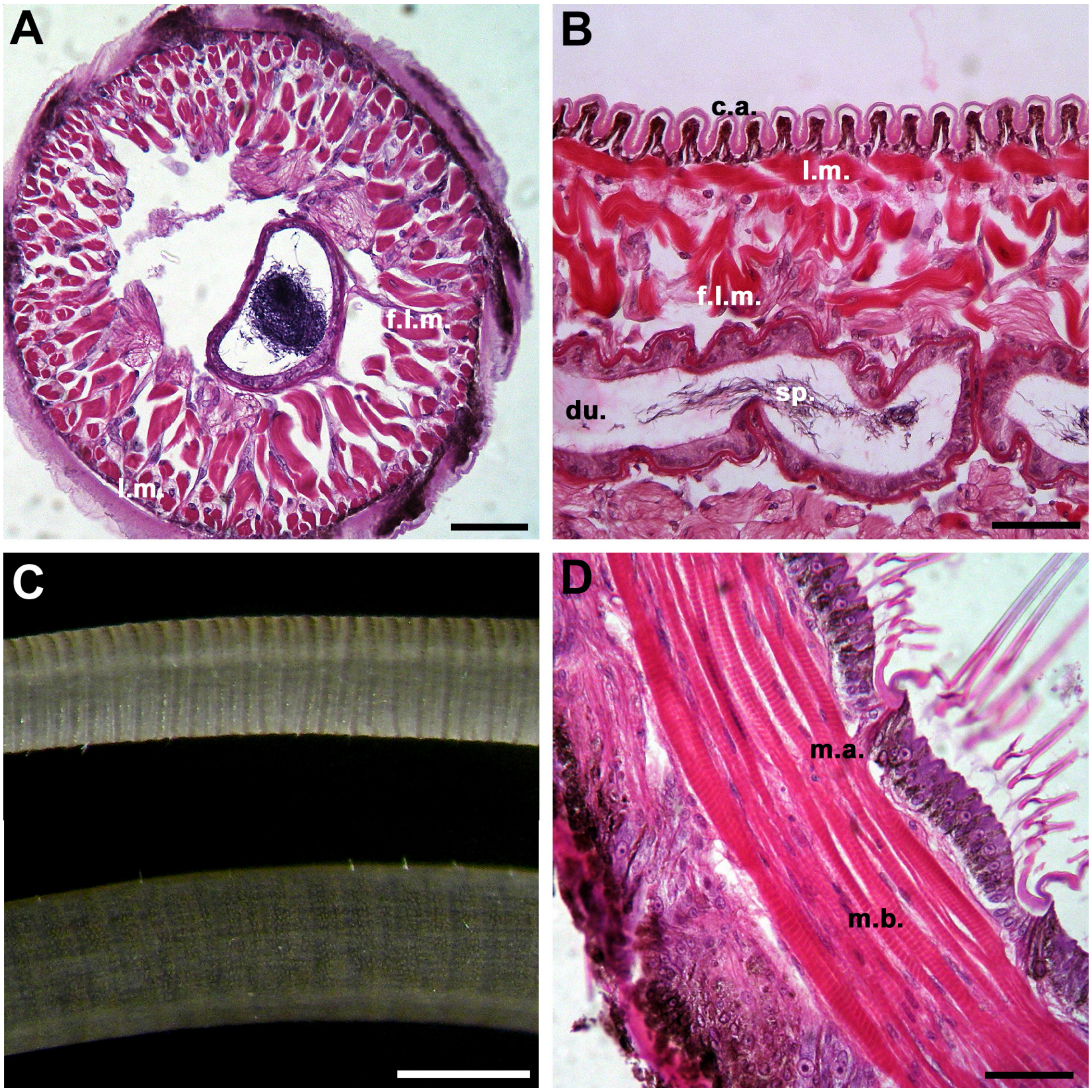

Figure 5-9 Photomicrographs of the penis and feeding leg structure of the acorn barnacle *Balanus glandula* from histological sections and artificial penis inflation. A) penis cross-section at the base from an exposed-shore barnacle (soma mass = 0.021 g; opercular diameter = 5 mm) showing longitudinal muscle bands (l.m.) in cross section at outer edge, and in oblique section (f.l.m.) near the centre; scale bar = 0.05 mm. B) longitudinal section at the base of a penis of a protected-shore barnacle (soma mass = 0.022 g; opercular diameter = 4.5 mm) showing folded cuticular annulations (c.a.) at the outer edge, as well as the taught longitudinal muscles (l.m.) near the edge, and loosely folded longitudinal muscle bands (f.l.m.) near the ductus (du.) at the centre with spermatozoa (sp.); scale bar 0.05 mm. C) base of partially (approximately 100 Pa, top) and fully (approximately 40 kPa, bottom) inflated penis of a protected-shore barnacle (soma mass= 0.023g; opercular diameter = 6 mm) showing extension of annulations and increase in diameter with increased pressure; scale bar 0.5 mm. D) longitudinal section near the middle of a feeding leg (single ramus) of an exposed-shore barnacle (soma mass = 0.048 g; opercular diameter = 6 mm) showing clearly striated muscle bands (m.b.) of longitudinal retractor muscle, and muscle attachment (m.a.) at the upper of two visible articulations; scale bar = 0.05 mm.

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CHAPTER VI. GENERAL CONCLUSION

Phenotypic plasticity can influence the strength and direction of interactions among populations and species as well as how organisms develop and behave, and even the rate and/or course of evolution. In the preceding chapters, I have explored how intertidal barnacles cope with, and respond to, environmental variation at multiple scales through such plasticity. Specifically, I expanded the suite of known plastic traits in barnacles to include their unusually long penises, and addressed a number of important questions related to the development and performance of barnacle feeding legs and penises in response to widespread spatial and temporal variation in water velocity and conspecific density. In this final chapter, I integrate these results with one another, and explore some of the broader implications of incorporating phenotypic plasticity into the study of ecology, development, and evolution.

