Phenotypic and Genetic Diversity in the Sea Urchin *Lytechinus Variegatus*

by

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University Program in Ecology
Duke University

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
Ecology in the Graduate School
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ABSTRACT

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Abstract

Diversity in coloration is a common phenomenon in marine invertebrates, although the ecological significance of the diversity is often unknown. Patterns of geographic variability, particularly with respect to color phenotypes, are evident in many organisms and may provide visual evidence of natural selection.

This dissertation examined the geographic and genetic variability of color patterns and morphology of the sea urchin *Lytechinus variegatus*. This study had 3 objectives: 1) to describe and quantify phenotypic diversity—color and morphology—throughout the geographic range; 2) to determine the heritability of color in genetic crosses between individuals with similar and differing phenotype; 3) to assess the degree of genetic divergence between and within the regions and congruence between the phylogenetic mitochondrial COI data and color phenotypes seen in the field.

The distribution of color phenotypes in the field is highly variable across the geographic range which stretches from Beaufort NC to southern Brazil and throughout the Gulf of Mexico and Caribbean. Urchins in each of the 5 regions sampled (Beaufort, Gulf, Keys, Panama and Brazil) have a distinct phenotypic composition despite the presence of similar color morphs. The two regions at the extremes—Beaufort and Brazil—demonstrate the most homogeneous phenotypes, each with a single dominant color morph. The Keys has the most heterogeneous composition with all 14 color morphs present. Morphological diversity mirrors color diversity in being highly variable

across the range. Urchins in Beaufort are significantly different morphologically from urchins in the central portion of the range, with thicker, flatter tests and longer more robust spines.

The heritability of color phenotypes and morphology suggests that genes rather than environment have a major role in color phenotype and patterning as well as morphology in test, spines and lanterns. F_1 and F_2 offspring of Beaufort and Tavernier Key crosses resemble the parental phenotypes in both morphology and color phenotype. Hybrids from crosses between regions have a mixed color palette and intermediate morphological characteristics. The crosses establish that in *L. variegatus* the white phenotype is a dominant autosomal trait and green and purple are recessive and co-expressed. Patterning of the test and spines is dominant to non-patterning.

Analysis of the mitochondrial COI gene in urchins from Beaufort, Gulf and Keys regions revealed two clades. Clade 1 composed exclusively of Keys urchins differed significantly ($F_{ST} = 0.89$, $P < 0.001$) from Clade 2 composed of urchins from Beaufort, Gulf and Keys. Genetic differentiation within Clade 2 was zero, indicating that urchins in these regions are genetically identical. The genetic split between phenotypically indistinguishable Keys urchins suggests cryptic species. Genetic differentiation does not concord with phenotypic and morphological differentiation. No structure was detected with regards to color phenotype in either clade or region of origin in Clade 2.

Contents

Abstract iv

List of Tables ix

List of Figures xi

Acknowledgements xiv

1. Introduction 1

2. Color variability in *Lytechinus variegatus* 9

2.1 Introduction 9

2.2 Materials and Methods 15

2.2.1 Sample sites and urchin habitat 15

2.2.2 Field surveys of *in situ* color morphs 17

2.2.3 *In situ* color scoring 18

2.2.4 Color variability 20

2.2.5 Test color after removal of spines and epidermis 21

2.2.6 Data analysis 22

2.3 Results 23

2.3.1 Field surveys of *in situ* color morphs 23

2.3.2 Color variability in live urchins 30

2.3.3 Color variability after removal of spines and epidermis 42

2.4 Discussion 53

3. Morphological variability in *L. variegatus* 63

3.1 Introduction 63
3.2 Materials and Methods 68
3.2.1 Sample Collection 68
3.2.2 Morphological measurements 69
3.2.3 After removal of spines and epidermis 72
3.2.4 Data analysis 73
3.3 Results 75
3.3.1 Urchin morphology 75
3.3.2 Morphology after removal of spines and epidermis 95
3.4 Discussion 102
4. Genetic crosses 114
4.1 Introduction 114
4.2 Materials and Methods 118
4.2.1 Urchin collection 118
4.2.2 Larval culture 119
4.2.3 Juvenile cultures 122
4.2.4 Crosses 123
4.3.5 F_2 and F_3 generations 125
4.3.6 Morphological and color variability 126
4.3 Results 128
4.3.1 Cross color phenotypes 128
4.3.2 Cross F_1 color morphs 129
4.3.3 Mendelian ratios 143

4.3.4 F_2 and F_3 offspring 153
4.3.5 Cross morphology 159
4.4 Discussion 174
4.4.1 Cross color phenotypes 174
4.4.2 Cross morphology 184
5. Genetic versus phenotypic variability 190
5.1 Introduction 190
5.2 Materials and Methods 194
5.3 Results 196
5.4 Discussion 205
5.4.1 Genetic diversity and population structure 205
5.4.2 Phylogenetic structure 207
5.4.3 Relationship between genetic and phenotypic structure 210
6. Summary 214
References 217
Biography 242

List of Tables

Table 2-1: *In situ* color morphs for the five regions.. 24

Table 2-2: Observed and expected (in parentheses) frequencies of color morphs for the five regions.. 26

Table 2-3: Total number of color categories and colors per region for all traits combined and for each of the three most visible traits: distal spine, proximal spine and test interambulacra 31

Table 2-4: Total count of the six color categories found in all four regions for all three traits combined. 41

Table 2-5: Total number of color categories and colors per location for all test areas combined and for each area individually. 44

Table 2-6: Total number of color categories and colors per site for all test areas combined and for each of the three areas: IA tubercules, IA wedge and 3rd color. 48

Table 3-1: Lists the ranges, means and standard deviations of all morphological characters measured from the 5 regions. 76

Table 3-2: Mean values and standard deviations of 5 ratios 85

Table 3-3: Mean values and standard deviations of spine length and width ratios.. 89

Table 3-4: Lists the ranges, means and standard deviations of all morphological characters measured on denuded tests from each of the Beaufort sites. 96

Table 3-5: Mean values and standard deviations of H/D ratio, weight ratio, periproct and peristome ratios for each of the Beaufort sites.. 99

Table 4-1: Within-site crosses. 124

Table 4-2: Between-site crosses. 125

Table 4-3: Color morphs of F_1 offspring. 130

Table 4-4: Number and color phenotype of all surviving F_1 offspring. 140

Table 4-5: Observed phenotypic ratios for F_1 offspring of white and purple urchins.. .. 144

Table 4-6: Observed phenotypic ratios of spine color for F_1 offspring of white and purple urchins. 145

Table 4-7: Observed phenotypic ratios of spine color for F_1 offspring of white, green and purple urchins.. 147

Table 4-8: Observed phenotypic ratios of test color for F_1 offspring. 149

Table 4-9: Observed phenotypic ratios for F_1 offspring of patterned (Tavernier) and non-patterned (Beaufort) urchins. 162

Table 4-10: Parental and offspring spine and test colors observed in 5 single color cross types.. 152

Table 4-11: Number and color phenotype of all surviving F_2 offspring..... 154

Table 4-12: Lists the ranges, means and standard deviations of all morphological characters measured on Beaufort, Tavernier and hybrid F_1 crosses.. 161

Table 4-13: Mean values and standard deviations of test, spine and lantern ratios for F_1 crosses. 163

Table 4-14: Relative difference in trait means for Beaufort and Keys field populations and Beaufort and Tavernier crosses. 169

Table 4-15: Lists the ranges, means and standard deviations of all morphological characters measured on Beaufort, Tavernier and hybrid F_2 crosses. 172

Table 5-1: COI summary statistics from Clade 1 (Keys) and Clade 2 (Beaufort, Gulf and Keys).. 198

Table 5-2: Pairwise F_{ST} values of genetic differentiation between Beaufort, Gulf and Keys populations in Clade 2. 199

Table 5-3: Hierarchical AMOVA comparing genetic variation of Beaufort, Gulf and Keys regions in Clade 2..... 199

List of Figures

Figure 2-1: Map showing the field sites for in situ color sampling and samples taken. ... 17

Figure 2-2: Typical color phenotypes from urchins across the geographic range. ... 19

Figure 2-3: Spine and test regions coded for color. ... 21

Figure 2-4: Distribution and relative frequency of color morphs in each of the five regions.. ... 28

Figure 2-5: Expected frequency distribution of color phenotypes across all regions assuming no association of color and location. ... 30

Figure 2-6: Frequency of color categories at the four regions. ... 33

Figure 2-7: Frequency of distal spine color categories by region. ... 35

Figure 2-8: Frequency of proximal spine color categories by region. ... 36

Figure 2-9: Frequencies of test interambulacral color categories by region. ... 38

Figure 2-10: Histogram showing the frequency of 139 colors for all three traits combined. ... 40

Figure 2-11: Test areas coded for color ... 42

Figure 2-12: Color category frequencies of denuded tests from Beaufort and Tavernier Key. ... 45

Figure 2-13: Color category frequencies of the three test areas for Beaufort and Tavernier Key. ... 47

Figure 2-14: Color category frequencies of denuded tests for all Beaufort sites. ... 49

Figure 2-15: Color category frequencies of the three test areas for all Beaufort sites. ... 52

Figure 3-1: Urchin test, lantern and spines showing morphological areas measured. ... 71

Figure 3-2: Histograms showing the size range of urchin test diameters from the 5 regions ... 77

Figure 3-3: Mean values (± SE) for test wall thickness for the (a) aboral, (b) ambital areas and (c) overall mean test thickness.. 80

Figure 3-4: Mean test thickness at the aboral end regressed on the mean test thickness at the ambitus..... 81

Figure 3-5: Urchin test wall thickness. 81

Figure 3-6: Comparison of H/D ratio.. 86

Figure 3-7: Mean values (± SE) for test height-diameter ratio (H/D), lantern length-width ratio and lantern wet weight per test diameter ratio. 87

Figure 3-8: Mean values (± SE) for test weight and peristome ratios 88

Figure 3-9: Mean values (± SE) for spine length and width ratios. 90

Figure 3-10: Multivariate analysis of variance canonical plot of test, spine and lantern ratios between the 4 regions.. 92

Figure 3-11: 3 views of the discriminant analysis planes.. 94

Figure 3-12: Histograms showing the range of test diameters for the four Beaufort sites. 97

Figure 3-13: Mean values (± SE) for ratios of test height-diameter, weight, test thickness, periproct and peristome across each of the four Beaufort sites..... 101

Figure 4-1: Color morphs of F_1 offspring..... 132

Figure 4-2: Color categories for spine and test traits combined for F_1 offspring 135

Figure 4-3: Color categories for individual spine and test traits for F_1 offspring 136

Figure 4-4: Histogram for all three traits combined.. 138

Figure 4-5: Phenotypes for F_1 and F_2 juveniles..... 156

Figure 4-6: Color categories for all traits combined for F_1 and F_2 juveniles..... 158

Figure 4-7: Mean values (± SE) for test height-diameter ratio (H/D), mean test thickness, test dry weight per test diameter and peristome ratio for F_1 crosses. 164

Figure 4-8: Mean values (± SE) for lantern length-width ratio and lantern wet weight per test diameter ratio.. 166

Figure 4-9: Mean values (± SE) for spine length and width ratios. 167

Figure 4-10: Discriminant analysis of F_1 crosses. 171

Figure 4-11: Discriminant analysis of F_2 crosses. 173

Figure 5-1: Neighbor-joining tree of *L. variegatus* based on 536 bp of the COI mitochondrial gene.. 201

Figure 5-2: Parsimony haplotype networks of Clade 1 and Clade 2 203

Figure 5-3: Parsimony haplotype network of color phenotype..... 204

Acknowledgements

There are a host of people and institutions to thanks for making this dissertation possible. Firstly, I'd like to thank my advisor Dan Rittschof for allowing me to follow my interest and change my dissertation project to something outside his expertise and for providing valuable guidance and support throughout the "journey". Thanks to my committee, Dave McClay, Bill Kirby-Smith and Mark Fonseca for their support and advice throughout the project and for patiently reading all that I had to say in these 200+ pages. Thanks to Dave for allowing me to steal a few urchins at a time from his yearly Florida winter supply to make the genetic crosses. Without them Chapter 4 would not be as interesting.

I am extremely grateful for the funding which allowed me to complete this project. The Duke Endowment Fund, the Duke Marine Lab and the Office of Naval Research provided fellowship, tuition and stipend money for me during my tenure here at the Duke Marine Lab. Field work would not have been possible without the generous support of the Duke International Travel Grant, the Sally Schrader-Hughes Travel Grant and the Oak Foundation. I am grateful for the use of facilities at the Duke Marine Lab and funds from the Oak Foundation which were essential to create and maintain the genetic crosses.

I would like to thank the Florida Department of Environmental Protection and their staff for providing me the necessary permits and lending logistical and personnel support to collect data and urchins in the Florida Keys and Gulf of Mexico. I would especially like to thank Janice Duquesnel for graciously providing maps and coordinates of sampling sites in the John Pennekamp State Park. I would also like to thank the Panama City NOAA lab for allowing me to collect urchins off their docks and to use their wet lab. John, Enric and Stacy it was great to see you all again. Thanks also to the Smithsonian Tropical Research Institute in Panama for permission to collect and export urchins and to Harilaos Lessios for collecting some hard-to-find *L. williamsi* for me.

The number of people who have helped me are many and I am truly grateful to them all. A very heartfelt thanks to the Watts lab at the University of Alabama, Birmingham for teaching me everything I needed to know about culturing urchins in the lab. I thank Steve Watts, Mickey Powell and the students for their hospitality, openness and friendship. Without them the number of crosses might have been a mere fraction of those actually made (with a good dose of frustration and despair thrown in). Tom Schultz was always very generous with his time and advice whenever I dropped by his office unannounced. His help in synthesizing the cross data and reading and editing Chapter 5 made for a much improved dissertation. Thanks also to Mark Hooper for being a great dive safety officer and dive partner in helping me collect my critters at "urchin city" and the jetty. A very special thanks to Sergio Souza and Soledad Lopez who went way out of their way to help me collect urchins and data in Arraial do Cabo

when it looked like I would come away with nothing. Their generosity and warmth made my stay in Brazil unforgettable.

The staff at the Duke Marine Lab have been wonderful throughout this whole journey. Patty Nolin made administrative details magically easy. Lanier Mitchum took care of all the boat details so that I didn't have to. To them both a very big thank you. Sly Murray and the staff in the dining hall made lunch infinitely more appealing than it would have been otherwise. To all the students who helped me collect urchins, clean out the urchin tanks and finally process the data, I owe you and will forever be thankful that you so cheerfully volunteered your time to my project.

To my many friends and colleagues past and present at the Duke Marine Lab I say thank you, thank you, thank you for making this a wonderful place to live and learn. It just would not have been the same without your support and friendship. To Jocelyn and Josh for being wonderful friends and neighbors. I am so glad our time at the lab coincided. I so miss our dinners.

And of course I want to thank my family for their love and support. To my nephews Josh and Gabriel, thank you for helping clean out tanks when all you really wanted to do was go straight to the beach. To my mom for her unwavering support and periodic packages of goodies. Thanks mom, I love you.

1. Introduction

Ecology is the study of the interactions of organisms with their physical and biological environment [Ricklefs, et al., 2000]. A primary goal in ecology is to describe and explain the underlying patterns observed in nature. Traditional ecological techniques of *in situ* sampling and controlled experiments allow us to identify patterns and/or differences between individuals within populations and infer the causal mechanisms. Current molecular genetic techniques allow us to examine the level of genetic diversity within populations and infer processes that lead to genetic divergence.

Variable coloration is one example of the kinds of patterns that have been investigated within species in both the terrestrial and marine environments. Coloration serves visual-effect functions such as concealment, advertisement and disguise [Bond, 2007; Cott, 1940; Needham, 1974]. Coloration may also have physiological functions such as thermoregulation, evaporative water loss and UV protection [Needham, 1974]. Both the visual effects and physiological functions have been amply characterized in many systems [Bond, 2007; Cott, 1940; Needham, 1974]. Despite the wealth of information on coloration in the animal kingdom there is still a scarcity of information on the functional significance of coloration for many taxa, especially in sightless animals. Often, color patterns may be highly variable within taxa, suggesting habitat-specific or genetic origins [e.g. Johannesson, et al., 2002; Wilbur, et al., 1997].

Color and color patterns are the most conspicuous aspect of many animals and thus are often the first indication of underlying physiological or ecological conditions. Color and the maintenance of color patterns within populations can be attributed to several ecological and physiological functions, sometimes working synergistically and at other times producing opposing effects [Cloudsley-Thompson, 1999]. Ecological aspects of color variability (i.e. sexual selection, aposematism and crypsis) are intricately linked with vision. Examples for each are numerous and can be found in both the terrestrial and marine realm. Color is used as a signaling mechanism and color variability is generally maintained through selection, either intraspecifically (e.g. female choice) or interspecifically (e.g. predation). Physiological functions of color operate at the individual level to maintain the physical integrity of the animal in specific environmental settings (e.g. thermoregulation, UV protection and evaporative water loss). Physiological differences that provide a selective advantage to individuals possessing the appropriate coloration are selected for and maintained.

In the marine environment the question of color and the significance of color patterns have been investigated in many taxa. Coloration in phenotypically diverse aggregations is due to selection, ecological adaptations, physiological adaptations and ontogenetic changes [Etter, 1988; McMillan, et al., 1999; Palma, et al., 2001; Sokolova, et al., 2000; Tollrian, et al., 2004]. Often, though, the functional significance of coloration remains obscure. In populations of *Pseudodistoma crucigaster* a colonial ascidian in the Mediterranean, color patterns between aggregations separated by only tens of meters correlate well with genetic divergence [Tarjuelo, et al., 2004] but the function and

significance of color is still poorly understood. Similarly, the polymorphic sea star *Linkia laevigata* demonstrates geographic divergence between color morphs throughout its geographic range in the Indo-West Pacific (blue in the West Pacific and orange in the Indian Ocean), but the functional significance of the color differences has yet to be fully investigated [Williams, et al., 1998]. Coloration and color patterns are complex and may be influenced by multiple factors and color polymorphism may be maintained through adaptive ecological or physiological selection.

Morphological variability, like color, is a function of phenotypic changes in response to genetic variability and changes in local environmental conditions. Variations in body size and shape can be induced through natural selection, phenotypic plasticity and genetic drift [Pigliucci, 2001b; Schluter, 2000]. Natural selection and phenotypic plasticity can modify the morphology of an organism through biotic and abiotic agents.

Changes in morphology induced through natural selection function at the population level by modifying gene frequencies of the trait/s under selection [Falconer, et al., 1996]. Selection acts over generations leading to differential survival of some individuals over others. In this manner the contribution of offspring to the next generation is driven by those individuals able to withstand changes in environmental conditions induced by biotic (predators, food resources) or abiotic (hydrodynamic forces, temperature, photoperiod and salinity) conditions. The change in gene frequency induces a corresponding change in phenotype frequency within the population. Conversely, phenotypic plasticity functions within the individual to modify their

morphology as a proximal response to environmental conditions [Pigliucci, 2001b; Scheiner, 1993].

Differences in phenotype between individuals of the same species inhabiting different habitats, often just meters apart reflect the genetic variability within a population. This variability allows divergent selection to act on genotypes leading to ecological adaptation. In the marine environment snails, mussels and barnacles inhabiting the rocky intertidal evolve morphologically in response to various ecological factors. Predation pressure, resistance to desiccation and strong wave action have been shown to affect survival of individuals leading to marked morphological differentiation between the upper (higher spires, thicker shells) and lower intertidal (flatter, thinner shells) [Boulding, et al., 1993; Johannesson, 1986].

Predator cues and increased wave action can also induce morphological changes on a more proximate timescale within the individual. Littorinid and patellid gastropods exposed to predator odors or increased water motion have developed thicker shells and a larger foot [Appleton, et al., 1988; Dalziel, et al., 2005; Trussell, 1997]. Predation and hydrodynamic forces similarly alter the length of feeding legs and operculum morphology in barnacles [Arsenault, et al., 2001; Jarrett, 2008; Li, et al., 2004].

Both color and morphology can be highly variable in echinoderms. Asteroids, ophiuroids and echinoids are particularly colorful even though the ecological relevance of color is unknown and little explored. Diet, ontogeny, behavior and habitat [Endean, 1966; Growns, et al., 1994; Jensen, 1974; Lewis, et al., 1984; Tsuchiya, et al., 1984, 1985]

have been proposed to account for the diversity seen in numerous urchin species but empirical evidence is lacking. Morphological features such as test shape, spine and lantern size can vary widely in response to environmental and microhabitat differences [Black, et al., 1982; Lewis, et al., 1984; McShane, et al., 1997].