From the results of past chapters across different structures, cues, and levels of organization, a number of important themes emerge relating to the evolution and maintenance of adaptive plasticity in variable environments. My work strongly supports the hypothesis that appendage form influences appendage performance. In Chapter II, I showed that penis form variation is highly predictable from a single cue (daily maximum water velocity), as past work has already shown for leg form variation (Arsenault et al. 2001). Furthermore, in Chapter V, I showed that muscle cross-sectional area and cuticle thickness increase on exposed shores, confirming that multiple components of leg and penis form vary simultaneously with variation in flow. Finally, numerous predictions from engineering theory provide additional support for adaptive variation in appendage form.

This work also supports the existence of fundamental biomechanical trade-offs in the allocation of limited material (Chapter V) yielding different optimal forms in different flow environments. For feeding legs, when water velocity is low, a larger feeding net (made up of longer legs) should allow the more efficient capture of limited food flowing over the fan; when water velocity is high, the increased drag should favour a smaller, stiffer fan composed of shorter, stouter legs yielding comparatively less drag (the simultaneous increase in particle flux in high flow should compensate for the smaller, lower-capacity filter). These results are consistent with changes in form in other filter-feeding animals that experience variable flows; both bryozoans and blackfly larvae grow smaller feeding fans in higher flows (Okamura and Partridge 1999, Zhang 2006). For barnacle penises, the tradeoff is similar; with limited material, barnacles face a tradeoff between maximizing the number of potential mates within reach (by increasing penis length) while maintaining the capacity to control penises under higher flow conditions (by increasing basal diameter, muscle strength and speed, and cuticle thickness). Of course, penis form is influenced only by turbulence and drag (and not by particle flux). Although recent work suggests exposed-shore barnacles are able to feed (Marchinko 2007) and successfully mate (Hoch 2009) in higher flows, direct measurements of drag and filter efficiency of different feeding leg forms (and reachable area for penises) could yield a more mechanistic understanding of the factors that contribute to such tradeoffs.