The sea urchin *Lytechinus variegatus* found in the western Atlantic from North Carolina and Bermuda to southern Brazil and throughout the Caribbean and Gulf of Mexico [Hendler, et al., 1995; Serafy, 1973; Watts, et al., 2007] is among a number of highly variable urchins. Others include *Paracentrotus lividus* [Boudouresque, et al., 2001] in the Mediterranean and eastern Atlantic coasts and *Heliocidaris erythrogramma* in the waters of south-eastern Australia and Tasmania [Growns, et al., 1994] just to name two.

L. variegatus mostly inhabits sheltered, shallow-water subtidal tropical seagrass beds and sand flats [Moore, et al., 1963; Serafy, 1973; Watts, et al., 2007] in densities between 0-40 ind/m^2. On rare occasions population explosions have driven densities to as many as 635 ind/m^2 [Camp, et al., 1973] resulting in severe overgrazing of the area. *L. variegatus* can also be found in deeper waters to 250 meters and on coarser crushed shell and quartz sand bottoms [Hill, et al., 2003; Serafy, 1979]. In Florida and the Caribbean the urchin is found most commonly in beds of the seagrasses *Thalassia testudinum*, *Syringodium filiforme, Halodule wrightii,* and *Cymodocea manatorum* [Watts, et al., 2007]. It is found on shell hash and in meadows of *H. wrightii* at the southern range of its distribution along the southeast coast of Brazil [Oliveira, et al., 1997]. At the northern limit of its range along the North Carolina coast it is mostly absent from *H. wrightii*

meadows [Sharp, et al., 1963, personal obs.], in large part because the seagrass beds often are exposed during low tide. Unlike *Arbacia puntulata*, the only other co-occurring urchin species in inshore waters, *L. variegatus* cannot tolerate even brief periods of exposure. Consequently, in Beaufort, *L. variegatus* lives almost exclusively on channel bottoms (2-6 m) composed of sand-mud-rubble mix. It is also found in deeper waters off the North Carolina coast on sand bottoms (personal obs.).

L. variegatus is a generalist omnivore, consuming a variety of plant and animal food [Beddingfield, 1997; 1998; Moore, et al., 1963; Valentine, et al., 1991; Watts, et al., 2007]. In shallow subtidal regions *L. variegatus* commonly feeds on blades of *T. testudinum*, although it appears to preferentially feed on decayed rather than fresh blades [Greenway, 1995]. Epibionts found on the seagrass seem to be preferred over the seagrass itself [Beddingfield, 1997]. In North Carolina *L. variegatus* may have a less herbivorous diet since it inhabits areas lacking in seagrass. Cursory investigation of stomach contents reveals very little plant material (personal obs.).

The morphological differences in *L. variegatus* have not been examined in great detail but have been noted along with color. Analysis of color and morphology by Serafy [1973] resulted in the assignment of 3 subspecies: purple *L. variegatus atlanticus* in Bermuda; red *L. variegatus carolinus* from North Carolina and along the Atlantic coast of Florida and in the Gulf of Mexico; and green *L. variegatus variegatus* in the Florida Keys, throughout the Caribbean down to southern Brazil. Morphological differences consisted of thicker spines and a greater number of interambulacral and ambulacral plates in *L. v.*

carolinus. However, there was considerable overlap in many characters to preclude using morphology for identification.

The subspecies distinction made by Serafy [1973] in regard to the geographical variability in *L. variegatus* stemmed from the difficulty in assigning a term to individuals within a species that differed morphologically but not sufficiently to be called a separate species [Mayr, 1982]. The use of the term was widely popular from the turn of the twentieth century until the 1950's [Mayr, 1982] when researchers studying polymorphic species such as birds, accorded even minor dissimilarities in individuals from different populations subspecies status. Much of the controversy that ensued resulted from differing interpretations of the term subspecies. Many considered the term to refer to "incipient species" while others understood subspecies to reflect localized adaptation [Mayr, 1982]. Inconsistent application of the subspecies concept led some to advise against using the trinomial to designate differences in geographical variations in populations [Wilson, et al., 1953]. The argument against subspecies rested on the issue that disjunct patterns of variation did not necessarily warrant additional taxonomic subdivision where poor geographic sampling may have failed to account for subtle clinal distributions. The issue has not been completely resolved but the advent of molecular techniques allows for greater discrimination between individuals and populations at the genetic level thus diminishing reliance on morphological features for species identification and potentially the need for the term subspecies.

In this dissertation I examine the geographic variability in *L. variegatus* from an ecological, genetic and phylogenetic perspective. In Chapters 2 and 3 I examine phenotypic variability (color phenotype and morphology respectively) and its relationship to the local habitat. Detailed color and morphological measurements are designed to quantify the level of phenotypic differentiation within and across regions. In Chapter 2 I conduct field surveys in 5 regions across the entire geographic range to identify and quantify the color phenotypes present in the species. Color variability is examined in greater detail on the spines and tests of a representative subsample of urchins from 4 of the 5 regions. In Chapter 3 I examine 16 morphological characters to quantify the level of variation present in all regions sampled. Mendelian-type genetic crosses in Chapter 4 between urchins from two regions (Beaufort and Keys) aim to elucidate the mode of inheritance of color and morphology. Crosses between urchins of similar and differing color morphs seek to evaluate both maternal and paternal contributions to offspring phenotype. Finally, in Chapter 5 I use molecular techniques to examine the level of genetic diversity between regions. A portion of the mitochondrial COI gene is amplified in urchins from 3 regions to probe the level of concordance between genetic and color diversity.

2. Color variability in *Lytechinus variegatus*

2.1 Introduction

The name given to the sea urchin *Lytechinus variegatus* is singularly apt considering the diversity in phenotypes that occurs. The diversity of colors has been noted but not studied in depth. Unlike many other systems in which color phenotypic diversity has been studied, i.e., flowers, insects, fish and birds, just to name a few, the diversity in urchin color has mainly been studied with the aim of identifying the chemistry of the pigments involved [Fox, et al., 1941; Vevers, 1963; 1966]. The functional significance of the pigments and by extension the colors, is poorly understood.

Diversity in color phenotype among plants and animals has been the focus of countless studies. Visible color polymorphisms such as seen in many brightly colored fish, insects, plants etc. are intrinsically intriguing because they suggest an underlying mechanism such as selection (natural or sexual) or plasticity. Being of the "right" color morph can be advantageous by increasing the chance for survival and reproduction. Examples of natural selection in the maintenance of color polymorphisms include classic studies of selection against predation through crypsis (reviewed by Bond [2007]) such as the classic study of the peppered moth *Biston betularia* [Kettlewell, 1958]. The use of color to advertise danger (aposematism) is also an effective anti-predator strategy [Harvey, et al., 1981; Lindquist, et al., 1996].

Selection for cryptic color phenotypes that increase survival and fitness have been proposed in disparate marine taxa: snails [Ekendahl, 1998; Johannesson, et al., 2002; Manriquez, et al., 2009], limpets [Mercurio, et al., 1985], bivalves [Todd, et al., 2006] and crabs [Palma, et al., 2001]. Color can be either a heritable but fixed trait [Ekendahl, 1998; Mercurio, et al., 1985; Palma, et al., 2001; Todd, et al., 2006] or plastic during early ontogeny [Manriquez, et al., 2009]. In many cases the color morphs are maintained by frequency dependent predation based on the contrast between prey and background coloration. In many systems intrapopulation variability is high owing to microhabitat differentiation [Johannesson, et al., 2002]. In areas of more homogeneous habitats color polymorphisms may vary over a wider geographic range. Predator mediated selection on differing phenotypes results in geographic variation.

Physiological factors contribute to color variability. Salinity tolerance was found to differ between color morphs of *L. saxatilis* and *L. obtusata* in the White Sea (Russia) [Berger, et al., 1997; Sokolova, et al., 2000]. Color polymorphisms are also common in terrestrial and intertidal gastropods and bivalves subjected to variable thermal and desiccation stresses (e.g terrestrial snails *Cepaea nemoralis* [Burke, 1989; Vicario, et al., 1988], intertidal snails *Nucella lapillus* [Etter, 1988] and *Littorina spp.* [Johannesson, et al., 2002], and bivalves [Mitton, 1977; Rose, 1984]). Physiological stress from high temperatures and desiccation during periods of emersion at low tide differs for the color morphs and having lighter colored shells appear to increase survival and fitness over dark morphs. Higher survival for darker morphs in submerged areas maintains the polymorphism. A similar mechanism may function with respect to exposure to UV

radiation. The deleterious effects of UV radiation on reproduction and development may select for individuals who possess greater amounts of UV-blocking pigments. In copepods and cladocerans varying levels of UV radiation induce a plastic response to increase levels of pigmentation [Hansson, 2004; Tollrian, et al., 2004].

Rather than carotenoids, hemoglobins, hematins and porphyrins which are some of the most common pigments in animals [Bandaranayake, 2006; Fox, 1947; Fox, et al., 1966], urchin colors are due to melanins and naphthoquinones. Melanin pigments are well characterized and are found in brown or black urchins such as *Diadema spp.* and *Arbacia spp*. Naphthoquinones, however, are rare as animal pigments, being found predominantly in fungi [Medentsev, et al., 2005; Wang, et al., 2009] and plants [Chen, et al., 1966; Verdan, et al., 2010] and tenebrionid beetles which store large quantities of a p-benzoquinone within their defensive glands [Kendall, 1974]. Naphthoquinones give urchins their characteristic purple, green and red color.

Color variability in echinoderms is especially widespread and has been examined in many species. Asteroids, ophiuroids and echinoids are particularly colorful and because they inhabit shallow water substrates are readily visible. Sea stars such as *Linkia laevigata* in the Indo-Pacific and *Pisaster ochraceus* on the Pacific coast of the US are conspicuous benthic invertebrates whose bright coloration stands out. The vivid blue and orange of *Linkia* and the purple and orange of *Pisaster* are due to carotenoid pigments linked to proteins [Fox, et al., 1941]. The distribution of color morphs within the species' ranges differs and the question of the underlying mechanism has yet to be

fully identified. It has been proposed that the color morphs in *Linkia* function as a warning signal due to toxic compounds in the animal [Zagalsky, et al., 1989].

Numerous studies examining the color frequency of *Pisaster* across a broad latitudinal gradient have found a consistent percentage of orange morphs in some areas but no causal mechanism maintaining the frequencies has yet been identified. Ontogenetic factors were proposed by Raimondi et al. [Raimondi, et al., 2007], who noticed that the frequency of the orange morphs increased with size of individuals in most populations. Harley et al. [Harley, et al., 2006] maintained that ecological factors (diet and salinity) were the likely reason for the observed frequency of the color morphs. Color variability in the ophiuroid *Amphipholis squamata* is also quite high with 11 recognized color morphs, some co-occurring in the same area but in different habitats [Deheyn, et al., 2000]. However, as is the case for asteroids the mechanism maintaining the variability is unknown.

Color variability has been noted in many echinoid species [Boudouresque, et al., 2001; Calderon, et al., 2010; Growns, et al., 1994; Vardaro, 2010] although the mechanisms underlying the variability remain poorly understood. Several hypotheses have been proposed: diet, ontogeny, behavior and habitat [Endean, 1966; Growns, et al., 1994; Jensen, 1974; Lewis, et al., 1984; Tsuchiya, et al., 1984] but empirical evidence is lacking. However, light intensity was shown to induce juvenile *Diadema antillarum* to change color [Millott, 1952] and the absence of light caused the absence of pigment on reconstituted spines in *Strongylocentrotus purpuratus* [Ebert, 1967].

Sharp and Grey [1963] demonstrated that *L. variegatus* responds negatively to sunlight and UV light, in particular, by moving away and covering itself with shells. The violently negative reaction to shorter wavelengths in *L. variegatus* versus *A. punctulata* suggested greater susceptibility in the lighter colored urchin. The experiments, conducted in Beaufort, presumably involved just the white phenotype. No comparative experiments were conducted on the different phenotypes within *L. variegatus* to see if greater pigmentation moderated the response. However, a similarly negative phototactic response was elicited from the green urchin *Strongylocentrotus droebachiensis* when exposed to UVA and UVB wavelengths [Adams, 2001]. A significant increase in the covering response behavior was seen in field trials of albino versus normally pigmented *Tripneustes ventricosus* in *Thalassia* beds in Jamaica [Kehas, et al., 2005]. The response by albino, white and green colored urchins indicates that UV radiation may be an important factor in urchin covering and shelter-seeking behavior. The behaviorally mediated responses may work in conjunction with the pigments in spines and test to protect the urchin from UV exposure. As these studies demonstrate, the proposed mechanisms maintaining color phenotypes are varied and differ depending on species and habitat but empirical data is needed for confirmation.

The color variability of *Lytechinus variegatus* has been noted but only superficially examined. The urchin has a broad latitudinal range occurring from North Carolina to Brazil. It is found throughout the Caribbean and Gulf of Mexico, even if somewhat patchily distributed [Moore, et al., 1963]. It is a shallow water echinoid, commonly found in seagrass beds, bare sand bottoms, rocky subtidal and areas of mixed seagrass

and coral rubble [Greenway, 1976, 1995; Hendler, et al., 1995; Moore, et al., 1963; Rivera, 1978; Serafy, 1979; Valentine, et al., 2000; Watts, et al., 2007]. The habitats, excepting North Carolina, are in clear waters, at shallow depths that are subject to high levels of solar radiation.

Color variability in *L. variegatus* prompted its name and designation into subspecies. Mortensen [1943] and Mayr [1954] both noted the geographic variability of *L. variegatus* with Mayr citing the genus *Lytechinus* as an example of allopatric speciation. Serafy [1973] supported the designation of subspecies status for *L. variegatus* through his examination of differences in color phenotype and morphology (Ch 3).

Though both spines and test are colored, color phenotype is usually denoted by the color of the spines as test color is less visible. White, green, purple, and red are the colors assigned by Serafy [1973] who partitioned the color morphs by subspecies assigning them color morph of greatest frequency. According to his classification *L. variegatus atlanticus* (Bermuda) is mostly purple, *L. variegatus carolinus* (US SE Atlantic coast) is mostly red and *L. variegatus variegatus* (Caribbean and Brazil) is mostly green.

In this chapter I reevaluate the previous classification of color phenotypes by conducting a series of field surveys in five geographically distant regions. I*n situ* color phenotypes will be quantified and observed phenotypic frequencies will be compared by contingency analysis. The surveys are designed to assess the overall variability across the geographic range and within each region. Differences in phenotypic frequencies are discussed within the framework of local habitat parameters.

2.2 Materials and Methods

2.2.1 Sample sites and urchin habitat

To determine the scope of variation in color morphs throughout the geographic range of *L. variegatus*, urchins from 11 locations were sampled and their live color phenotype documented. The 11 locations were grouped into 5 regions related to the geographic range of *L. variegatus*: Beaufort, Gulf of Mexico (Gulf), Florida Keys (Keys), Panama and Brazil. Beaufort is the northern limit of the range, Gulf, Keys and Panama encompass the central part of the range and Brazil is the southern limit of the range.

Beaufort samples were urchins collected in Bogue, Back and Core Sounds and on artificial reefs a few miles offshore in Onslow Bay (Bft 34.72 N, 76.65 W). Gulf samples were urchins collected in Saint Andrews Bay (SAB 30.15° N, 85.67° W) and Saint Joseph's Bay (SJB 29.80° N, 85.36° W) on the northern Florida Gulf coast. Keys samples were from Key Biscayne (Mia 25.69° N, 80.17° W) in South Florida, Indian Key (Ind 24.89° N, 80.68° W), Pigeon Key (Pig 24.70° N, 81.16° W), and Tavernier Key (Tav 25.02° N, 85.51° W) in the Florida Keys. Panama samples were from Bocas del Toro (BDT 8.45° N, 82.15° W) and Galeta Point (GP 9.40° N, 79.87° W). Brazil samples were from Arraial do Cabo (ADC 22.96° S,42.03° W) and Cabo Frio (CF 22.53° S, 42.1° W)(Fig. 2-1).

The habitat in Beaufort is a sand-shell hash mix on sand flats at 1.5–4 m depth bordering channel basins within Bogue, Back and Core Sounds and sand substrate near offshore artificial reefs at a depth of 16–20 m. Urchin habitat in St. Joseph Bay and Galeta

Point consisted of large meadows of the seagrass *Thalassia testudinum* at depths of 1–2 m. In St. Andrews Bay, Key Biscayne, Indian Key, and Pigeon Key habitat was a mix of *T. testudinum* and *Syringodium filiforme* also at 1–2 m depth. Habitat in Bocas del Toro was comprised of variable size patches of *T. testudinum* within coral rubble 1–2 m in depth abutting on mangroves. In Arraial do Cabo and Cabo Frio the habitat was rocky subtidal covered with a mixture algal species (e.g. *Sargassum sp*., *Padina sp*., and other macrophytes), large sea fans (*Gorgonia sp*.) as well as the zoanthid *Palythoa caribeaorum* at 1–3 m depth.

I conducted field surveys in all locations listed above except Tavernier Key. Urchins from this site were collected live by a third party and routinely sent to the McClay lab at Duke University and a portion were transported to the marine lab where they were assessed for color and morphology. Habitat at Tavernier Key was large meadows of *T. testudinum* at 5–6 m depth [Ken Nedimyer, Sea Life Florida, pers. comm.]. Color data on urchins from Saint Joseph's Bay and Saint Andrews Bay were obtained both from *in situ* surveys and from samples sent to DUML via a third party.

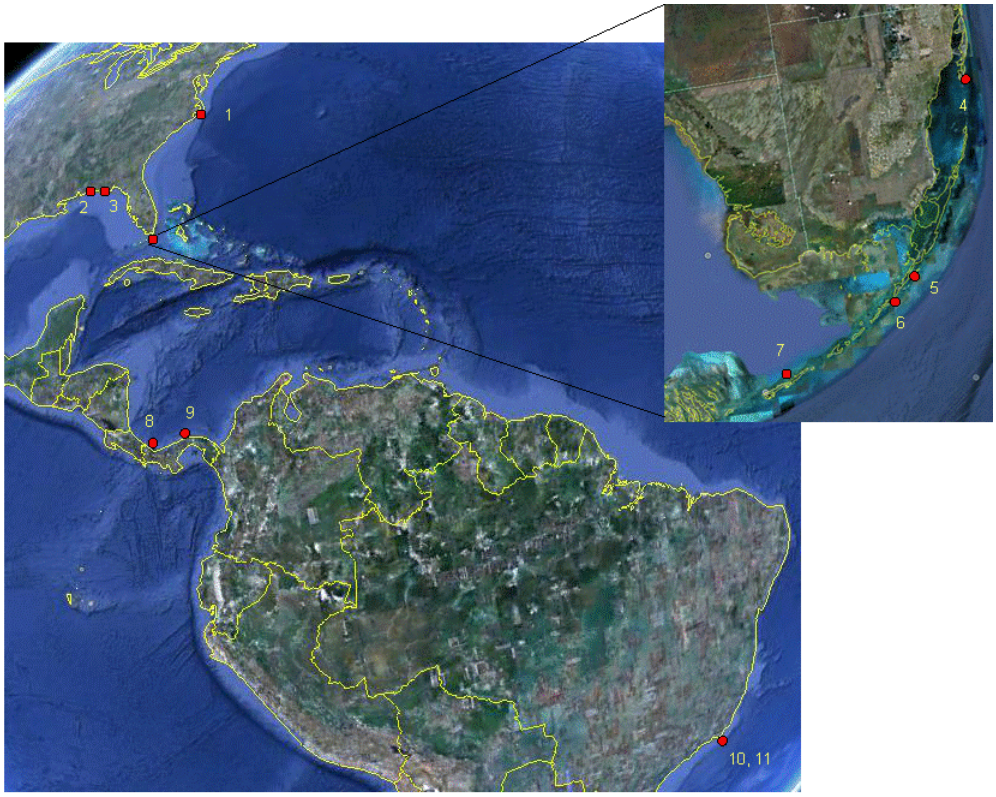

Figure 2-1: Map showing the field sites for in situ color sampling and samples taken. Beaufort: (1) Beaufort, NC; Gulf: (2) St. Andrews Bay, (3) St. Joseph Bay; Keys: (4) Key Biscayne, (5) Tavernier Key, (6) Indian Key, (7) Pigeon Key; Panama: (8) Bocas del Toro, (9) Galeta; Brazil: (10) Arraial do Cabo, (11) Cabo Frio. The distance between Arraial do Cabo and Cabo Frio is approximately 10 km and therefore indicated by a single dot.