6.1 Is Barnacle Appendage Form Evolving?

Although much of the variation in overall size and shape of barnacle appendages among sites is clearly due to phenotypic plasticity (Chapter II; Marchinko 2003), it is not known whether variation in other traits (e.g., cuticle and muscle, Chapter V; leg and penis form with conspecific density, Chapter III) are also plastic. Indeed, the transplant experiment between an exposed shore and a quiet bay (Chapter IV) revealed a sizeable effect of origin location, and the magnitude of the effect depended on the trait measured. Such an origin-site effect can be due to two causes: i) plastic responses that became fixed during an earlier stage of development (Hoverman and Relyea 2007), or ii) true genetic differences. Regardless of their ultimate cause, these origin-site effects imply responses at any one site may be restricted to a narrower window than the full range of developmental variation possible in the species as a whole. Presuming some of this variation in leg and penis form among sites is due to variation in underlying genes, this would imply the reaction norms of barnacles are evolving separately in different wave-force regimes. For barnacles, one could test the full extent of divergence by studying gene flow and then comparing leg form plasticity of barnacles among genetically distinct populations across broader geographic areas known to experience limited gene flow. For example, in a common-garden transplant experiment using populations from largely open coast regions like Oregon and California and largely wave-protected regions like Puget Sound, one could ask whether variation in wave force over wide geographic scales has selected for variation in reaction norms among populations.

6.2 Similar Plasticity in Other Barnacles?

Surprisingly (at least initially to me), while I was conducting my PhD research on the Pacific coast, another PhD student (J. Matt Hoch) was studying penis form variation using the common Atlantic barnacle *Semibalanus balanoides* (Linnaeus), enabling comparison of penis form variation between acorn barnacles experiencing independent selection in different environments (Atlantic vs. Pacific oceans)[8].

In the Pacific, I showed that *B. glandula* changes both penis length and basal width (and hence shape) in a highly predictable manner consistent with adaptation to maximum water velocity (Chapter II), while in the Atlantic, only penis basal diameter varied with plaster dissolution rate (a proxy for average water flow) in *S. balanoides* (Hoch 2008). In both cases variation in penis form was largely due to phenotypic plasticity, and appears to be adaptive in *S. balanoides* (Hoch 2009) as it presumably is for *B. glandula* as well (Chapter V). Barnacles also differ in their responses to conspecific density; *B. glandula* grows shorter (relaxed and extended) penises when more neighbours are within reach, while *S. balanoides* increases the number of annulations (though shows no variation in relaxed penis length; extended penis length was not measured). Despite the different methods, these combined results raise two interesting possibilities: i) variation in selection pressure has led to variation in patterns of plasticity in different species inhabiting different environments, or ii) costs and limits to

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[8] Given that the first papers were published within months of each other (Hoch 2008, Neufeld and Palmer 2008), this concurrent discovery of barnacle penis form plasticity by multiple researchers adds yet another example to the growing list of multiple simultaneous discoveries (Ogburn and Thomas 1922) started nearly 90 years ago.

plasticity vary between these species and environments restricting the evolution of plasticity in different ways (e.g., the absence of penis length plasticity in response to flow in *S. balanoides*). Regardless, this work on barnacle penises in multiple species provides a rare example of likely adaptive plasticity in genital form in multiple species.

This rare example of within-species variation in penis form also sheds light on the role of natural selection in shaping penis form. In other animals that must copulate, genitalia are famously variable even among closely-related species, and this variation *among* species is thought to have arisen primarily due to sexual selection (female choice, sexual conflict, and male-male competition; Hosken and Stockley 2004). In contrast, here I provide a compelling example of genital form variation *within* a single species, likely reflecting a tradeoff between reaching more mates, and controlling penises in high flow (both examples of natural selection). The natural selection acting on penis form in barnacles can be thought of in a similar way to that acting on traits that help migrating animals reach their breeding grounds (e.g., endurance, navigation ability) except that in barnacles — one of few sessile animals to copulate — only the penis must be adapted to reach potential mates. Another example of natural selection on genital form comes from mosquitofish (Langerhans et al. 2005) where genital form appears to reflect a tradeoff between natural and sexual selection; larger genitals (modified anal fins called gonopodia) are preferred by females, while smaller gonopodia enable faster escapes from predators. This suggests that both natural selection and sexual selection can act simultaneously on genital form, and this interaction is worthy of study in other groups, especially those that either simply cannot retract their genitalia or must keep them extended for long periods of time.

Barnacles offer a number of interesting opportunities to study this balance between natural and sexual selection. Although this thesis focused on genital form variation within a single species due to natural selection, barnacle penis form varies considerably and consistently among species as well (Darwin 1854, Klepal et al. 1972, Klepal et al. 2010). One striking difference, especially among the stalked barnacles, is the arrangement and number of setae and other projections that extend from the penis. This variation could reflect a tradeoff between resisting turbulence or high velocity on exposed shores, and mate-competition with other nearby males. For example, in *Lepas anatifera* Linnaeus (a pelagic gooseneck barnacle that often forms dense clusters on floating objects)*,* my preliminary observations show that the penis is covered with many cuticular projections around its entire circumference (whether these are in fact setae is currently unknown). These projections also form a dense bottle brush-like ring at the tip of the penis and extend into a scoop-like fan near the tip (Fig. 6-1a, b). In contrast, the intertidal gooseneck barnacle *Pollicipes polymerus* Sowerby (a wave-exposed species with few reachable neighbours; Lewis and Chia 1981) has a penis that is unusually short with short setae in four rows along its length and virtually free of any projecting setae or other structures at the tip (Fig. 6-1c, d). Studies of penis form (flexural stiffness and material properties), sperm competition, and mating ability among different species will shed light on the potential balance between reaching more mates and competing with rival males. These are exciting research questions I will pursue in the future.

6.3 Plasticity and Development

In Chapters III and IV, I tested for a number of possible developmental limits to plasticity in barnacles, and found evidence both for and against such limits. Specifically, I identified an important developmental limit to leg-length response time, likely mediated by energetics (Chapter IV), while in Chapter III I showed that developmental coupling does not restrict the plastic responses to multiple conflicting cues. However, a more mechanistic study of developmental plasticity incorporating gene expression could offer much new insight into these and other patterns relating to the development and evolution of phenotypic plasticity. Barnacles are ideal for this type of work because: a) variation in multiple appendages can be easily generated through transplants, b) barnacles remain plastic throughout adulthood, and c) barnacles must change external appendage form through molting, which restricts the intervals when internal developmental processes are manifested in external changes in form. These characteristics should facilitate a number of interesting avenues for future research.

First, one could ask questions about the evolution of segmentation patterns, as discussed in more detail in Chapter IV. In barnacles, the number of leg segments is developmentally plastic (Chapter IV) while in many other arthropods this number is fixed. Do leg segments develop using similar pathways in barnacles and other arthropods? At least two genes seem like candidates for further study. First, the gene *Distal-less* is important in distal leg elongation in many arthropods (Williams 1998) and may serve a similar function in barnacles, perhaps influencing the length of each ramus articulation. Second, the Notch pathway is also worth further investigation, as it is required for development of all cuticular joints regardless of whether these fall at the boundaries of true segments or merely between successive smaller subdivisions within true segments (called annuli) (de Celis et al. 1998, Kojima 2004).

Second, one could ask how the development of legs and penises is related and how sharing developmental genes might influence patterns of variation available to selection. Although I found no evidence for developmental coupling to restrict the direction of concurrent responses (Chapter III), this does not imply the development of legs and penises is completely independent. Knowing whether the same genes are involved in the development of both structures should allow a better understanding of how development may restrict future adaptation. Furthermore, one could ask numerous other important questions using a developmental genetic approach. Do genes that control annulation formation in penises also control article differentiation in legs? More broadly, a survey of patterns of articulation across arthropods may offer new insight into how such articulation patterns evolve on longer timescales. Can other groups change the number of articles during adulthood? Does article number often vary among individuals of the same size? Is this variation adaptive in other groups? Finally, one could compare normal development in a single environment with development during plastic changes in leg and penis form during adulthood. In other words, are the genes that control the initial development of leg and penis form the same as those that control plastic responses later in life?