2.2.2 Field surveys of *in situ* color morphs

Color was recorded in two ways: *in situ* surveying and on urchins sent to DUML. For *in situ* surveying color data from a minimum of 4 and a maximum of 10 30 m^2 transects per site was collected from all sites except Beaufort and Tavernier Key.

Transect lines 30 m in length and between 1–5 m in depth were placed haphazardly through urchin habitat. Two observers swam the length of the lines and recorded the color morph of each urchin encountered in 60 m^2 area within 2 m of the transects. Transect length at Pigeon Key was reduced to 15–20 m due to substrate topology and limited seagrass cover.

Color data for Beaufort urchins was collected during dredge surveys. The surveys were conducted with a dredge basket 0.75 m in diameter and for approximately 100 m resulting in an area of approximately 75 m^2 per pass. The strong tidal regime of Bogue Sound and poor visibility precluded transect surveys. Urchins from offshore artificial reefs were collected by hand using SCUBA.

2.2.3 *In situ* color scoring

To compare within- and among-site phenotypes and to quantify their relative frequencies, I recorded the color phenotype *in situ*. From a distance urchin color is due to spine color. Test color had no bearing on the color phenotype since it is not readily evident from a distance. Each urchin encountered along the transects was assigned a color morph based on the color obvious to the eye. Urchins sent from Saint Joseph's Bay, Saint Andrews Bay and Tavernier Key were color coded as with *in situ* surveying.

Color morphs were grouped into broad categories based on observed local color patterns. In some instances the color morph encompassed spines of a single color and in other cases spines on individuals were of two and sometimes three colors (Fig. 2-2).

Single color morphs signify that all spines around the test were a single color. In bicolored morphs all the spines have a color on the proximal end different from that on the distal end. In dual colored morphs the spines are uniform in color but the color differs on different areas of the test (i.e., fully green colored spines on the ambulacral areas and fully white colored spines on the interambulacral areas). Triple color morphs are a combination of bicolored and dual colored spines.

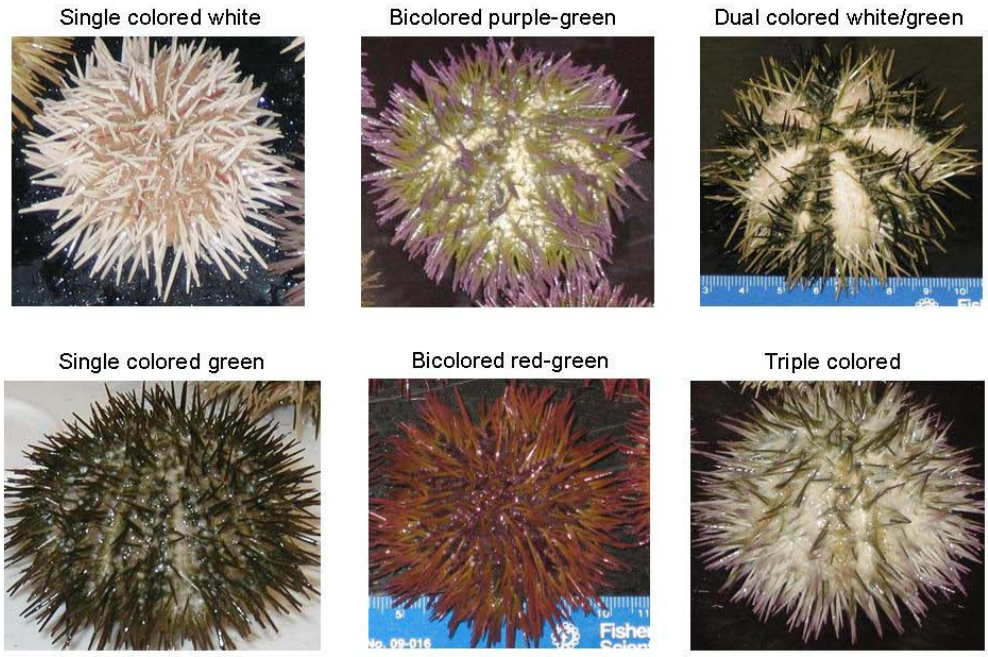

Figure 2-2: Typical color phenotypes from urchins across the geographic range.

A subsample of urchins was collected from the sites and brought back to DUML for a more in-depth comparison of color and morphology. Color variability was scored on sacrificed urchins at DUML to insure homogeneity in light levels and by only two people to insure consistency and minimize variability.

2.2.4 Color variability

To quantitatively assess the difference in urchin color between and among sites, color variability was broken down into discrete units on the spines and the test. Examined closely, most color morphs were a composite of colors. To account for this variability I coded spine and test colors separately and visually matched them to standard color paint cards from Lowe's Home Improvement Store. The paint cards were selected from different companies (American Tradition, Eddie Bauer, Laura Ashley and Waverly Classics) to insure a reasonably accurate representation of the colors found on the urchins. The colors were then sorted from light to dark within each color category (e.g. white, green, purple etc.) and numerically coded.

There are sixteen color categories: white, light pink, pink, red, purple, light lavender, lavender, beige, light brown, brown, tan, grey, light green, green, dark green and orange with a variable number of colors per category.

Spine color was coded from the tip (distal) to the base (proximal) by scoring the change in color. In most cases, when the spines were examined closely the tip was a different color from the base, even for urchins classified as single color. Test color was scored from the interambulacral areas (Fig. 2-3). In cases where the test was more than one color, color was also scored from the ambulacral areas (see dual colored urchin in Fig. 2-2).

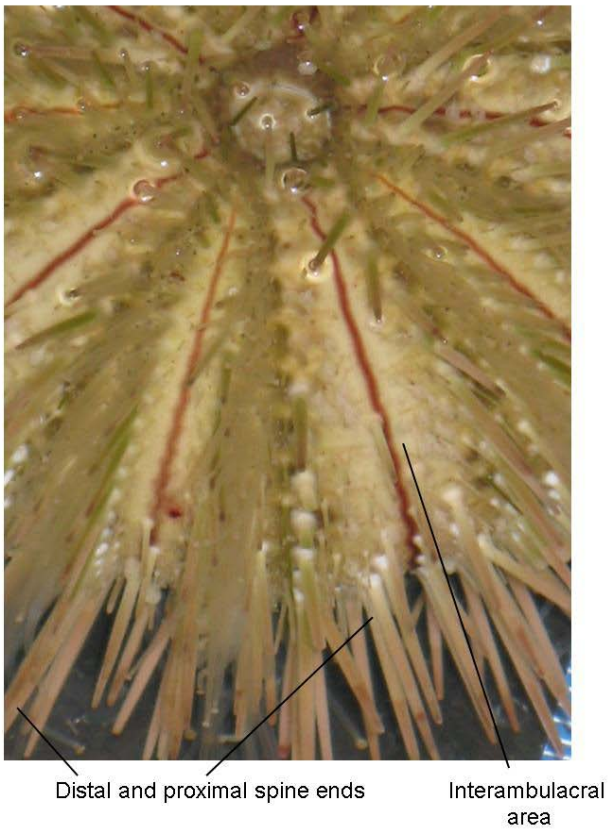

Figure 2-3: Spine and test regions coded for color.

2.2.5 Test color after removal of spines and epidermis

Due to restrictions on the number of urchins permitted to be collected at most sites, analyses on tests were limited to Beaufort and Tavernier Key. Color was taken on tests after the spines and epidermis were removed. Urchins (20–73 mm TD) from Beaufort and Tavernier Key were sacrificed by cutting through the peristomial membrane, removing the lantern and emptying out the contents. They were then placed in seawater for several days to allow for the removal of the spines and epidermis. Once

the tests were cleaned they were rinsed in fresh water and allowed to air dry for several days. Several samples taken from Brazil and St Joseph Bay were collected as denuded tests *in situ*.

Tests from Beaufort originated from four sites—3 inshore from Bogue, Back and Core Sounds and one from an offshore site in Onslow Bay. The substrate for two of the three sites—Oscar Shoal (OS) and Turning Basin (TB)—is a sand-shell hash mix on sand flats at 1–4 m depth bordering the channel basins of Back and Bogue Sounds respectively. The site at Cape Lookout (CL), at the far eastern edge of Core Sound, differed slightly from the other inshore sites as it had patches of the seagrass *Zostera marina*. The offshore site (Off) was a sand substrate at a depth of 16–20 m. Habitat at Tavernier Key was large meadows of *T. testudinum* at 5-6 m depth.

Test color/s was scored using the same standard color paint cards from Lowe's Home Improvement Store. Color was scored from the area surrounding the spine tubercules and from the interambulacral areas.

2.2.6 Data analysis

Color variation (both color phenotype and spine and test color variability) was compared with an *r* x *c* contingency table. The observed frequencies were used to determine the expected frequencies for each color morph or color category per region. Deviation from the null hypothesis of no association between region and color was determined by the chi-square statistic. The large size of the Beaufort field sample biased the statistic and the Beaufort sample size was adjusted downward by reducing the

sample size from 1864 to 1000, in line with the Gulf sample. All analyses were done in JMP ver. 8 (JMP IN 8.0.1, SAS Institute, Cary, NC, USA, 2008).

2.3 Results

2.3.1 Field surveys of *in situ* color morphs

A total of 3954 urchins were counted and scored for color at the 11 locations. Due to inter-site variation in the number of urchins counted and scored, the data were grouped into five regions, Beaufort, Gulf, Keys, Panama and Brazil. 1864 urchins were counted in Beaufort, 1076 urchins in the Gulf, 288 urchins in the Keys, 88 urchins in Panama and 638 urchins in Brazil.

Color morphs were grouped into broad categories based on observed color patterns of the spines. Test colors were not considered. The final tally of color morphs was 14 (Table 2.1). Five of the color morphs were single color: green, white, pink, purple and red; 7 were dual colored: pink-green, purple-green, red-green, red-purple, white/green, white/pink and white/purple; and 2 were triple color: green/white/purple and green/white/other (red or pink).

Table 2-1: *In situ* color morphs for the five regions. Numbers indicate the number per color morph counted at each site. Single color morphs followed by bicolor, dual and triple colored. Bicolored spines are listed with a hyphen, whereas, dual color morphs in which the spines are uniform in color but the color differs on different areas of the test are listed with a slash. Triple color morphs are also listed with a slash.

	Region					
Color phenotype	**Beaufort**	**Gulf**	**Keys**	**Panama**	**Brazil**	**Row total**
green	2	20	42	16	60	140
white	1503	39	36	7	0	1585
pink	138	166	22	0	6	332
purple	213	7	23	0	23	266
red	0	246	22	0	0	268
pink-green	0	199	39	0	11	249
purple-green	8	26	34	8	535	611
red-green	0	363	10	0	3	376
red-purple	0	10	4	0	0	14
white/green	0	0	36	17	0	53
white/pink	0	0	3	0	0	3
white/purple	0	0	4	2	0	6
green/white/other	0	0	2	0	0	2
green/white/purple	0	0	11	38	0	49
Total	1864	1076	288	88	638	3954

The presence and frequency of color morphs differ across the regions. Of the 14 color morphs only two are present in all 5 regions—green and purple-green. White, pink and purple are present in four regions: white in Beaufort, Gulf, Keys and Panama; pink and purple in Beaufort, Gulf, Keys and Brazil. Pink-green and red-green are present in Gulf, Keys and Brazil. The remaining seven color morphs occur in at most two regions.

Color frequency is highly skewed across the regions. Color morphs white and purple-green were found in the highest numbers, 1585 and 611 urchins respectively, in a single region at opposite ends of the geographic range: white in Beaufort and purple-green in Brazil. White was found in four regions but the vast majority, 94.8%, was in Beaufort whilst being completely absent in Brazil. Conversely, 87.6% of the purple-green morph was found in Brazil but relatively rare in Beaufort and Panama at 1.3% each. The rarest color morphs with counts in the single digits—white/pink, white/purple and green/white/other—were found in the Keys and/or Panama.

Contingency analysis (r x c) of the full data set of 14 color morphs across regions cannot be undertaken due to the unacceptably low expected frequencies for some of the rare color morphs. Therefore, to test the hypothesis that the frequency of color morphs is independent of region I dropped the least frequent 4 color morphs (red-purple, white/pink, white/purple and green/white/other). Since these color morphs are rare (each having a total count < 15) the expectation that they would occur in sufficient numbers at all locations can be discounted.

The frequency of observations for color morphs found in all regions is highly significantly different. Contingency analysis on the revised data set shows that color morph is not independent of region ($\chi^2 = 8105$, df = 36, $P < 0.0001$). Table 2.2 is a revised version of Table 2.1 listing the expected frequencies (in parentheses) alongside the observed frequencies of all but the rare color morphs listed above. The expected frequencies are calculated based on the data assuming independent distribution. From the table we see that all color morphs should be present in all regions.

Table 2-2: Observed and expected (in parentheses) frequencies of color morphs for the five regions. Contingency analysis (r x c) indicate highly significant differences in the frequencies of the color morphs across the regions $\chi^2 = 8105$, $P < 0.0001$. All color morphs should be present in all regions.

	Region				
Color phenotype	**Beaufort**	**Gulf**	**Keys**	**Panama**	**Brazil**
green	2 *(66)*	20 *(39)*	42 *(10)*	16 *(3)*	60 *(23)*
white	1503 *(747)*	39 *(431)*	36 *(115)*	7 *(35)*	0 *(256)*
pink	138 *(157)*	166 *(90)*	22 *(24)*	0 *(7)*	6 *(54)*
purple	213 *(125)*	7 *(72)*	23 *(19)*	0*(6)*	23 *(43)*
red	0 *(126)*	246 *(73)*	22 *(20)*	0*(6)*	0 *(43)*
pink-green	0 *(117)*	199 *(68)*	39 *(18)*	0 *(6)*	11 *(40)*
purple-green	8 *(288)*	26 *(166)*	34 *(45)*	8 *(14)*	535 *(99)*
red-green	0 *(177)*	363 *(102)*	10 *(27)*	0 *(8)*	3 *(61)*
white/green	0 *(25)*	0 *(14)*	36 *(4)*	17 *(1)*	0 *(9)*
green/white/purple	0 *(23)*	0 *(13)*	11 *(4)*	38 *(1)*	0 *(8)*

The combination of single, bicolored, dual or triple color morphs within a region varied (Figure 2-4). Single color urchins are common to all five regions but they are synonymous with Beaufort. 99.5% of the color groupings in Beaufort were single color morphs white, pink and purple. Gulf and Keys regions also had a sizeable portion of single color morphs, 44.5% and 50.3% respectively. Bicolored morphs predominate in Brazil and the Gulf where they comprise 85.6% and 55.5% of the population respectively. In Brazil the color morph purple-green comprised 83.9% of the dual color

morphs, with pink-green and red-green accounting for the remaining 1.7%. Pink-green, purple-green, red-green and red-purple were the dual color groupings in the Gulf. In the Keys 4% of the urchins were tricolored and the rest were split between single, bicolored and dual color morphs. Triple color morphs predominate in Panama with the green/white/purple morph comprising 43.2% of the population. The overall color palette of Panama is the narrowest with just three colors present—white, green and purple. Colors on the red end of the spectrum (including pinks) are absent.

The distribution and frequency of color morphs varies dramatically between regions. Figure 2-4 graphically illustrates the disparity between sites. Beaufort and Brazil each have low variability in color, having few color morphs and being dominated by one. White accounts for 80.6% of the urchins in Beaufort. Purple-green accounts for 83.9% of the urchins in Brazil. In contrast, the central sites are more variable both in terms of the number of color morphs and the proportion of each within the site. Despite the fewer number of urchins counted in the Keys the phenotypic composition is broadest of the five regions. It appears to be the hotspot for color variability with all 14 color morphs present and the least unbalanced distribution with none over 14.6% of the total. The Gulf has nine color morphs with 4—pink, red, pink-green and red-green—making up 90% of the population. Panama, like Brazil has only six color morphs but the relative frequency of each is greater.

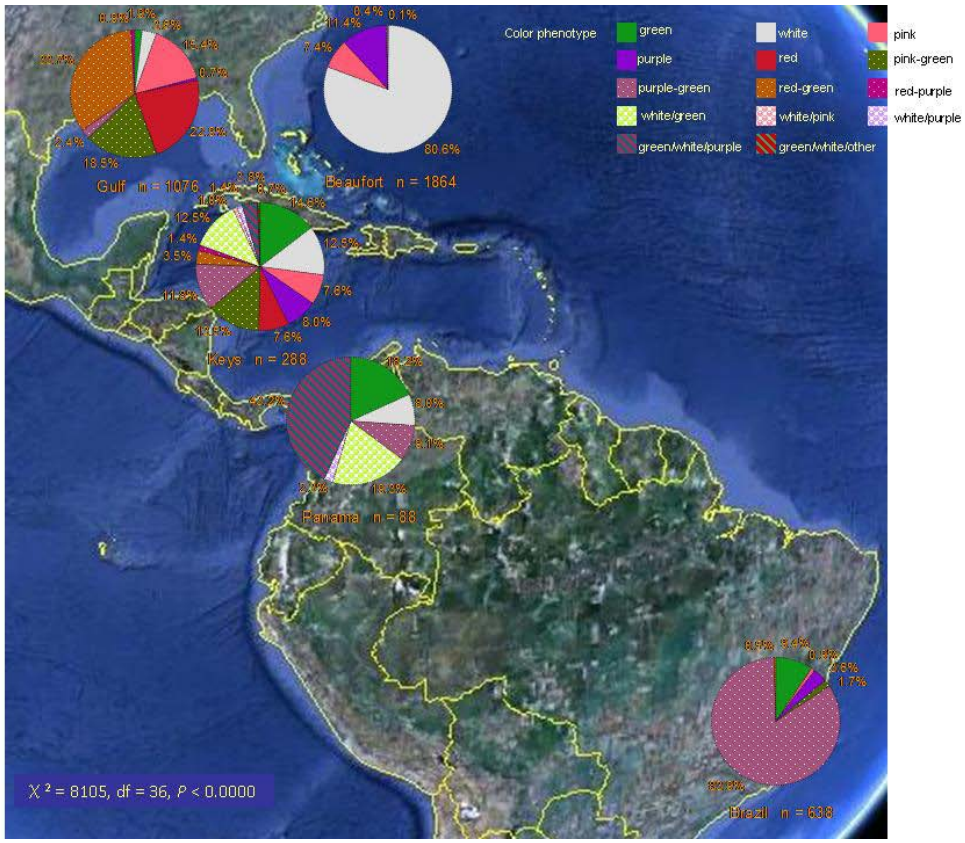

Figure 2-4: Distribution and relative frequency of color morphs in each of the five regions. Single color morphs shown as full colors, stippled colors are bicolor, colored spheres are dual color and diagonal bars are triple color. Beaufort and Brazil each have low variability in color, having few color morphs with one dominant. Keys has the greatest variability with all fourteen color morphs and the least unbalanced distribution. Single color morphs predominate in Beaufort, bicolor morphs in Brazil and triple color morphs in Panama.

Contingency analysis of color morph frequencies in Table 2.2 illustrates that given the observed frequencies the expectation is for all color morphs to be present at all 5 sites. Figure 2-4 demonstrates that many color morphs are completely absent in some regions, thus belying the expectation. The very high number of samples from Beaufort compared to the other regions increases the expected frequencies of the white morph relative to the other morphs. To reduce this bias, I randomly decreased the Beaufort sample to 1000, in line with Gulf numbers, so that the expected frequencies generated by the data better reflect the comparative diversity between regions. The expected frequencies should result in the frequency distribution of phenotypes as seen in Fig. 2-5. White, purple-green, and red-green would become the most frequent phenotypes across all regions.

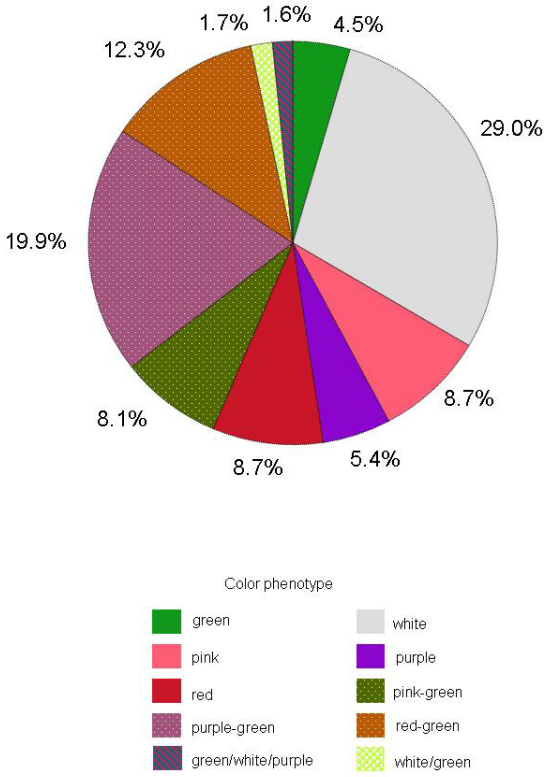

Figure 2-5: Expected frequency distribution of color phenotypes across all regions assuming no association of color and location.

2.3.2 Color variability in live urchins

Detailed analysis of color variability using color paint cards was conducted on the spines and test of 297 urchins: 78 from Beaufort, 86 from the Gulf, 88 from the Keys and 45 from Panama. Frozen urchins were thawed but not dried before color measures were taken. Color is found within the carbonate matrix of the test and spines and

remains the same after death. The thin layer of epidermis in *L. variegatus* is clear and does not appreciably alter the underlying color.

From the 297 urchins a total of 139 different colors were observed. These colors are grouped into seven categories: white (35), pink/red (31), purple/lavender (27), brown (30), grey (1), green (14) and orange (1). Urchins from Beaufort, Gulf and Keys each have 6 color categories and Panama has 4 categories. The number of colors is greatest for Beaufort and Keys at 91 each. Gulf has 65 colors and Panama the fewest with 48. Despite the fewer number of colors in the Gulf, there is no significant difference in color category diversity between the regions ($\chi^2 = 7.60$, df=3, $0.10>p>0.05$). The three most prominent traits—distal spine, proximal spine and test interambulacra varied in both the number of categories and the number of colors across the four regions. Table 2.3 lists the total number of categories and colors scored for all traits combined and separately for distal spine, proximal spine and test interambulacra.

Table 2-3: Total number of color categories and colors per region for all traits combined and for each of the three most visible traits: distal spine, proximal spine and test interambulacra.

		Region			
Trait		Beaufort n = 78	Gulf n = 86	Keys n = 88	Panama n = 45
Overall	*Categories*	*6*	*6*	*6*	*4*
	Colors	*91*	*65*	*91*	*48*
Distal	Categories	5	6	5	4
	Colors	37	29	45	16
Proximal	Categories	5	5	5	3
	Colors	42	32	39	23
Test IA	Categories	5	6	6	3
	Colors	44	25	43	23

The number of colors and categories were uncorrelated. The regions with the greatest number of categories often did not have the greatest number of colors. The Gulf region had the most color categories for the distal spine trait at 6 but had only 29 colors compared to the Keys which had one less category but the most colors at 45. For the proximal spine trait, three regions—Beaufort, Gulf and Keys—had the same number of categories at 5 each but the number of colors varied. Beaufort had the most colors (42) followed by Keys (39) and Gulf (32). Gulf and Keys had the same number of categories for test interambulacra (6) but Keys had almost twice as many colors, 43 versus 25. Beaufort with one less category had the most colors at 44. Panama had the fewest categories and the fewest colors for all traits. Contingency analysis revealed no significant difference in the number of colors for each of the traits across the regions (χ^2 = 3.17, df = 6, P = 0.79).

The palette of categories for all combined traits for the regions differs (Figure 2-6). Despite the equal number of categories in Beaufort, Gulf and Keys the composition and frequency vary dramatically. In Beaufort the bulk of color is shared in roughly equal proportion by four categories—purple/lavender, pink/red, white and brown. In contrast, Gulf and Keys each have one dominant category—purple/lavender in the Gulf and green in the Keys. Green is also the dominant category in Panama. Purple/lavender, dominant in the Gulf, is also amply represented in the other regions. Brown makes up a sizeable share of the overall color in Beaufort, Gulf and Keys (~20%) but is present as

only a tiny fraction in Panama. Grey is present in the Gulf and Keys but absent in Beaufort and Panama. Pink/red is an important component of Beaufort color but is completely absent in Panama and comprises a small share of the color in the Gulf and Keys. Orange is a very rare category that is present only in Beaufort. The frequency of color categories between regions differed significantly ($\chi^2 = 335$, df = 15, $P < 0.0001$).

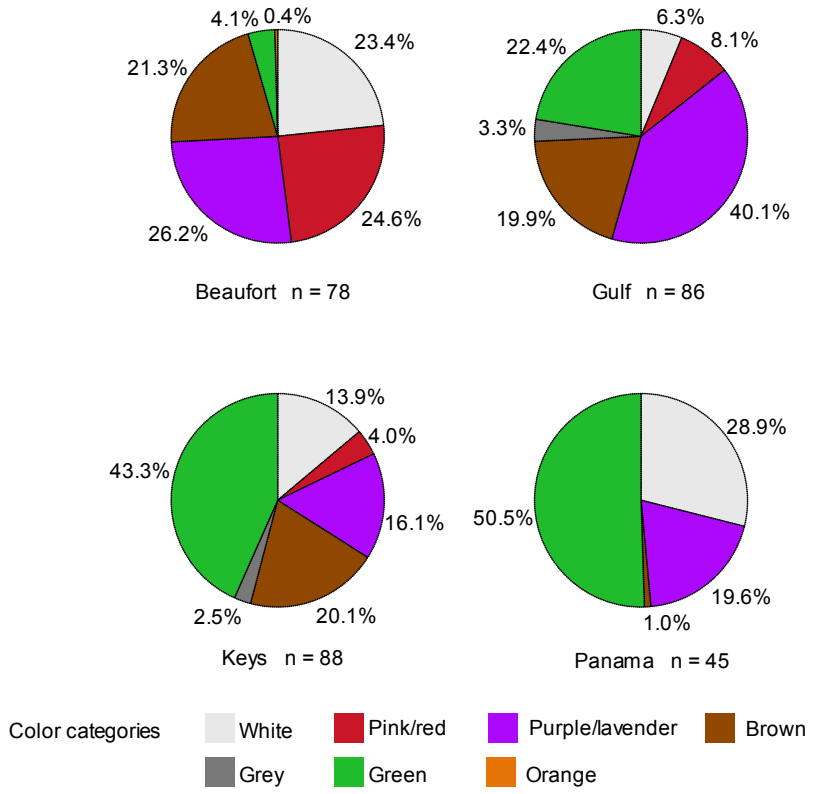

Figure 2-6: Frequency of color categories at the four regions. All spine and test colors are combined. Beaufort, Gulf and Keys regions each have 6 categories while Panama has 4. Beaufort and Keys each have 91 total colors, Gulf has 65 and Panama has 48. Contingency analysis demonstrates a significant difference ($\chi^2 = 35$, df = 15, $P < 0.0001$) for the six categories—white, purple/lavender, brown, grey and green. Orange, found only in Beaufort, was not included in the analysis.

Distal spine, proximal spine and test interambulacra are the most conspicuous of the traits examined, with color data for all 297 urchins. Examining spine and test colors for the three traits separately, the relative frequency of categories differs by region. The predominant distal spine color category for all regions is purple/lavender (Fig. 2-7). The frequency across the regions differs such that for Panama purple/lavender comprises the greatest proportion of total distal color at 86.7%, whereas, the smallest proportion at roughly half, 43.6%, is in Beaufort. White and pink/red combined comprise the same proportion, 43.6%, as purple/lavender of distal spine color in Beaufort but neither category is very abundant in the other regions. White is found at very small frequencies and pink/red is completely absent in Panama. Panama has the narrowest palette with 4 categories and the most unbalanced distribution with purple/lavender comprising the vast majority, 86.7%, of distal spine color. In contrast, Beaufort color categories are less skewed resulting in overall greater variability in distal spine color.

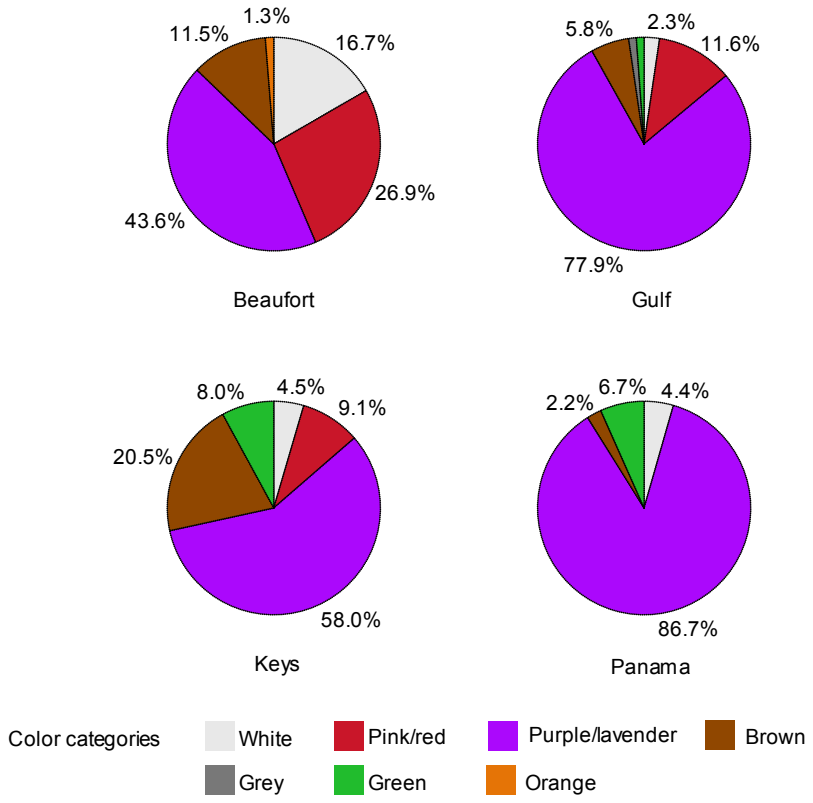

Figure 2-7: Frequency of distal spine color categories by region. Gulf has the greatest number of categories with 6, whereas, Panama has the fewest at 4. Beaufort and Keys both have 5. Gulf colors grey and green at a frequency of 1.2%.

For proximal spine color the number of color categories decreases to 5. Grey and orange are missing. The frequency of proximal spine color categories also differs within and across regions, as shown in Figure 2-8. In all regions one category encompasses the largest share of proximal spine color. In Panama and Beaufort white is the predominant category at 71.1% and 53.8% respectively. In the Keys and Gulf green is the dominant category at 63.6% and 53.5% respectively. In Beaufort green occurs at a much smaller frequency, 3.8%. Pink/red is sizeable proportion of proximal spine color in Beaufort

(26.9%) but a minor component in the Gulf and Keys and nonexistent in Panama. Purple/lavender, the dominant category in distal spine color is present in all regions but greatly reduced. The remaining category, brown, is present in the greatest proportion in the Gulf (10.5%) and absent in Panama. Proximal spine color in Panama is essentially reduced to two categories: white and green. Interestingly, the distribution of white and green proximal spine color in Panama and the Keys is almost a mirror image.

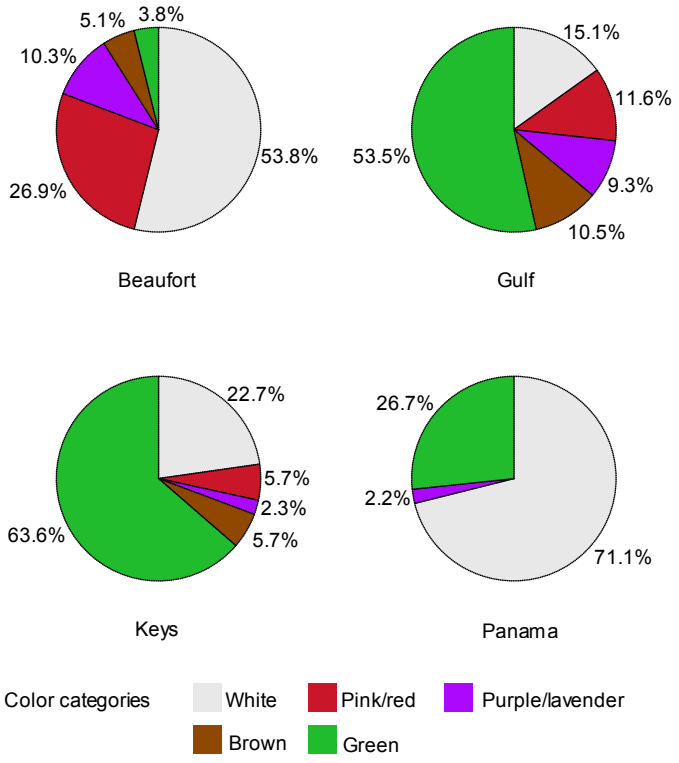

Figure 2-8: Frequency of proximal spine color categories by region. Beaufort, Gulf and Keys each have 5 categories. Panama has the fewest at 3.

Test interambulacral colors show the most interesting pattern. Each region has a different distribution of color categories as seen in Figure 2-9. Panama has two dominant categories—white and green—in roughly equal proportion comprising the bulk (97.8%) of the total. Likewise, the Gulf has two dominant categories, purple/lavender and brown, comprising 79.1% of the total. The Keys has 3 categories—green, brown and white—at similar frequencies totaling 91%. Beaufort also has three categories—brown, pink/red and purple/lavender —comprising 94.8% of the total but brown is at twice the frequency of pink/red and purple/lavender. Purple/lavender, very prominent in distal spine color in all regions is essentially restricted to Beaufort and the Gulf in the tests. It is not factor at all in Panama test color and is rare in the Keys. Pink/red is an important test color category in Beaufort, but is inconsequential in the Gulf and Keys and absent in Panama.

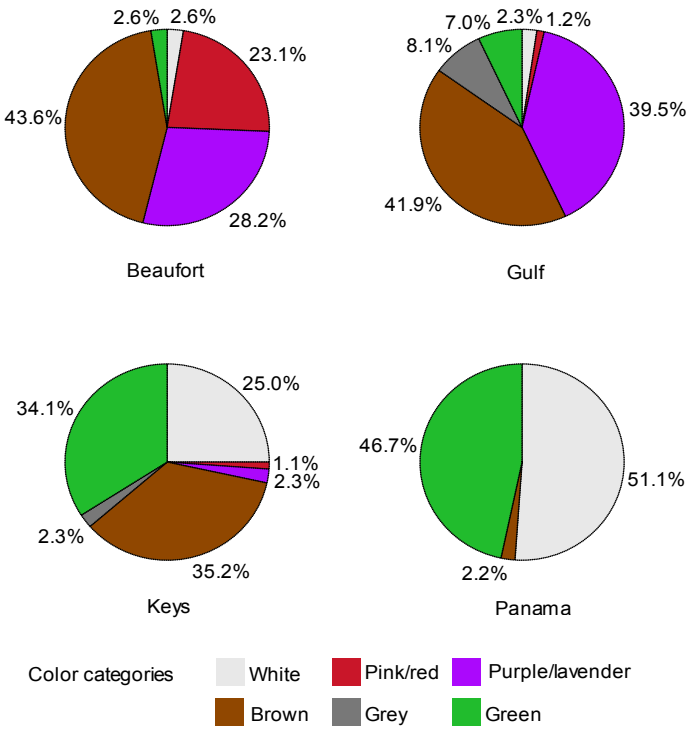

Figure 2-9: Test interambulacral color category frequencies by region. Gulf and Keys each have 6 categories, Beaufort has 5 and Panama has 3.

Partition of the categories from 7 to 15 (white, light pink, pink, red, purple, lavender, light lavender, beige, brown, grey, tan, light green, green, dark green and orange) allows for a finer scale examination of colors. Unlinking categories pink/red and purple/lavender and subdividing pink, lavender, brown and green into lighter and darker shades demonstrates how the distribution and frequency of colors differs considerably within and among regions for all combined traits (Figure 2-10). The majority of colors occur once or in few urchins (3–5) resulting in a shallow and broad distribution within a category. Categories such as white, light pink and beige have a

fairly wide but shallow distribution of colors in one or several regions. For other categories the frequent occurrence of one or a few colors results in a narrower condensed distribution. Purple, lavender and light green are the three categories with such a distribution—fewer colors but greater frequency for the colors present, some with counts in the double digits.

Partition of categories also allows for greater discrimination of colors within and across regions. Differences in the identity and frequency of individual colors demonstrate the inherent geographic variability of the species. The categories pink/red, purple/lavender, brown and green—subdivided and partitioned into lighter and darker shades—emphasize the differences in color phenotype between the regions. Pink hues, prevalent in Beaufort, are relatively rare in other regions but make up the entirety of the pink/red category. In the Gulf and Keys, pink and red are equal contributors. Purple and lavender are encountered in all regions but in the Gulf and Keys, purple is much more prevalent. In Beaufort and Panama purple and lavender are roughly equally abundant. Green is a significant contributor to urchin color in the Gulf, Keys and Panama but the composition differs—light green colors predominate in the Gulf and Keys and dark green colors predominate in Panama. Brown, partitioned into 4 subcategories—beige, light brown, brown and tan—demonstrates much inter-site variability. Beige is prevalent in Beaufort and rare in other regions, whereas, brown has a strong presence in the Gulf and Keys. Tan is most abundant in the Keys. The relative paucity of colors in Panama is readily evident.

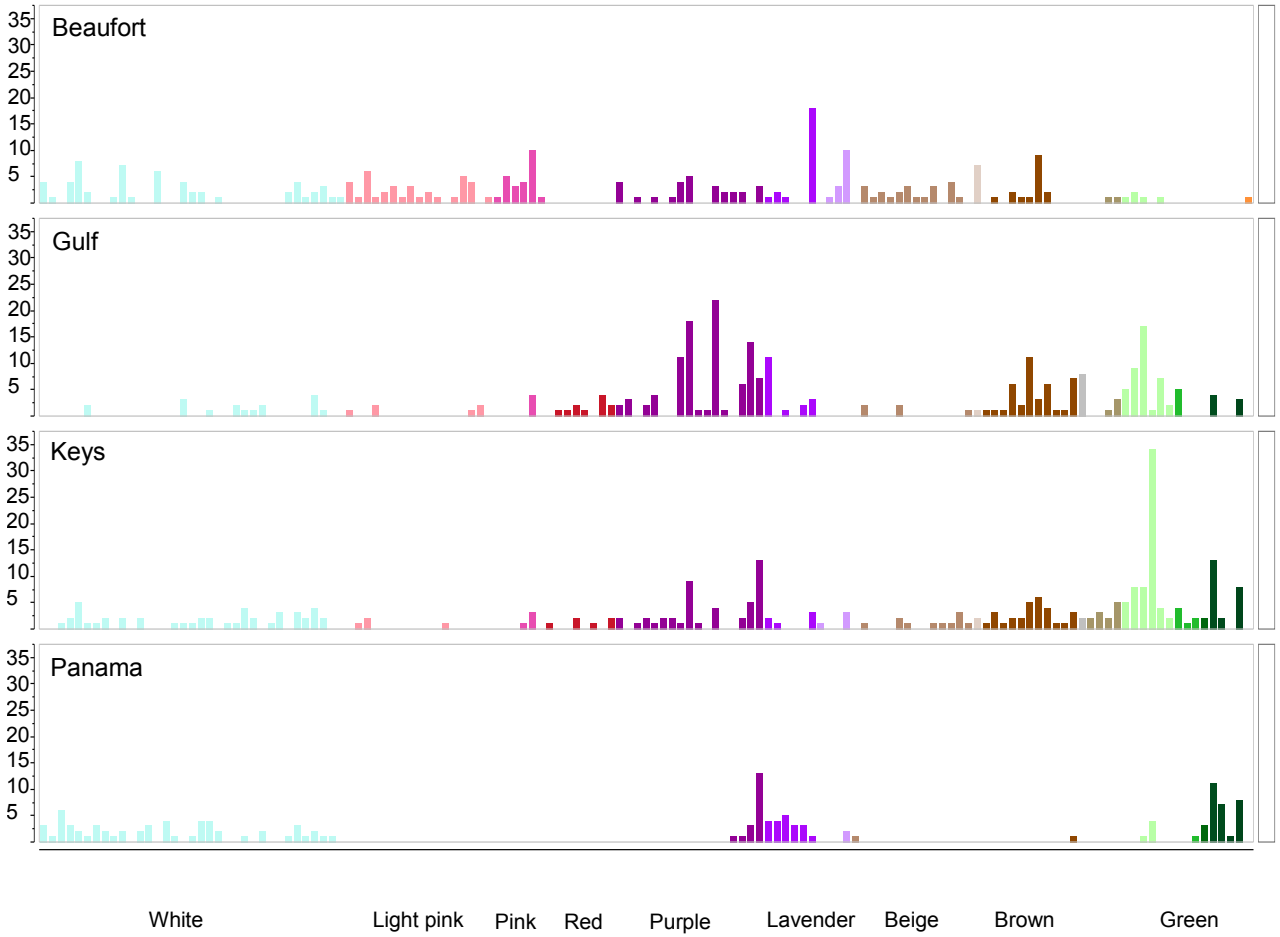

Figure 2-10: Histogram showing the frequency of 139 colors for all three traits combined. Colors are partitioned into 15 categories for each region. From top to bottom: Beaufort, Gulf, Keys and Panama.