6.4 Plasticity and Ecology

Studies of plasticity can also shed light on a number of important ecological questions (for some recent reviews see: Agrawal 2001, Miner et al. 2005). For example, in Chapter IV, I showed that plastic responses in leg form are slower under conditions where energy acquisition is expected to be lower. In other words, barnacles appear to suffer an ecological limit to plasticity. Under optimal conditions barnacles are able to develop a new leg form quickly, but in the wild response times are limited, likely because barnacles are restricted to less favourable habitat higher on the shore due to interactions with competitors and predators. This result has two general implications for the study of response time-lags: i) resource acquisition under natural conditions may be an important and underappreciated limit on morphological response times in natural systems, and ii) laboratory studies of response times (where animals are well fed) may fail to detect such limits. An integrated view of ecology, development and evolution will be important in detecting the presence and impact of such limits in other systems.

Knowing how much, how quickly, and to what cues organisms respond are important also for predicting the impact of the rising number of introduced species around the globe (Carlton and Geller 1993). Organisms colonize new environments with the traits they have when they get there (Agosta and Klemens 2008). Although seemingly obvious, this statement has an important implication for the study of plasticity and invasion; what cues organisms use to detect their environment and how they sense and interpret these cues will determine whether plasticity is beneficial or harmful in the new environment.

Consider the following example involving induced defense in response to the arrival of a novel predator. As mentioned in Chapter I, many organisms develop a more-predator-resistant form in the presence of potential predators. For example, the Pacific whelk *Nucella lamellosa* (hereafter simply called whelks) develops a thicker shell with a narrower aperture in the presence of cues from a native predatory crab (Appleton and

Palmer 1988). However, on the Pacific coast, a novel crab predator, the European green crab *Carcinus maenas* Linnaeus (hereafter *Carcinus*), has recently colonized some areas (Cohen et al. 1995, Carlton and Cohen 2003, Yamada et al. 2005, Gillespie et al. 2007). This effectively establishes two environments for the whelk: the “old” environment without *Carcinus,* and the “new” environment with *Carcinus*. Experiments in the laboratory confirm that *Carcinus* readily consumes whelks when offered, and defensive changes in shell form developed in the presence of the native crab are adaptive against *Carcinus* (Edgell and Neufeld 2008). Therefore, the capacity of the whelks to persist in the “new” environment with *Carcinus* should depend only on the ability to sense and correctly interpret cues from *Carcinus*. Unfortunately (for the whelks at least), a subsequent experiment clearly shows whelks do not thicken their shells when exposed to cues from *Carcinus* (Edgell and Neufeld 2008).

This lack of response, however, may be due to two causes. Whelks may simply lack the sensory equipment to detect cues from the novel crab, but this is doubtful because whelks can clearly distinguish among numerous other predatory and non-predatory crabs (Marko and Palmer 1991). Alternatively, whelks may perceive cues from *Carcinus* but simply not interpret these cues as dangerous. Testing these two alternatives is important because, while evolving additional sensory equipment may be difficult, changing the way existing cues are interpreted (whether through learning or genetic change, or their interaction) should happen much more quickly (Neufeld and Palmer in press.).

Barnacles, too, may offer some insight into the role of plasticity in aiding establishment in new environments. Barnacles are prime candidates to be moved around the globe due to their habit of settling on the hulls of ships, and many barnacles have been introduced and become established in new habitats (Carlton and Geller 1993). For example, the Pacific barnacle *B. glandula* that has been the focus of my thesis, has recently become established in Argentina, South Africa, and Japan (Kado 2003, Schwindt 2007, Geller et al. 2008, Laird and Griffiths 2008, Savoya and Schwindt 2010). The presence of the same species in multiple locations could allow one to ask questions about how plasticity may facilitate or limit establishment in these new areas (as well as how reaction norms may have evolved in the new environments as discussed above). Furthermore, a comparison of traits among barnacle species that have succeeded in new environments with those that have not may offer an excellent case study for how plasticity facilitates or hinders the colonization of new environments.

6.5 Plasticity and Evolution

This idea that plasticity can somehow facilitate adaptation is far from new. Although Lamarck is often discredited because of his second "law" of evolution suggesting that all acquired characteristics are inherited (Lamarck 1809), he clearly recognized that use of a structure can often improve its function, and idea that very closely parallels what we now call phenotypic plasticity. He sums up this idea in his first "law" of evolution (Lamarck 1809):

> In every animal which has not exceeded the limit of its development, the more frequent and sustained use of any organ gradually strengthens this organ, develops it, makes it larger, and gives it a power proportional to the duration of this use; whereas, the constant lack of use of such an organ imperceptibly weakens it, makes it deteriorate, progressively diminishes it faculties, and ends by making it disappear.