Contingency table analysis carried out on the frequency of categories common to all four regions demonstrates the extent of inter-region differentiation. Table 2.4 lists the total count for each of the six categories occurring in all four regions—white, purple, lavender, beige, brown and green. Categories light pink, pink, red, grey and orange were not considered since all were absent in one or more regions. For statistical purposes lavender, brown and green included all light and dark subcategories. The data indicate that there is a significant difference in color distribution and thus color is region dependent ($\chi^2 = 226$, df = 15, $p < 0.0001$).

Table 2-4: Total count of the six color categories found in all four regions for all three traits combined. Contingency table analysis of category per region demonstrates that the distribution of color is related to region ($X^2 = 226$, df = 15, $p < 0.0001$).

	Region			
Color categories	Beaufort	Gulf	Keys	Panama
White	57	17	46	57
Purple	28	91	45	19
Lavender	26	17	10	21
Beige	22	5	11	1
Brown	25	43	33	1
Green	5	54	93	36

2.3.3 Color variability after removal of spines and epidermis

Analysis of color variability using color paint cards was conducted on the dried tests of 508 urchins. 366 from Beaufort and 142 from Tavernier Key were scored for color. The three areas coded for color are: interambulacral (IA) tubercules, IA wedge and 3rd color. IA tubercules refers to the area around both ambulacral and interambulacral tubercules. As they both generally have the same color only the IA area is referenced. IA wedge refers to the area between the interambulacral tubercules. 3rd color refers to any other color seen on the test regardless of its location (Fig. 2-11).

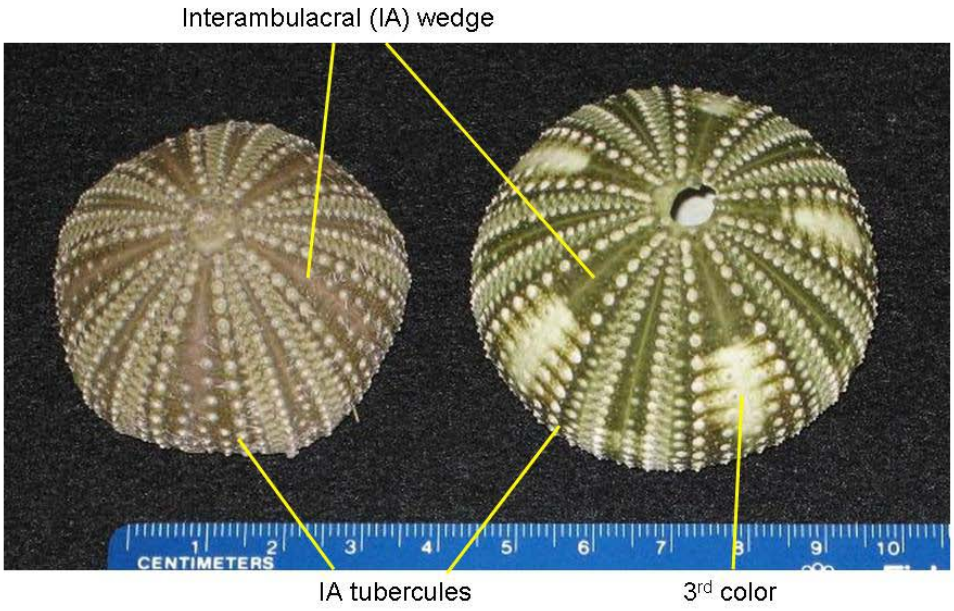

Figure 2-11: Test areas coded for color. Beaufort test on the left and Tavernier Key test on the right.

Color on tests is most pronounced on the aboral side and can be one uniform color or up to three separate colors. If multicolored, darker colors are generally in the area immediately surrounding the ambulacral and interambulacral tubercules. Lighter colors are found in the ambulacral and interambulacral wedge areas. Often these lighter colors can be a lighter shade within the same category (e.g. dark green and light green) or a lighter shade within a different category (e.g. dark green and lavender). A third color is sometimes visible and can be found bordering the darker color of the tubercules or in the ambulacral wedge area. In some cases, the interambulacral wedge areas may have two colors (Fig. 2-11 Tavernier Key test). The more abundant color is listed as the IA wedge color and the secondary color is listed as the 3rd color.

A total of 93 different colors were observed. The colors were grouped into the same seven categories as for spine and test colors: white (20), pink/red (21), purple/lavender (9), brown (25), grey (1), green (16) and orange (1).

Table 2.5 lists the total number of categories and colors scored for all test areas combined and for each of the areas scored for color. Contingency analysis revealed no significant difference in the number of colors for each of the test areas at the two locations ($\chi^2 = 0.69$, df = 2, $p = 0.71$). Each location was equally variable in the number of distinguishable colors for the three test areas.

Table 2-5: Total number of color categories and colors per location for all test areas combined and for each area individually: IA tubercules, IA wedge and 3rd color. IA tubercules refers to the area around both ambulacral and interambulacral tubercules. As they both generally have the same color only the IA area is referenced. IA wedge refers to the area between the interambulacral tubercules. 3rd color refers to any other color seen on the test regardless of its location.

		Location	
Trait		Beaufort n = 366	Tavernier Key n = 142
Overall	*Categories*	*7*	*6*
	Colors	*82*	*48*
IA tubercules	Categories	6	5
	Colors	36	18
IA wedge	Categories	6	5
	Colors	61	37
3rd color	Categories	5	6
	Colors	37	17

Color on Beaufort tests includes all seven categories. Tavernier Key tests have six categories. A total of 82 colors were counted in the Beaufort sample, 48 in the Tavernier Key sample. 21% of Beaufort tests and 9% of Tavernier Keys tests are single colored: 289 (79%) of the 366 Beaufort tests examined had a different IA wedge color and 81 (28%) of these had a third color. Of the 142 Tavernier Key tests examined 129 (91%) had a different IA wedge color and 26 (20%) of these had a third color.

The palette of categories for colors between Beaufort and Tavernier Key tests is similar (Fig. 2-12). Green, brown, white and purple/lavender are found at both locations and with similar distributions. However, contingency analysis of the frequencies of the categories reveals a significant difference ($\chi^2 = 33$, df = 5, $p < 0.0001$). Thus despite the

apparent similarity—shared categories at comparable frequencies and an equally variable number of distinguishable colors per area—test coloration between the two locations differs.

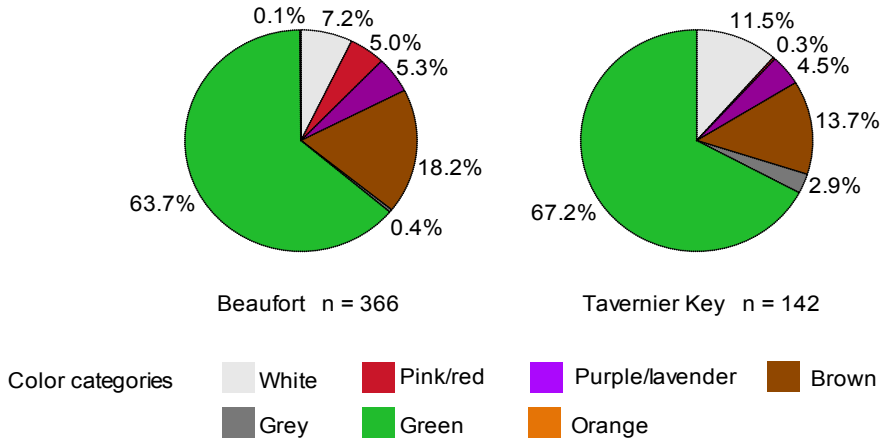

Figure 2-12: Color category frequencies of denuded tests from Beaufort and Tavernier Key. All test area colors are combined. Beaufort has 7 categories and 82 total colors, whereas, Tavernier Key has 6 categories and 48 total colors. The frequencies of the four categories shared by both sites—white, purple/lavender, brown and green—are significantly different ($\chi^2 = 33$, df = 5, $p < 0.0001$).

Separating the data by test area for each location (i.e., IA tubercules, IA wedge area and 3rd color), allows us to ascribe the source of variability more accurately (Fig. 2-13). Frequency charts for each of the test areas show similar distributions for IA tubercules and IA wedge but divergent distributions for 3rd color. Categories green and

brown are at strikingly similar frequencies for IA tubercule color, differing by 1.1% for green and 3.1% for brown between the two locations. Analysis indicates that the frequencies of these two categories are not significantly different across the two locations ($\chi^2 = 0.52$, df = 1, p = 0.47). Categories white, purple/lavender, brown, grey and green for IA wedge area are also very similar in distribution. If we compare the frequencies of these five shared categories, they do not differ ($\chi^2 = 7.1$, df = 4, p = 0.13). Distribution of 3rd color categories diverges between the two locations. Color between the two locations for this trait is significantly different ($\chi^2 = 18.5$, df = 3, p = 0.0003). In Beaufort, three categories at similar frequencies comprise 88.9% of the total. In contrast, Tavernier Key has one category comprising 60.5% of the total.

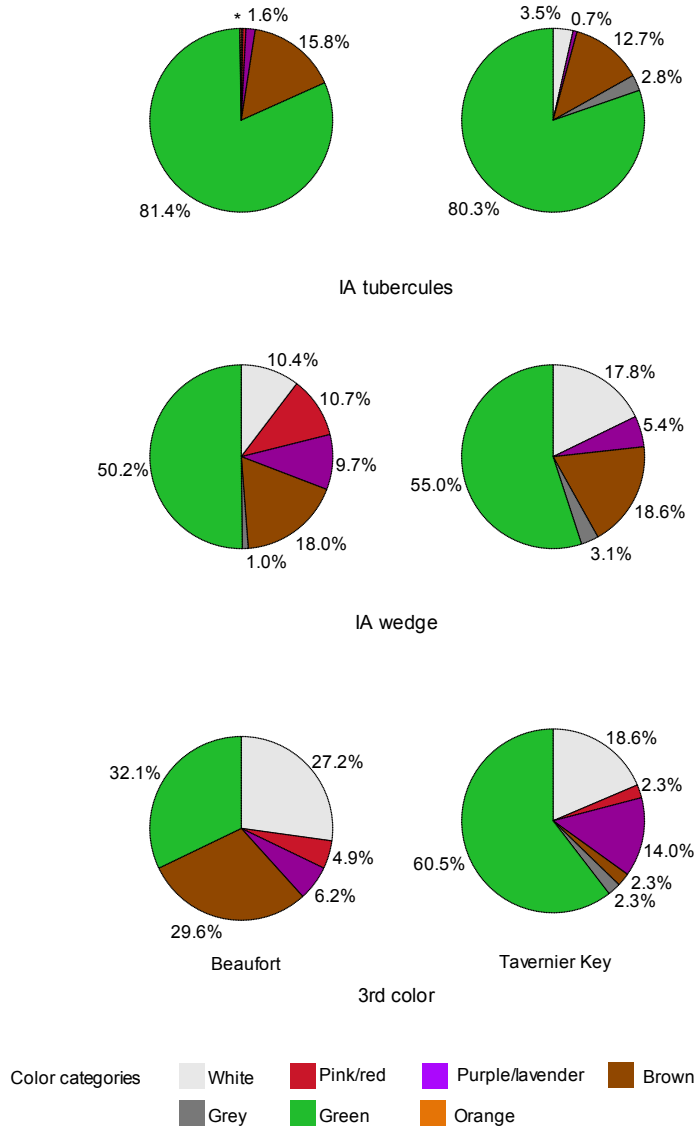

Figure 2-13: Color category frequencies of the three test areas for Beaufort, left, and Tavernier Key, right. Top pie charts show the categories for IA tubercules, middle pie charts are IA wedge categories and bottom pie charts are the 3rd color. *Categories white, pink/red and orange for Beaufort IA tubercules are at frequencies less than 0.05%. Distribution of the green and brown categories for IA tubercules is not significantly different between the two locations (χ^2 = 0.52, df = 1, p = 0.47) and neither are the five shared categories for IA wedge area (χ^2 = 7.1, df = 4, p = 0.13). The four 3rd color categories, however, are significantly different (χ^2 = 18.5, df = 3, p = 0.0003).

The Beaufort sample comes from inshore (Cape Lookout (CL), Oscar Shoal (OS) and Turning Basin (TB)) and offshore (Off) sites. Table 2.6 lists the overall number of categories and colors for all test areas combined and each area separately for all sites. Sites with the same number of categories had varying numbers of colors. Despite this variability, contingency analysis indicated no significant difference in the number of colors for each of the three areas across the sites ($\chi^2 = 4.8$, df = 6, p = 0.57).

Table 2-6: Total number of color categories and colors per site for all test areas combined and for each of the three areas: IA tubercules, IA wedge and 3rd color.

		Site			
Trait		Cape Lookout n = 88	Oscar Shoal n = 130	Turning Basin n = 95	Offshore n = 53
Overall	*Categories*	*5*	*5*	*6*	*6*
	Colors	*26*	*29*	*45*	*55*
IA tubercules	Categories	3	3	4	3
	Colors	7	12	23	20
IA wedge	Categories	5	5	6	5
	Colors	20	27	32	31
3rd color	Categories	4	4	4	5
	Colors	12	9	18	13

Separating the Beaufort data by site shows overall test color for the 3 inshore sites is very similar (Fig. 2-14). The largest category for all three inshore sites is green followed distantly by brown. In contrast the largest category for the offshore site is brown followed by pink/red. Analysis of the frequencies between all sites for the shared categories white, purple/lavender, brown and green shows that the distribution of

categories across the four sites is highly significantly different ($\chi^2 = 178$, df = 9, $p < 0.0001$). The magnitude of the difference is driven in large part by the color make-up of the offshore site. However, if we eliminate the offshore site from the analysis the difference between sites is reduced but it does not change the outcome: inshore sites are significantly different in their color composition ($\chi^2 = 17.2$, df = 6, $p = 0.0087$).

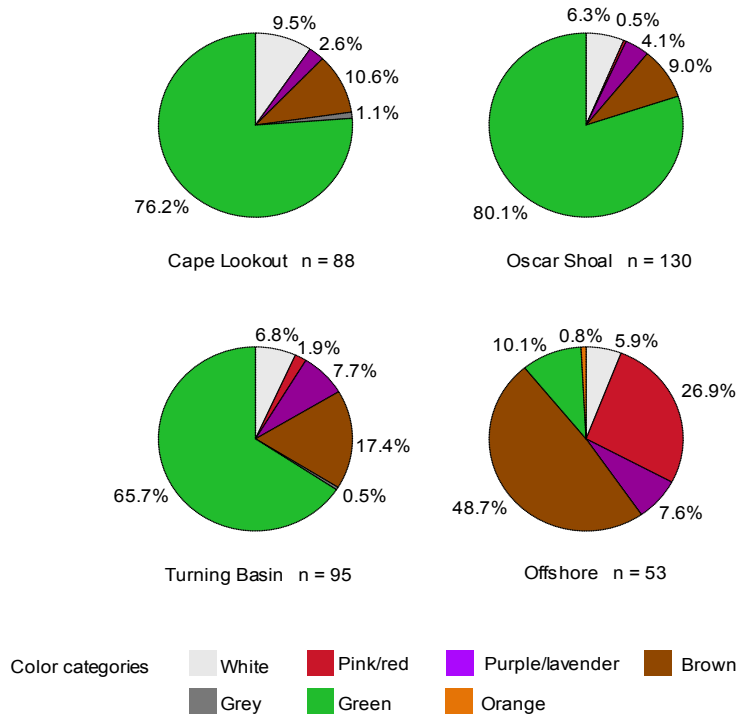

Figure 2-14: Color category frequencies of denuded tests for all Beaufort sites. Cape Lookout, Oscar Shoal and Turning Basin are inshore. All test area colors are combined. Analysis of the frequencies between sites for the categories white, purple/lavender, brown and green shows that the distribution of categories across the four sites is highly significantly different ($\chi^2 = 178$, df = 9, $p < 0.0001$). The offshore site contributes overwhelmingly to the difference. If taken out the difference between sites remains ($\chi^2 = 17.2$, df = 6, $p = 0.0087$) indicating small scale local variability.

Separating the data by test area for each site (i.e., IA tubercules, IA wedge area and 3rd color), shows that the inshore sites differ dramatically from the offshore site (Fig. 2-15). The overwhelming category for IA tubercule color for inshore sites is green but brown for the offshore site. Interestingly, frequencies for green and brown are almost mirror images for Turning Basin and Offshore. Analysis of the frequencies for green and brown shows that the distribution is highly significantly different ($\chi^2 = 225$, df = 3, $p < 0.0001$). The difference is driven in large part by the color make-up of the offshore site. However, the same analysis limited to the three inshore sites cannot be undertaken as some of the values fall below acceptable limits. Brown IA tubercule color for both CL and OS is miniscule.

Green is also the major IA wedge category for inshore sites. Offshore pink/red is the dominant category. The four shared categories across all sites remain white, purple/lavender, brown and green. Analysis of the frequencies shows that the distribution is highly significantly different ($\chi^2 = 31$, df = 9, $p = 0.0003$). Again, this difference is driven in large part by the color make-up of the offshore site. Eliminating the offshore site from the analysis reduces the magnitude of the difference but does not change the outcome ($\chi^2 = 8.5$, df = 6, $p = 0.02$).

The distribution of categories for 3rd color is less uniform. All inshore sites are composed of the same 4 categories—white, purple/lavender, brown and green. However, each site differs in their respective proportions. Each site has a different principal category—white at CL, brown at OS and green at TB. The offshore site differs

by having 5 categories instead of four and three principal categories instead of one. The three most common 3rd colors for all inshore sites are white, brown and green versus white, pink/red and brown for the offshore site. Due to the overall small sample sizes across the four sites for this trait, contingency analysis on the four shared categories cannot be undertaken as many of the values fall below acceptable limits.

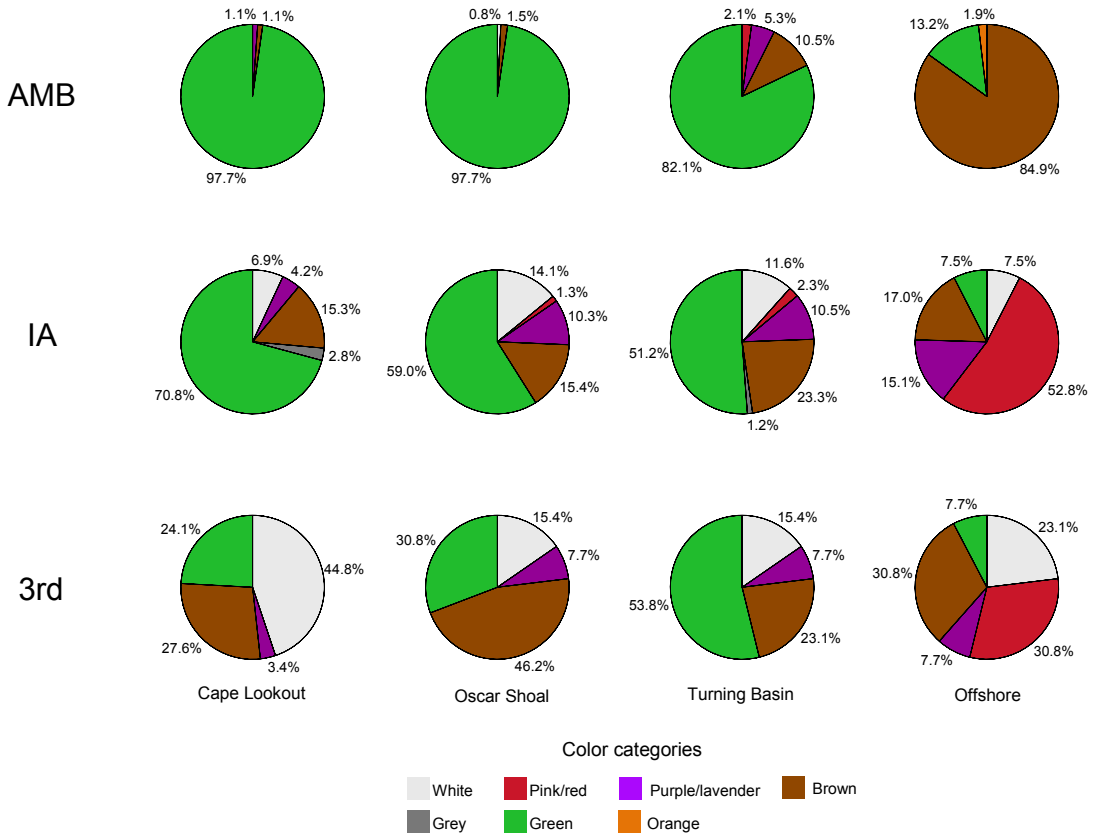

Figure 2-15: Color category frequencies of the three test areas for the four Beaufort sites. Top pie charts show the categories for IA tubercules, middle pie charts are IA wedge categories and bottom pie charts are the 3rd color.

2.4 Discussion

The data presented in this chapter demonstrate the great variability in color phenotypes found in *L. variegatus* across the range. The data show that color morph is dependent on region ($\chi^2 = 8105$, df = 36, $P < 0.0000$). However, color morphs do not correspond to subspecies as previously assigned because many color morphs are common among urchins from disparate regions of the range. Color variability is more extensive in the spectrum of phenotypes present but their occurrence is geographically circumscribed as evident in the frequency of color morphs at the extremes of the range. The distribution of color phenotypes in the field survey data is highly variable across the geographic range (Table 2.1). Urchins in each of the regions have a distinct phenotypic composition despite the presence of similar color morphs (Fig. 2-4). The two regions at the extremes—Beaufort and Brazil—demonstrate the most homogeneous phenotypic composition with a dominant color morph each. The Keys has the most heterogeneous composition with all 14 color morphs present.

The difference in the phenotypic composition of each region belies the connection between subspecies and color morph. This is evident for *L. v. carolinus*. The red phenotype ascribed to the subspecies is completely absent in Beaufort where 80% of the urchins are white. Red urchins are only 23% of the Gulf population whereas the dual colored red-green and pink-green color morphs comprise 52%. Bermuda urchins were not sampled so their phenotype cannot be quantified but apparently they most resemble

Gulf urchins (D. McClay, pers. comm.). This suggests that their phenotype is more complex than previously proposed and makes their subspecies designation based on phenotype dubious. Urchins from the Keys, Panama and Brazil demonstrate that the single phenotype ascribed to *L. v. variegatus* is wholly insufficient. The high diversity in the Keys region compared to the narrower phenotypic ranges of Panama and Brazil point to regional phenotypic clusters. The heredity of color phenotype is demonstrated in Chapter 4 and based on this the clusters would be constituted by the frequency of the alleles coding for the phenotypes. From the data we see that the overall level of phenotypic variability is high across the entire range but levels of intrapopulation variability differ. This suggests that color phenotype is a trait that varies over a smaller spatial scale than the subspecies.

The homogeneity of phenotypes seen in Beaufort and Brazil is due to the dominance of a single and different color phenotype. The prevalence of white (80.6%) in Beaufort is similar to the prevalence of the purple-green phenotype (83.9%) in Brazil. The very uneven distribution of phenotypes at the geographic extremes suggests that the genetic variability for color in these two regions is diminished compared to the central regions. The greater number of phenotypes in the Gulf, Panama and especially the Keys suggests increased genetic diversity. The most frequently encountered phenotypes were about half as abundant—red-green (33.7%) in the Gulf and green/white/purple (43.2%) in Panama with several other phenotypes in the double

digits. In the Keys none of the 14 phenotypes was greater than 15% of the total thus increasing the representation of all phenotypes.

A slightly different picture emerges from the data on the color variability in the spines, tests and denuded tests. Despite the divergence of phenotypes across the regions, the number of colors and color categories per region was not statistically different (Table 2.3), indicating equal levels of color diversity within regions. This seems counterintuitive given the divergence in phenotypes seen from the field data. But the phenotypes were based on first impression classification, whereas, the color variability data captured the nuances. Phenotypes such as white, purple, green and others subsumed variations in the color categories. Many lavender urchins were classified as purple, other phenotypes ranged from light to dark hues (e.g. green ranged in color from pea green to forest green, white urchins could be stark white to white with pinkish, tannish or greenish overtones etc.). The clustering of phenotypes into broad groups facilitated classification and subsequent analysis. The color variability data was designed to compare the types of colors present within and across regions since similar phenotypes could differ in the appearance of the colors. Hence the color variability data measured the range of colors both within and across regions.

The details can be seen in each of the spine and test frequency pie charts. The distribution of color categories for both spine and test areas differed dramatically highlighting regional differences. Proximal spine (Fig. 2-8) and test IA (Fig. 2-9) areas

provided the greatest contrast between the regions attesting to differences in urchin coloration at the coarse (color phenotypes) and fine (spine and test colors) scales.

Despite the differences in the frequency of color categories the multiple counts per color show that many colors are common to all regions and occur repeatedly (Fig. 2-10). Some of the phenotypes—both single color and dual color—are common and are found in more than one region (Fig. 2-4). The broad representation of colors within and across regions implies that the colors and the genes coding them are present in all populations to varying degrees. This is likely true despite the apparent discrepancy with the phenotype data. Data from genetic crosses in Chapter 4 demonstrate that the expression of certain colors is dependent on interactions between alleles and their frequency within a population. Thus while certain phenotypes are underrepresented in some regions, the alleles for the colors may be present but remain at very low frequencies. An example is the rarity of green urchins in Beaufort. The color data demonstrates the presence of green and its low frequency.

The factors that underlie the discrepancy between the observed and expected phenotypic frequencies creating the patterns in geographic variability remain unknown. The ecological relevance of the phenotypes and whether stochastic or adaptive forces are at play in their maintenance is also poorly understood. However, if we assume that colors have a functional significance we can posit several hypotheses for the observed geographic patterns.

Color phenotype may be an adaptive trait that is subject to selection [Hoekstra, et al., 2004; McMillan, et al., 1999]. The mechanism maintaining the frequencies may differ at each location given the level of habitat differentiation. The two regions at the extreme—Beaufort and Brazil—differ in substrate type, hydrodynamics, mean monthly temperature and likely faunal assemblages from the central regions. Both regions border biogeographic breaks where conditions change dramatically [Avise, 2000; Gaylord, et al., 2000; Ventura, et al., 1995]. This could have important impacts on the survival of recruits and adults. From the data the difference in color morph frequency evident at the two geographic extremes far surpasses the difference seen in other regions. The predominance of the white color morph and the absence of other morphs in Beaufort, especially, suggest an adaptive response. This could include a response to predation (crypsis) and/or to UV radiation. Beaufort urchins inhabit a pale, featureless, largely monochromatic substrate. The whitish, grayish sandy substrate offers very little protection against visual predators. Being white or off-white may be advantageous to urchins as it diminishes the contrast between them and the background effectively allowing them to hide in plain sight (add to this the practice of covering themselves with bits of shell and other debris which makes them less visible on the substrate). Many urchins of the same color can be a way of limiting one's exposure to predators [Bond, 2007; Cott, 1940]. Greater predation on greenish-red colored *L. variegatus* compared to white colored *Tripneustes ventricosus* on the white limestone background within a barrier-reef lagoon off Andros Island, Bahamas suggests that the more conspicuous

color may have exposed them to increased predation risk [Aseltine, 1982]. The chromatic background in Brazil is more varied than Beaufort. Macroalgae of various sorts and large lavender colored gorgonian fans are common in the rocky habitat of Arraial do Cabo. The green and lavender colors may be an advantageous chromatic background for the mostly purple-green color morphs. Refuge from predation (crypsis) may be a common mechanism between the two regions in interpreting the lopsidedness of the phenotypes.

By the same token, urchins in the Gulf, Keys and Panama are found in a polychromatic landscape dominated by seagrass beds (*T. testudinum, S. filiforme and H. wrightii*) and often bordered by other habitat (sand, coral reef and mangrove stands) may benefit from having more varied phenotypes. The seagrass canopy itself along with the accompanying suite of invertebrates provides a fuller palette that may help camouflage individuals of certain phenotypes. Having green colored spines, either fully or partially, may make urchins less conspicuous when enveloped in the seagrass blades [Bond, 2007].

Alternatively, pigmentation may be examined as a response to UV radiation. Highly pigmented urchins in clear waters may be at a selective advantage if the pigments provide protection against the deleterious effects of UV exposure (suggested from the resonant structure of the pigment molecules). The difference in the reaction of *L. variegatus* compared to *A. punctulata* in response to ultraviolet light suggests that darker somatic pigmentation may reduce the harmful effects [Sharp, et al., 1963]. In

Beaufort *A. punctulata* is often found in shallower water on pilings and sea walls and exposed at low tide (personal obs.), whereas, *Lytechinus* is in deeper water and often covered in shells. Coastal waters in North Carolina are high in suspended organic material that renders the water opaque. Waters high in dissolved organic material attenuate UV penetration within several meters [Smith, et al., 1979], which may obviate the need for *L. variegatus* to be heavily pigmented. In the Gulf, Caribbean and Brazil the waters are often very clear and may allow the passage of elevated levels of UVB radiation [Dunne, et al., 1996; Hader, et al., 2007] to the shallow depths in which the urchins are generally found. The effect of UV exposure on Beaufort urchins may be diminished to a level that allows for the reduction (not elimination) of pigmentation on the exposed spines while maintaining it on the test. While I did not quantify or compare the amount of pigment in the spines and test across regions its presence is attested to in the color variability data and shown in Fig. 2-10, even for urchins in Beaufort where the dominant phenotype is white.

The likely importance of pigmentation can be inferred from data on the denuded tests. For both Beaufort and Tavernier Key urchins pigmentation of the tests is quite high, even if the proportion of colors differs between locations (Figure 2-12). A lightly pigmented test (i.e. white color in one or more areas) was reduced to being 3rd color which renders it the least abundant category (Fig. 2-13). Given that the echinoid test is a highly porous matrix of calcium and magnesium carbonate [DuBois, et al., 1989; Magdans, et al., 2004] having a pigmented test may be important in protecting gonadal

tissue [Walker, et al., 2001]. Because test coloration is not apparent in the live animal it may not be under any selective pressure due to predation, allowing for its variability, especially in the Beaufort population (Figs. 2-14, 2-15). Here, competing selective pressures of predator avoidance through crypsis (little pigmentation) versus protection from harmful UV radiation through pigmentation may be relaxed compared to other regions.

Variation in color phenotype seen across the range may be maintained by factors unrelated to color. Our understanding of the genetic architecture underlying phenotypic and morphological features in *L. variegatu*s is rudimentary. As such, undetected physiological traits may be the basis of selection. If genes coding for other traits are linked to those for color phenotype then correlation between them will be driven by pleiotropy [Falconer, et al., 1996]. In such cases, the overdominance of phenotypes is maintained for reasons having little to do with color per se. Likewise, if color genes are in close physical proximity to the physiological trait under selection then linkage disequilibrium will alter the frequencies of the color genes, as well, potentially producing the phenotypic differences seen [Falconer, et al., 1996].

If color phenotype is a neutral trait, the patterns of geographic variability may be due to stochastic factors such as larval dispersal, recruitment and genetic drift. The extensive dispersal potential of *L. variegatus* has been assumed given its planktonic larval phase. This life history trait, common to many marine invertebrates, suggests high levels of gene flow creating a large panmictic population [Palumbi, 1994]. As such, the

expectation is that color phenotypes should be randomly distributed. Calculating the expected frequency distributions from the field data (Table 2.2) lends support for this interpretation. Adjusting the large sample size in Beaufort downward to lessen the bias, the revised distribution indicates that all color morphs should be present in all regions, with three color morphs predominating: white at (29%), followed by purple-green (20%) and red-green (12%). The remaining phenotypes would be below (10%) (Fig. 2-5).

The long development time of larvae (from 2 weeks to > 60 days, [McEdward, et al., 2001; Strathmann, 1978], 2 weeks to 30 days in genetic crosses Ch. 4) allows for their dispersal far from their point of origin. This should increase the representation of phenotypes across the regions, especially since it is a heritable trait (Ch. 4). However, little is known about actual larval development time *in situ* and potential larval behavioral patterns that may influence dispersal patterns. Larval aggregation in response to food cues in echinoid larvae [Burdett-Coutts, et al., 2004; Metaxas, et al., 1998] and active transport and orientation through behavioral mechanisms in larval crabs and fish have been demonstrated [Cronin, et al., 1986; Leis, et al., 2007; Olmi, 1994]. How widespread these behaviors are in other taxa with pelagic larval phases is unclear. However, these behaviors indicate that larvae are not always just passive particles transported by chance but actively participate in their dispersal. This active participation may influence population differentiation at very small scales leading to variation in phenotypes along a clinal gradient allowing for more local recruitment, and thus reinforcing the presence of native over novel phenotypes.

Genetic drift may factor in the maintenance of divergent phenotypes especially in small populations. Die offs of *L. variegatus* have occurred in Beaufort in 2007 and 2010 (pers. obs.), in Tampa in 2007 (D. Rittschof, pers. comm.) and in St. Joseph Bay in 1994 [Beddingfield, et al., 1994], and may severely reduce populations such that bottleneck-like conditions may alter the genetic diversity within the populations [Mladenov, et al., 1997; Pastor, et al., 2004]. Fluctuations in allele frequency, especially in small populations, leads to fixation of alleles and population differentiation [Falconer, et al., 1996]. In the case of *L. variegatus*, this may lead to drastic fluctuations in the alleles for color phenotypes causing some to be lost or severely reduced. This would lead to the overrepresentation of some colors with respect to others.

The difference in the classification of phenotypes between my study and Serafy's [1973] is most likely one of degree rather than substance. I chose to expand the categories to spotlight the diversity. The distribution of phenotypes cuts across the presumed subspecies boundaries and seems to be more a matter of location. The observed frequency distribution compared to the expected frequency distribution of color morphs listed in Table 2.2 demonstrates their apparent grouping by region. Regions such as Beaufort and Brazil have very narrow phenotypic ranges even though they fall within the boundaries of *L. v. carolinus* and *L. v. variegatus* respectively, whereas the Florida Keys have a very broad phenotypic range. Given this, color is not a good measure to differentiate subspecies.

3. Morphological variability in *L. variegatus*

3.1 Introduction

Morphological variability in organisms is a function of phenotypic changes in response to genetic variability and changes in local environmental conditions. Variations in body size and shape can be brought about through several mechanisms: natural selection, phenotypic plasticity and genetic drift. Differences in phenotypes as a result of natural selection and phenotypic plasticity are generated through biotic and abiotic agents.

Biotic agents of morphological change may be predators and food resources. Differential predation increases the representation of morphs that are resistant to predation increasing their fitness relative to other morphs. Differences in food resources may induce alterations in the food gathering apparatus to better exploit available resources [Ebert, 1980; Hagen, 2008; Levitan, 1991] Abiotic agents include hydrodynamic forces, temperature, photoperiod and salinity. Increased wave action or strong steady tidal currents impose flow-induced forces that increase the likelihood of dislodgement or injury [Denny, 1994; Koehl, 1984; Vogel, 1994]. Animals that are able to alter body shape and tenacity of adhesive structures are better able to mitigate the effects of drag and avoid dislodgment [Denny, 1994; Koehl, 1984]. Abrupt or sustained changes in temperature, photoperiod and salinity impose physiological and metabolic stresses

eliciting physiological (e.g. osmoregulation) and behavioral responses (e.g. migration) [Jansen, et al., 2007; McCormick, et al., 1998; Somero, 2002; Todd, 1964].

Differences in phenotype between individuals of the same species inhabiting different habitats, often just meters apart reflect the genetic variability within a population. Morphological differences suggest divergent selection on genotypes due to ecological adaptation. For sessile and benthic organisms with limited mobility the differences in phenotype may signal abrupt changes in habitat. For organisms in which morphological differences are genetically determined geographic variability is attributable to differential survival among individuals of differing genotypes such that certain genotypes increase in frequency and spread within a population [Falconer, et al., 1996].

On rocky shores the morphological variability of snails, mussels and barnacles in response to predators and hydrodynamic forces has been widely examined [Janson, 1987; Jarrett, 2008; Johannesson, 1986; Leonard, et al., 1999; Rolán-Alvarez, et al., 1997; Seeley, 1986; Trussell, 1996, 1997]. The variability between individuals in the upper versus lower intertidal is ascribed to natural selection [Conde-Padín, et al., 2009; Janson, 1987; Johannesson, 1986; Rolán-Alvarez, et al., 1997] and phenotypic plasticity [Leonard, et al., 1999; Trussell, 1996, 1997]. Morphological differences between upper and lower intertidal can be dramatic. Individuals in the upper intertidal are generally larger and have thicker shells than individuals in the lower intertidal. Predation pressure and resistance to desiccation are implicated in the differential survival of individuals in the upper intertidal. Conversely, increase hydrodynamic forces due to wave action operate

in the lower intertidal. Snails inhabiting the lower intertidal compensate for the higher drag forces of increased wave exposure with smaller, thinner shells, larger apertures and lower spires [Janson, 1987; Johannesson, 1986]. Mussels respond by also increasing the number of byssal threads [Young, 1985]. Barnacles shorten the length of the feeding ciri in response to increased wave exposure [Arsenault, et al., 2001; Li, et al., 2004]. These compensatory mechanisms reduce instances of damage or dislodgement.

Phenotypic plasticity is the ability of genotypes to produce different morphologies in response to environmental conditions [Pigliucci, 2001a, 2001b] and has been widely documented for both plants and animals. Changes in morphology are mediated by external conditions that change within the lifetime of the individual. The ability to alter morphology in the face of changing environmental conditions would be highly advantageous, increasing the fitness of those organisms relative to organisms whose morphology is less plastic. This has been demonstrated for invertebrate larvae in relation to predator cues and food abundance [Boidron-Metairon, 1988; Hart, et al., 1994; Strathmann, 1978].

Predation pressure and stronger hydrodynamic forces can also induce plastic changes in shell morphology for sessile or slow moving organisms like mussels and snails living in the intertidal. Predator cues and increased wave action have been shown to induce shell thickening and increases in foot size in littorinid and patellid gastropods [Appleton, et al., 1988; Dalziel, et al., 2005; Trussell, 1997]. Predation and hydrodynamic forces similarly alter the length of feeding legs and operculum morphology in barnacles [Arsenault, et al., 2001; Jarrett, 2008; Li, et al., 2004]. These studies demonstrate that

morphological variability may arise through both selection and plasticity depending on biological constraints of the organism (e.g. limited or no mobility) and on the spatial and temporal scales of the stresses.

Urchins are a diverse group whose morphology differs from species to species and also within species. Test shape and thickness, spine lengths and widths and lantern size are characters that vary widely depending on habitat. In areas of differing wave exposure and substrate structure differences in test shape between conspecifics have been documented in tropical and temperate urchins [Dix, 1970; Lewis, et al., 1984; 1965; Moore, 1935]. Flatter tests have been documented in urchins from more dynamic areas where wave energy is greater. Individuals of *Echinus esculentus* [Moore, 1935], *Echinometra lucunter* [Lewis, et al., 1984] and *Tripneustes ventricosus* [McPherson, 1965] from exposed sites had flatter tests than in more sheltered sites. Similarly, *Psammechinus miliaris* with rounder tests were more common in seagrass beds than those dredged from exposed deeper sites [Lindhal, et al., 1929].

Urchins from higher energy habitats have thicker test walls. *Echinometra lucunter* in Barbados [Lewis, et al., 1984], and *Evechinus chloroticus* in New Zealand [Dix, 1970] have thicker, more robust tests in areas of greater wave exposure. However, test thickness was also correlated with lower water temperatures [Dix, 1970]. Similarly, urchins in areas of greater wave energy have more robust spines [Dix, 1970].

The observations reported above were from field populations so it is unknown whether the differences are from habitat-driven selection on phenotypes or a plastic response to environmental cues. For feeding structures such as urchin lanterns, plasticity

in the size of the lantern in response to food limitation occurs. Urchins grown in conditions of food limitation have larger lanterns compared to urchins with abundant food [Ebert, 1980, 1996; Hagen, 2008; Levitan, 1991]. In the field, differences in lantern size were seen in urchins from habitats of contrasting food availability and increased density [Black, et al., 1982; McShane, et al., 1997].

Experimental studies have demonstrated the plastic nature of test shape and thickness in response to environmental and microhabitat differences. In all cases, urchins modified the shape of the test in response to external cues within a relatively short period of time (from a few days to <23 weeks). Test height in urchins grown in pits was greater than urchins grown on a flat surface [Hernandez, et al., 2010]. Tensile mechanical stress due to the activity of the ambulacral tube feet compressed urchin test growth in the vertical direction thus making it flatter [Dafni, 1986]. Waterborne cues from crab predators induced thicker skeletal growth in *S. droebachiensis* [Selden, et al., 2009].