Darwin, although focused primarily on natural selection of heritable variation, also believed in the principles of use and disuse, though he clearly placed them in a subordinate role to natural selection of innate variation. In The Origin of Species, Darwin (1872) writes,

> On the whole, we may conclude that habit, or use and disuse, have, in some cases, played a considerable part in the modification of the constitution and structure; but that the effects have often been

> largely combined with, and sometimes overmastered by, the natural selection of innate variations.

However, the first to clearly outline how plasticity (use and disuse) and natural selection might reinforce one-another was Spalding (1873). To explain certain instinctual behaviours, Spalding suggested that survival and reproduction of the quickest learners would eventually lead to a completely instinctual action where learning was no longer required[9]. Baldwin (1896) extended this idea to suggest that plastic traits in general could keep certain animals alive (something he termed 'organic selection') and thus direct the course of future evolution (what he termed 'orthoplasy'). Later, this concept was formulated in a genetic context by Waddington (1942) and independently by Schmalhausen (1949). Specifically, Waddington coined the term "genetic assimilation" to describe the process where traits that were initially environmentally induced become constitutively produced in later generations, and in a series of elegant laboratory selection experiments on *Drosophila* provided the first evidence for its occurrence (Waddington 1952, 1953, Waddington et al. 1954). Many examples of genetic assimilation (and the related concept of genetic accommodation; West-Eberhard 2003) now exist both from the laboratory (Suzuki and Nijhout 2006) and from nature (Aubret and Shine 2009). Remarkably, in the instances where traits have evolved independently multiple times and allow such comparisons, this 'genes as followers' mode of evolution appears to have occurred with approximate equal frequency as the conventional mode of evolution from new mutations (Palmer 2004, Schwander and Leimar 2011).

The best known example of how environmentally-induced changes in form could later become inherited comes from the study of heat shock proteins, particularly Hsp90. Hsp90 acts as a molecular chaperone, maintaining the correct three-dimensional structure of many signaling molecules and other proteins and correcting small conformational changes in proteins caused by mutations (Sorensen et al. 2003).

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[9] This paper contains many interesting accounts of experiments conducted in Spalding's kitchen involving blindfolded chickens and other peculiarities and is well worth the read.

However, Hsp90 production is limited, so when an organism is placed in a stressful thermal environment (i.e., “heat shocked”) Hsp90 may be in short supply as it is so tied up dealing with stress-induced conformational changes in proteins. Suddenly, mutations that were previously hidden by Hsp90 become expressed in the phenotype and thus are exposed to selection for the first time. This is one of many ways that development can act as a capacitor of evolution, allowing hidden mutations to accumulate, and then periodically exposing them to selection (Rutherford and Lindquist 1998, Gibson and Dworkin 2004, Suzuki and Nijhout 2006, Moczek 2007).

Perhaps most surprising, in the last ten years the budding new field of epigenetics (Gilbert and Epel 2009, Hall and Hallgrímsson In press) has started to provide experimental and mechanistic support for stable inheritance of environmentally-induced changes, suggesting that Lamarck’s second law on the inheritance of acquired characteristics may not be so far off after all. Transgenerational plasticity has now been documented across a wide range of experimental systems (Agrawal et al. 1999, reviewed in Gilbert 2001) and some of the mechanisms behind the stable inheritance of environmentally-induced traits are starting to be understood (the most widespread and long-lasting appear to be DNA methylation, and chromatin remodeling; Gilbert and Epel 2009).

Darwin’s major scientific contribution was the identification of natural selection as the driving force behind phenotypic evolution, yet he lacked any explanation for the *origin* of such variation. Throughout most of the 20th century, scientists have focused almost exclusively on genes as the only important source of variation upon which selection can act. In this thesis, I have shown that the (internal and external) environment deserves an equal place alongside genes in the generation of such variation, and that environmentally-induced variation in form and behaviour can have a large impact on an organism’s performance. In this last chapter, I provide evidence for how environmentally-induced variation in form can and does ultimately become heritable, and suggest a number of promising avenues for future research.