Differences in trait morphology within and between populations may be adaptive. If phenotypic differences enhance function then they become an adaptation and can lead population differentiation and ultimately to speciation [Reznick, et al., 2001]. Such a scenario is likely responsible for the difference in lantern size between two recently diverged sympatric *Strongylocentrotid* species. Functional specialization for durophagy (consumption of hard food) led to speciation between *S. pallidus* which feeds on hard-shelled *Mytilus edulis* and *S. droebachiensis* which feeds on macroalgae [Hagen, 2008].

Morphological variability within *L. variegatus* is high. Serafy [1973] documented differences in morphological characters between the subspecies. Spine diameter (thickness) and the number of interambulacral (IA) plates were found to be the most distinguishing characters. Morphologically *L. v. atlanticus* from Bermuda was more similar to *L. v. carolinus* than to the Caribbean *L. v. variegatus* but as there was considerable overlap in all characters between the subspecies he concluded that morphology was not a reliable indicator of subspecific distinction.

In this chapter I evaluate the morphology of urchins from the different regions with respect to location and concordance with color phenotype. Samples from each region were collected in field surveys for detailed morphometric analysis at the Duke Marine Lab. Analysis of data are designed to assess the overall variability across the geographic range, within each region in relation to the subspecies.

3.2 Materials and Methods

3.2.1 Sample Collection

Samples for morphological measurements were collected at the same 11 locations listed in Chapter 2 for color phenotype data. Beaufort (Bft 34.72 N, 76.65 W), Gulf: Saint Andrews Bay (SAB 30.15° N, 85.67° W) and Saint Joseph's Bay (SJB 29.80° N, 85.36° W), Keys: Key Biscayne (Mia 25.69° N, 80.17° W), Indian Key (Ind 24.89° N, 80.68° W), Pigeon Key (Pig 24.70° N, 81.16° W), and Tavernier Key (Tav 25.02° N, 85.51° W), Panama: Bocas del Toro (BDT 8.45° N, 82.15° W) and Galeta Point (GP 9.40° N, 79.87° W)

and Brazil: Arraial do Cabo (ADC 22.96° S,42.03° W) and Cabo Frio (CF 22.53° S, 42.1° W)(Fig. 2.1).

3.2.2 Morphological measurements

Ten characters—test diameter (TD), test height (TH), test wall thickness (TW), the diameter of the peristome (PS), the length and width of both ambital (AMSL and AMSW) and apical (ABSL and ABSW) interambulacral spines and the height (LH) and width (LW) of the Aristotle's lantern—were measured with digital calipers taken to the nearest 0.01 mm. Two characters, the ambulacral (AMB) and interambulacral (IA) plates were counted. Wet weight measurements were taken of the whole live animal, the emptied test with attached spines and the lantern.

After the morphological measurements the urchins were frozen and transported to DUML, for detailed determination of spine and test color. Dry weight measurement of the test and lantern was taken after air drying for several days.

Test diameter (TD) was measured as the widest region from one ambulacral suture to the opposite interambulacral suture. The calipers were positioned between the rows of spines in both the ambulacra and interambulacra. Two measurements were taken, the second measurement was taken by rotating the urchin 90° from the first measurement. The mean was calculated. Test height (TH) was measured by centering the calipers over the apical disc and peristome of the urchin. Again, two measurements 90° apart were taken and the mean calculated. The lantern was removed by cutting through the thick skin surrounding the peristomial opening. The diameter of the peristome (PS) was measured by placing the inside jaws of the calipers in the peristomial

opening taking care not to include the buccal notches. Two measurements, 90° apart, were taken and the mean calculated (Fig. 3-1 A).

Test wall thickness was measured by splitting the test into two or more sections along the interambulacral suture. The mean width of the test wall (TW) was measured at the interambulacral suture at both the ambital and apical regions since a difference in the thickness between apical and ambital regions has been noted for Beaufort urchins. Again, two measurements were taken at each section and the mean for each section calculated. The overall test wall mean was calculated by averaging the values from the ambital and apical sections. Lantern height (LH) was measured from the top of the lantern to the tips of the teeth. Lantern width (LW) was measured as the widest region of the top of the lantern. Two measurements were taken for both the length and width and the mean calculated for each (Fig 3-1 A, B).

The length and width of spines at the ambitus and near the apical disc were measured. Five of the longest primary spines were selected from the interambulacral plates at the ambitus. Length (AMSL) from tip to base and width (AMSW) at the base of the spine just above the collar were measured. The mean was calculated for both the length and width. The same procedure was done for five of the longest interambulacral spines at the aboral region near the apical disc (ABSL and ABSW) (Fig. 3-1C). Ten more randomly selected spines were taken and preserved in 95% ethanol for use in genetic analyses.

Two characters, the ambulacral (AMB) and interambulacral (IA) plates were counted. The plates were counted by counting the number of primary spines in each

section from the peristome to the apical disc. As there is one primary spine per plate, these counts represent the number of interambulacral and ambulacral plates in a column from the peristome to the apical disc.

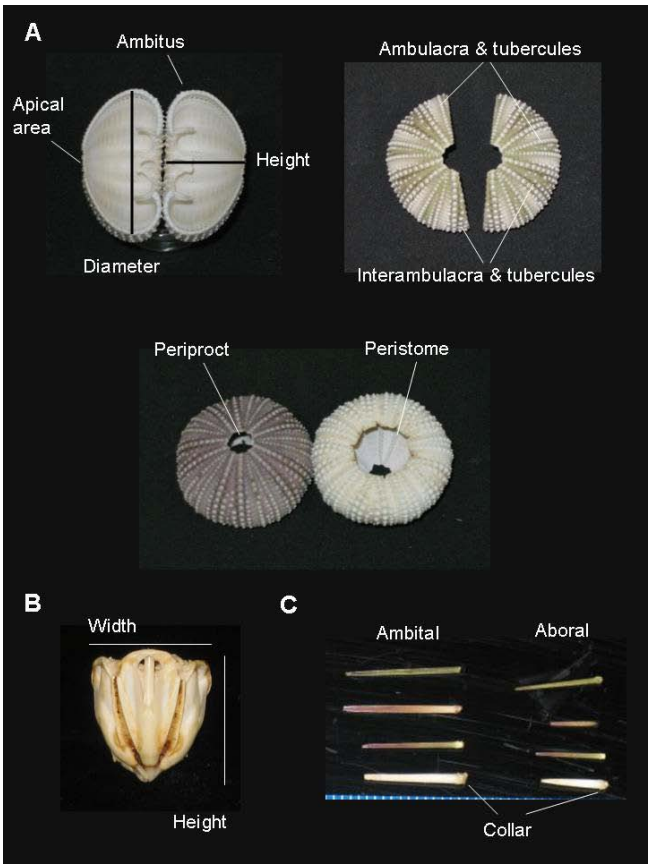

Figure 3-1: Urchin test, lantern and spines showing morphological areas measured. Denuded test shown since it demonstrates morphological features more clearly. Spines are attached at the tubercules. The number of ambulacral and interambulacral plates was counted by counting the spines on intact urchins and the tubercules on denuded tests.

Morphological measurements for urchins collected at Arraial do Cabo and Cabo Frio, Brazil are incomplete and are limited to whole wet weight, test diameter and height, lantern wet weight, height and width. Other measurements could not be completed *in situ* and due to export restrictions the urchins remain at the Instituto de

Estudos do Mar Almirante Paulo Moreira (IEAPM) in Arraial do Cabo. Live urchins sent to DUML from Saint Joseph's Bay, Saint Andrews Bay and Tavernier Key were weighed, measured and scored for color upon arrival.

3.2.3 After removal of spines and epidermis

Due to restrictions on the number of urchins permitted to be collected at most sites, analyses on tests were limited to Beaufort and Tavernier Key. Morphology measures were taken on tests after spines and epidermis were removed. Urchins (20–73 mm TD) from Beaufort and Tavernier Key were sacrificed by cutting through the peristomial membrane, removing the lantern and emptying out the contents. They were then placed in seawater for several days to allow for the removal of the spines and epidermis. Once the tests were cleaned they were rinsed in fresh water and allowed to air dry for several days. Several samples taken from Brazil and St Joseph Bay were collected as denuded tests *in situ*.

Tests from Beaufort originated from four sites—3 inshore from Bogue, Back and Core Sounds and one from an offshore site in Onslow Bay. The substrate for two of the three sites—Oscar Shoal (OS) and Turning Basin (TB)—is a sand-shell hash mix on sand flats at 1–4 m depth bordering the channel basins of Back and Bogue Sounds respectively. The site at Cape Lookout (CL), at the far eastern edge of Core Sound, differed slightly from the other inshore sites as it had patches of the seagrass *Zostera marina*. The offshore site (Off) was a sand substrate at a depth of 16–20 m. Habitat at Tavernier Key was large meadows of *T. testudinum* at 5-6 m depth.

Morphological characters measured include many of the same ones as on live urchins: test diameter (TD), test height (TH), the diameter of the peristome (PS), the mean width of the test (TW) and the number of ambulacral (AMB) and interambulacral (IA) plates (in this case determined by counting the number of primary spine tubercules in each section) from the peristome to the apical disc. One additional character was included: the diameter of periproct (PP), measured as the area occupied by the genital plates, ocular plates and madreporite in the live animal (Fig. 3-1 A).

3.2.4 Data analysis

All morphological character frequency distributions were assessed for normality and homoscedasticity. In cases where one or both conditions were not met, data were ln–transformed. If this proved ineffective I compared the means using the nonparametric Kruskal-Wallis test of means and Welch's ANOVA test. One-way analysis of variance (ANOVA) test was used when all assumptions were met. Due to the high number of comparisons a Bonferroni correction factor was employed to adjust the overall experimentwise error rate of 0.05 (Sokal and Rohlf, 1995). In all comparisons (test, spine, lantern measures), the observed p-value remained well below the corrected significance cutoff level for each comparison.

Several measures were converted to ratios to give better indication of the true nature of the difference in test and spine morphology by eliminating the effect of size since larger urchins have comparatively larger structures. Differences in the means of

the ratios between regions were also assessed for normality and homoscedasticity. Comparisons were tested as outlined above.

Multivariate analysis of variance was used to analyze the characters in aggregate since each character is not independent of the others and a change in one may prompt a change in the others. Nine ratios along with test thickness were examined. Discriminant analysis was used to visualize the differentiation of regions and assess the classification capacity of morphological characters. All analyses were done in JMP ver. 8.

Several morphological features were compared and differences between regions were examined through analysis of variance (ANOVA). The nonparametric Kruskal-Wallis test of means and Welch's ANOVA test will be used should the assumptions of normality and homoscedasticity not be met. Results of wild caught urchin morphology will be compared to lab reared crosses (Ch 4) to assess the relative contribution of genetic versus environmental factors.

3.3 Results

3.3.1 Urchin morphology

Morphological measurements were taken on the spines, test and lantern of 417 urchins: 112 from Beaufort, 113 from the Gulf, 104 from the Keys, 45 from Panama and 43 from Brazil. Measurements on samples from Brazil were limited to the diameter and height of the tests and the wet weight, height and width of the lanterns.

All test and lantern character frequency distributions were normally distributed. All except aboral test wall thickness and overall mean test wall thickness were heteroscedastic or had unequal variances. All 16 characters listed in Table 3.1 were highly significantly different across the regions (*** = $P < 0.0001$). 12 of the 16 characters had their greatest mean values at the regions on the edge of the geographic range—Beaufort and Brazil. Seven of the characters (test dry weight, aboral, ambital and mean test thickness, aboral spine width and ambital spine length and width) were greatest in the Beaufort population. Five characters (test diameter and height, lantern wet weight, height and width) were greatest in the Brazil population. Only one character—the number of ambulacral plates—was highest in the central range, in Panama. For the remaining characters (peristome, aboral spine length and interambulacral plates) the greatest mean values were shared among two or more regions.

Table 3-1: Lists the ranges, means and standard deviations of all morphological characters measured from the 5 regions. Measurements on samples from Brazil are limited to the diameter and height of tests and the wet weight, length and width of lanterns. Differences between regions for all test, lantern and spine characters were significantly different (ANOVA, Kruskal-Wallis, Welch test *** = $P < 0.0001$).

	Character		Beaufort n = 112	Gulf n = 113	Keys n = 104	Panama n = 45	Brazil n = 43		χ^2	F
Test	diameter (mm)	range	31.97–74.00	34.67–61.64	33.14–90.16	24.59–82.96	31.18–86.20	***	100	
		mean	49.59	45.00	51.16	51.66	65.07			
		st dev.	11.34	5.78	8.64	13.12	9.50			
	height (mm)	range	17.54-47.83	21.25–37.48	19.31–66.24	11.68–49.66	20–41.78	***	62	
		mean	27.74	29.20	31.75	30.49	34.69			
		st dev.	7.30	3.29	6.32	8.52	4.38			
	aboral	range	0.44–1.06	0.45–1.06	0.43–1.24	0.36–0.82		***		23
	thickness (mm)	mean	0.74	0.69	0.63	0.61				
		st dev.	0.12	0.11	0.14	0.12				
	ambital	range	0.57–1.50	0.55–1.20	0.44–1.31	0.38–0.85		***	134	
	thickness (mm)	mean	0.91	0.79	0.67	0.66				
		st dev.	0.17	0.13	0.17	0.11				
	mean	range	0.55–1.22	0.53–1.11	0.45–1.22	0.37–0.82		***		51
	thickness (mm)	mean	0.82	0.74	0.65	0.63				
		st dev.	1.28	0.11	0.15	0.11				
	dry weight (g)	range	2.84–40.10	3.07–16.12	1.77–53.09	1.66–27.50		***	41	
		mean	13.39	7.14	9.28	9.47				
		st dev.	8.63	2.46	7.52	6.64				
	peristome (mm)	range	12.65–24.47	12.38–19.46	12.76–25.70	11.97–23.90		***	105	
		mean	18.39	14.81	17.88	17.54				
		st dev.	2.76	1.55	2.09	2.90				
	IA	range	17–31	18–30	18–30	16–32		***	37	
		mean	22	24	22	23				
		st dev.	3.12	2.16	2.78	3.66				
	AMB	range	20–41	23–40	20–45	22–48		***	20	
		mean	30	31	31	34				
		st dev.	4.70	3.46	4.06	6.64				
Lantern	length (mm)	range	10.5–25.64	10.05–19.56	10.62–22.74	9.90–19.28	11.68–26.90	***	170	
		mean	17.47	13.72	15.65	14.79	22.02			
		st dev.	3.17	1.59	2.13	2.49	2.57			
	width (mm)	range	10.59–22.88	9.96–19.59	10.19–23.81	9.86–20.56	10.20–23.40	***	138	
		mean	16.76	13.43	15.70	15.46	19.79			
		st dev.	2.97	1.79	2.11	2.95	2.32			
	wet weight (g)	range	0.70–6.16	0.30–2.53	0.50–4.10	0.46–2.80	0.54–5.62	***	75	
		mean	2.61	1.07	1.59	1.55	3.63			
		st dev.	1.30	0.43	0.61	0.69	0.99			
Spines	ABSL (mm)	range	3.63–9.05	3.87–7.62	4.73–10.92	3.75–10.50		***	144	
		mean	5.96	6.03	7.90	7.62				
		st dev.	1.16	0.69	1.24	1.67				
	AMSL (mm)	range	9.49–18.09	8.80–14.34	8.14–15.90	7.11–14.40		***	115	
		mean	14.08	12.11	12.81	11.37				
		st dev.	1.52	1.17	1.38	1.85				
	ABSW (mm)	range	0.50–1.22	0.48–0.79	0.44–0.91	0.39–0.73		***	173	
		mean	0.80	0.65	0.66	0.54				
		st dev.	0.11	0.06	0.08	0.09				
	AMSW (mm)	range	0.79–1.86	0.72–1.12	0.60–1.14	0.50–0.82		***	261	
		mean	1.15	0.89	0.83	0.65				
		st dev.	0.18	0.07	0.10	0.08				

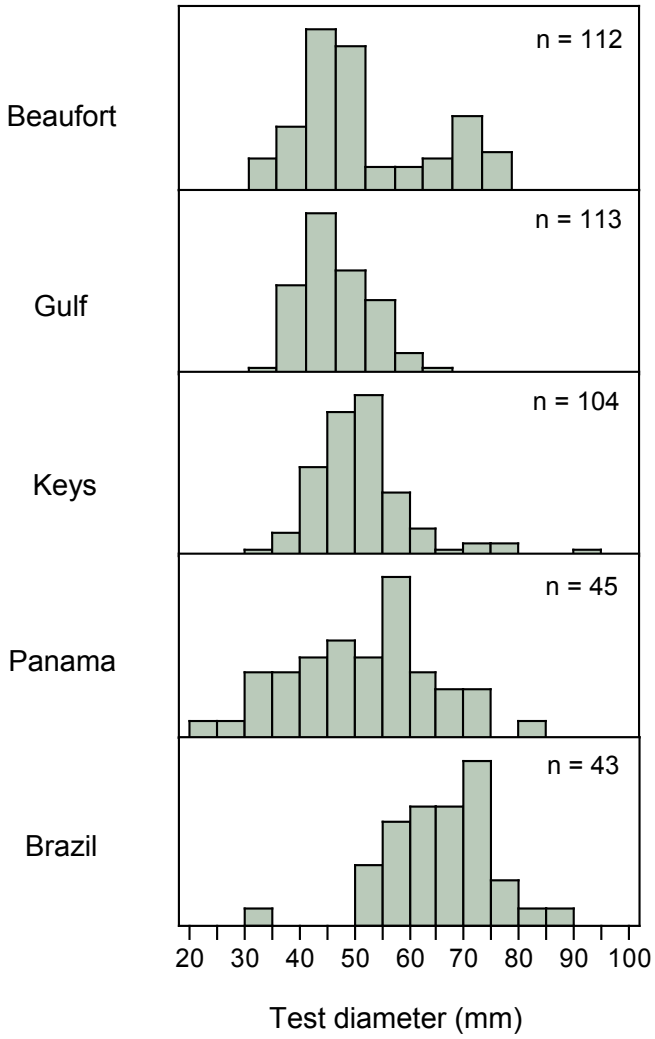

Figure 3-2: Histograms showing the size range of urchin test diameters from the 5 regions. Mean test diameter: Beaufort = 49.59 mm, Gulf = 45.00 mm, Keys = 51.16 mm, Panama = 51.66 mm and Brazil = 65.07 mm. Urchins in Brazil tended toward larger sizes, whereas, the range of test diameters in Panama was the broadest. Beaufort test size is bimodal owing to the larger sizes of offshore urchins. A Kruskal-Wallis comparison test of mean test diameter $\chi^2 = 99.7$, $P < 0.0001$. Beaufort = Gulf ≠ Keys ≠ Panama ≠ Brazil.

Urchins in Brazil were larger on average than elsewhere. Mean test diameter in Brazil was 65 ± 9.5 mm, 20 mm larger than urchins from the Gulf which had the smallest mean diameter 45 ± 5.78 mm. The overall size range of urchins was largest in Panama (55 mm) and smallest in the Gulf (35mm) (Fig. 3-2). Beaufort test size shows a bimodal distribution. Offshore urchins are significantly larger than those inshore. Mean test diameter for inshore urchins is 43.49 ± 4.3 mm versus offshore urchins 63.92 ± 8.9 mm ($t = 13.41$, $P < 0.0001$). Beaufort, Keys and Panama mean diameters do not differ but are significantly different from Brazil and the Gulf (Kruskal-Wallis $\chi^2 = 99.7$, df = 4, $P < 0.0001$).

Mean test height was greatest for Brazil urchins (34.69 ± 4.38 mm) and least for Beaufort urchins (27.74 ± 7.3 mm). The difference in height was modest, only 6.95 mm in mean height separated Brazil from Beaufort urchins but the difference across the regions was highly significantly different (Kruskal-Wallis $\chi^2 = 62$, df = 4, $P < 0.0001$). Panama (30.49 ± 8.52 mm), Gulf (29.20 ± 3.29 mm) and Beaufort (27.74 ± 7.3 mm) mean heights do not differ but are significantly smaller than Keys (31.75 ± 6.32 mm) and Brazil (34.69 ± 4.38mm).