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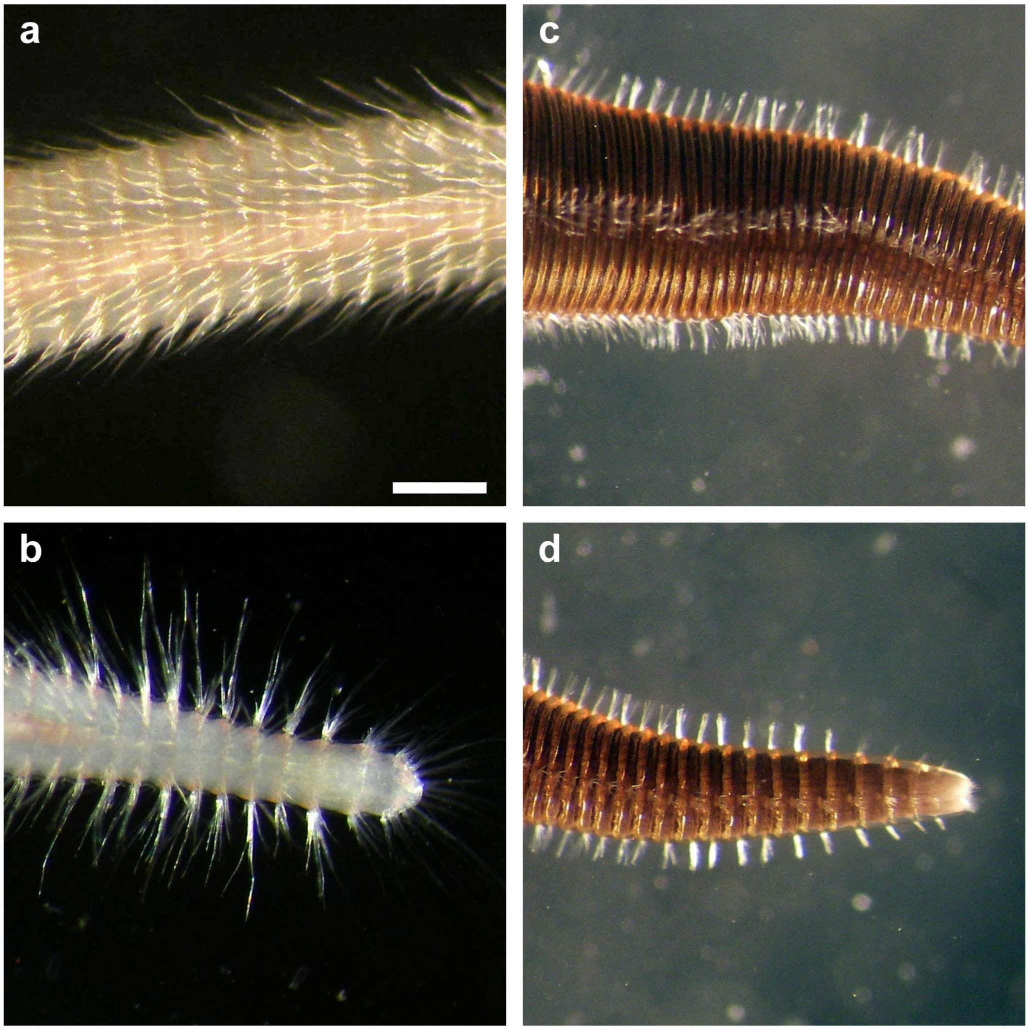

Figure 6-1 Relaxed penis form of the pelagic gooseneck barnacle *Lepas anatifera* (a, b) and the intertidal gooseneck barnacle *Pollicipes polymerus* (c, d), both common in Pacific waters. In *Lepas* the penis is covered in backwards-facing projections originating at each annulation (a), and longer projections form a bottle-brush-like ring at the tip terminating in a scoop-like fan (b). In *Pollicipes*, cuticular projections are shorter and are present only along four longitudinal lines (c) with only a ring of very small setae visible at the tip (d). Barnacles are approximately equal-sized (2.5 cm carinal-rostral diameter); scale bar approximately 0.6 mm.