Urchins in Beaufort were heavier, had thicker test walls, stouter spines and longer ambital spines than urchins in the Gulf, Keys or Panama (comparisons with Brazil could not be made). Mean test dry weight was significantly different (Kruskal-Wallis $\chi^2 = 41$, df = 3, $P < 0.0001$) with the greatest value in Beaufort (13.39 ± 8.63 g) and least in the Gulf (7.14 ± 2.46 g). Test weight did not differ between the Gulf, Keys (9.28 ± 7.52 g) and Panama (9.47 ± 6.64 g)

The mean value of test wall thickness is the average of the mean values measured on the aboral side near the apical plate and around the ambital area. The thickness of both the aboral and ambital regions is greatest for Beaufort urchins. The relationship mirrors exactly the overall mean test thickness (Fig. 3-3). Beaufort urchins had significantly thicker test walls (0.82 ±1.28 mm) than urchins in other regions (Gulf = 0.74 ± 0.11 mm, Keys = 0.65 ±0.15 mm, Panama = 0.63 ± 0.11 mm) (ANOVA F = 51, $P < 0.0001$). Urchins from the Keys and Panama had the thinnest test walls and Gulf urchins were intermediate. Comparing aboral and ambital test wall thickness, Beaufort and Gulf test walls are asymmetrical, as seen in the regression plot in Figure 3-4. The slopes of the lines differ for each region with Panama (1.00) and Keys (0.86) near isometric and Gulf (0.77) and Beaufort (0.54) being thinner at the aboral end than at the ambitus (Kruskal-Wallis $\chi^2 = 81$, df = 3, $P < 0.0001$). The difference in thickness is 0.18 mm for Beaufort and 0.10 mm for Gulf urchins. Keys and Panama urchins have tests of almost uniform thickness (0.04 mm and 0.05 mm respectively). Figure 3-5 illustrates the difference in overall test thickness between Beaufort and Keys urchins and more specifically shows the marked difference in aboral versus ambital thickness typical of the Beaufort population.

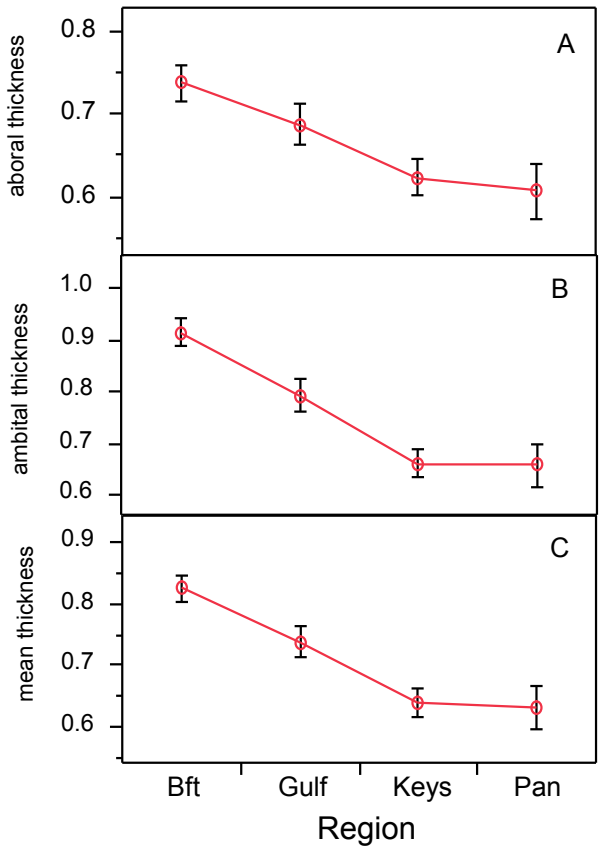

Figure 3-3: Mean values (± SE) for test wall thickness for the (a) aboral, (b) ambital areas and (c) overall mean test thickness. In all cases, Beaufort urchins have thicker test walls than other regions, on average 25% thicker than Keys or Panama tests. Test wall thickness for Keys and Panama does not differ. Beaufort ≠ Gulf ≠ Keys = Pan.

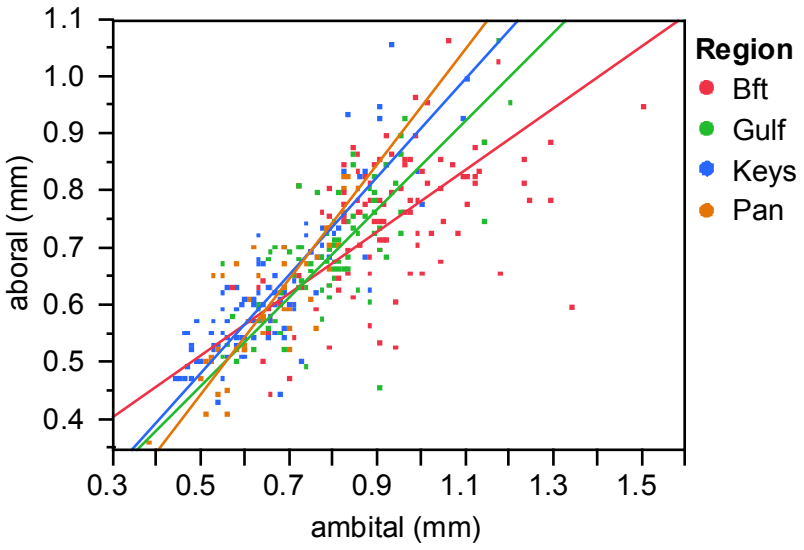

Figure 3-4: Mean test thickness at the aboral end regressed on the mean test thickness at the ambitus (the curve leading towards the peristome). Beaufort and Gulf tests are asymmetrical, being thinner at the top and thicker at the bottom. The difference is greater for Beaufort urchins (0.18 mm) than for Gulf urchins (0.10 mm). Keys and Panama have tests of near uniform thickness differing by only 0.04 mm and 0.05 mm respectively. Beaufort ≠ Gulf ≠ Keys = Panama.

Figure 3-5: Urchin test wall thickness (denuded test demonstrates more clearly the difference in test thickness at both the aboral and oral ends). Beaufort urchin on the left and Keys urchin on the right. Beaufort urchins have thicker tests around the ambital region. Keys urchins have nearly uniform tests.

Mean spine length was significantly different for both aboral (ABSL Kruskal-Wallis $\chi^2 = 144$, df = 3, $P < 0.0001$) and ambital (AMSL Kruskal-Wallis $\chi^2 = 115$, df = 3, $P < 0.0001$) spines across the regions. Beaufort had the longest ambital spines (14.08 ± 1.52 mm) but the shortest aboral spines (5.96 ±1.16 mm). Although the latter were not different from the Gulf (6.03 ± 0.69 mm) they were significantly different from the Keys (7.90 ± 1.24 mm) and Panama (7.62 ± 1.67 mm), which had the longest aboral spines. Ambital spine length differed among all 4 regions with the longest spines in Beaufort followed by Keys (12.81 ± 1.38 mm), Gulf (12.11 ± 1.17 mm) and Panama (11.37 ± 1.85 mm).

Mean spine width differed significantly for both aboral (Kruskal-Wallis $\chi^2 = 173$, df = 3, $P < 0.0001$) and ambital spines (Kruskal-Wallis $\chi^2 = 261$, df = 3, $P < 0.0001$) across the regions. Spine width was greatest for Beaufort for both aboral and ambital spines. Beaufort aboral spines were 32% stouter than Panama spines (0.80 ± 0.11 mm versus 0.54 ± 0.09 mm), which were the most slender. Gulf (0.65 ± 0.06 mm) and Keys (0.66 ± 0.08 mm) had spines of equal thickness. Ambital spine width differed among all 4 regions with the stoutest spines in Beaufort (1.15 ± 0.18 mm) followed by Gulf (0.89 ± 0.07 mm), Keys (0.83 ± 0.10 mm) and Panama (0.65 ± 0.08 mm). Beaufort ambital spines were 43% thicker than Panama spines.

All lantern measures had their greatest mean values in the Brazil population. Lantern wet weight was significantly different across the regions (Kruskal-Wallis $\chi^2 = 175$, df = 4, $P < 0.0001$) and greater at the edges of the range (wet weight instead of dry

weight is used in comparisons because there were no dry weight measures for Brazil samples). The heaviest lanterns were in Brazil (3.63 ± 0.99 g) followed by Beaufort (2.61 ± 1.3 g). Lantern weight in the Keys (1.59 ± 0.61 g) and Panama (1.55 ± 0.69 g) did not differ. Lanterns were lightest in the Gulf (1.07 ± 0.43 g). Lantern height and width have identical patterns having the greatest mean values in Brazil (22.02 ± 2.57 mm and 19.79 ± 2.32 mm respectively) and the smallest values in the Gulf (13.72 ± 1.59 mm and 13.42 ± 1.79 mm respectively). Lantern height and width did not differ in the Keys (15.65 ± 2.13 mm and 15.70 ± 2.11 mm respectively) and Panama (14.79 ± 2.49 mm and 15.46 ± 2.95 mm respectively) but they did differ from Beaufort (17.47 ± 3.17 mm and 16.76 ± 2.97 mm). Differences were highly significantly different across the regions for height (Kruskal-Wallis $\chi^2 = 170$, df = 4, $P < 0.0001$) and width (Kruskal-Wallis $\chi^2 = 138$, df = 4, $P < 0.0001$).

The number of ambulacral plates had the greatest mean value within the central portion of the range. Panama urchins had significantly more AMB plates (34 ± 7) than the Keys (31 ± 4), Gulf (31 ± 3) or Beaufort (30 ± 5) (Kruskal-Wallis $\chi^2 = 20$, df =3, $P < 0.0001$). The number of interambulacral plates was greatest for the Gulf (24 ± 2) and Panama (23 ± 4) differing from Beaufort and Keys, each having 22 ± 3 (Kruskal-Wallis $\chi^2 = 37$, df = 3, $P < 0.0001$). The peristomial opening was not significantly different between Beaufort (18.39 ± 2.76 mm), Keys (17.88 ± 2.09 mm) and Panama (17.54 ± 2.9 mm) but did differ from the Gulf (14.81 ± 1.55 mm) (Kruskal-Wallis $\chi^2 = 105$, df = 3, $P < 0.0001$).

Three test and two lantern measures were converted to ratios to give better indication of the true nature of the difference in test and spine morphology by

eliminating the effect of size since larger urchins have comparatively larger structures (Table 3-2). Test diameter and test height separately give linear measures of test shape but a height-diameter ratio (H/D) gives a better indication of the overall spherical nature of the test. Test ratios close to 1 indicate a very round urchin, whereas, a ratio close to 0.50 indicates a flatter test, the diameter twice the length of the height. Test H/D ratio is greatest in the central portion of the geographic range and smallest at the edges (Kruskal-Wallis $\chi^2 = 233$, $P < 0.0001$). The H/D ratio is greatest for Gulf urchins (0.65 ± 0.058) and smallest for Brazil (0.54 ± 0.038) and Beaufort (0.56 ± 0.035) (Fig. 3-5). Despite Brazil urchins having the largest mean test diameter and height and Gulf having the smallest, Gulf urchins have the most rounded, dome-shaped test, whereas, Brazil urchins are flattest. Keys (0.62 ± 0.04) and Panama (0.59 ± 0.04) urchins have intermediate ratios (Figs. 3-7 A).

The ratio between lantern length and width (L/W ratio) gives an indication of the overall size of the structure in 2 dimensions much like the test H/D ratio. Brazil has proportionately the longest lanterns with a ratio of 1.11 ± 0.047, whereas, Panama has the smallest ratio at 0.96 ± 0.053 (Fig 3-7 B). Lantern ratios for Beaufort (1.04 ± 0.058) and Gulf (1.02 ± 0.047) are not statistically different but differ from the Keys (1.00 ± 0.058) and each of the other sites (ANOVA F = 54, $P < 0.0001$).

Lantern wet weight was heaviest for Brazil and Beaufort (3.63 g and 2.61 g respectively). Lantern weight in relation to the diameter of the test is given by the lantern weight ratio (g/mm). Brazil and Beaufort have heavier lanterns relative to the size of their tests than urchins from the other regions (0.055 ± 0.011 g/mm and 0.049 ±

0.015 g/mm respectively) (Kruskal-Wallis $\chi^2 = 175$, df = 4, $P < 0.0001$). Gulf urchins have the lightest lanterns (0.024 ± 0.007 g/mm). Beaufort and Gulf urchins have similar size lanterns (H/W ratio) but they are heavier in Beaufort than in the Gulf (Fig. 3-7 C).

Test weight ratio, measured as the dry weight per test diameter, and peristome ratio, measured as the diameter of the peristome per test diameter show that Beaufort urchins have heavier tests (Kruskal-Wallis $\chi^2 = 69$, df = 3, $P < 0.0001$) with a larger peristomial opening (Kruskal-Wallis $\chi^2 = 47$, df = 3, $P < 0.0001$) relative to their size than urchins from the other regions (Table 3-2, Fig. 3-8). Beaufort urchins are a third heavier than urchins from the other locations, weighing 0.25 g/mm versus 0.16–0.17 g/mm for Gulf, Keys and Panama. Test weight and peristome size do not differ in the Gulf, Keys and Panama.

Table 3-2: Mean values and standard deviations of 5 ratios. Numbers in bold indicate the greatest values for the character. Ratios include test height-diameter (H/D), test dry weight per diameter, peristome diameter per test diameter, lantern length-width and lantern wet weight per test diameter. Results of one-way ANOVA (F) and Kruskal-Wallis (χ^2) comparisons of means: *** = $P < 0.0001$.

		Beaufort n=112		Gulf n=113		Keys n=104		Panama n=45		Brazil n=43				
	Characters	mean	*st. dev.*	mean	*st. dev.*	mean	*st. dev.*	mean	*st. dev.*	mean	*st. dev.*		χ^2	F
Test	H/D ratio	0.56	*0.035*	**0.65**	*0.058*	0.62	*0.040*	0.59	*0.040*	0.54	*0.038*	***	233	
	Test ratio (g/mm)	**0.25**	*0.100*	0.16	*0.040*	0.17	*0.090*	0.17	*0.080*			***	69	
	Peristome ratio	**0.37**	*0.030*	0.34	*0.012*	0.35	*0.030*	0.35	*0.040*			***	47	
Lantern	Lantern L/W ratio	1.04	*0.058*	1.02	*0.047*	1.00	*0.058*	0.96	*0.053*	**1.11**	*0.047*	***		54
	Lantern weight ratio (g/mm)	0.049	*0.015*	0.024	*0.007*	0.031	*0.009*	0.028	*0.007*	**0.055**	*0.011*	***	175	

Figure 3-6: Comparison of H/D ratio (dome shape). Beaufort urchin on the left and Gulf urchin on the right. Mean H/D ratio for Gulf tests are 15% larger than for Beaufort tests.

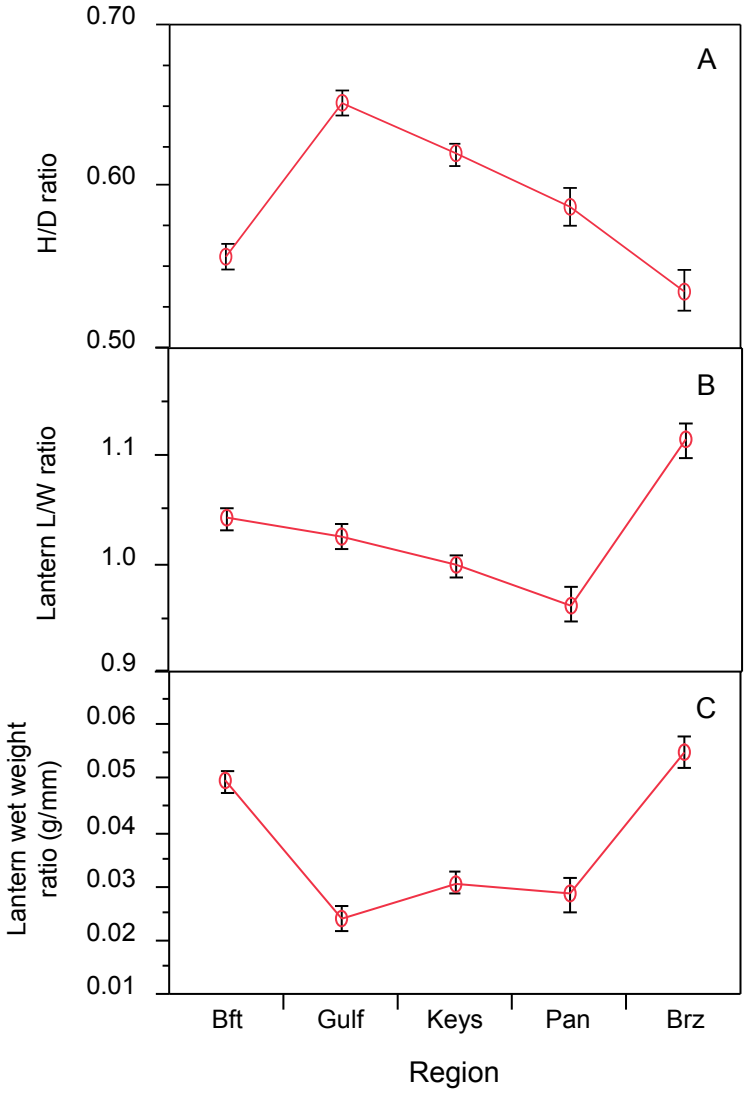

Figure 3-7: Mean values (± SE) for test height-diameter ratio (H/D), lantern length-width ratio and lantern wet weight per test diameter ratio. A) Gulf urchins have the most dome-shaped tests, whereas, urchins from Beaufort and Brazil are flattest. Beaufort = Brazil ≠ Gulf ≠ Keys ≠ Panama. B) Brazil has the largest lanterns and Panama the smallest. Beaufort = Gulf ≠ Keys ≠ Pan ≠ Brazil. C) Brazil and Beaufort have the heaviest lanterns per body size, whereas, lanterns for Gulf, Keys and Panama are much lighter. Brazil ≠ Beaufort ≠ Gulf ≠ Keys = Panama = Gulf.

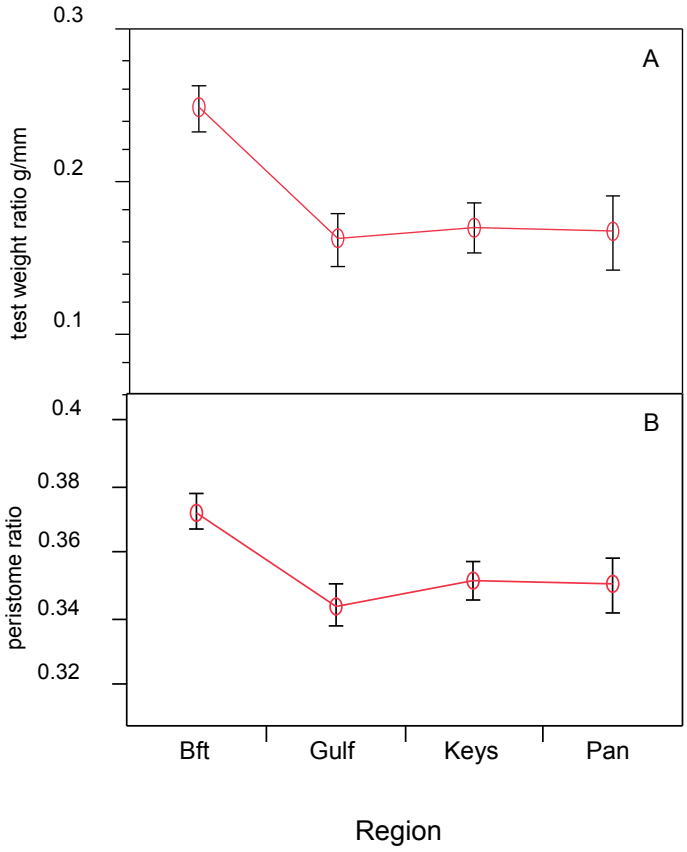

Figure 3-8: Mean values (± SE) for test weight and peristome ratios. A) Ratio of test dry weight to diameter. Beaufort tests are a third heavier than tests from the other regions. Beaufort ≠ Gulf = Keys = Pan. B) The peristomial opening is larger in Beaufort urchins. Beaufort ≠ Gulf = Keys = Pan.

Spine length in *Lytechinus variegatus* is not uniform across the test. Spines on the aboral surface are shorter than spines along the ambitus. The difference in length is especially evident in Beaufort where aboral spines are quite short. Direct comparison of spine length between regions must eliminate the effect of urchin size, since spines grow as urchins grow. To make direct comparisons I calculated spine length as a ratio to test diameter. This ratio converts spine length into a fraction of test diameter. The same

approach was taken to examine spine width since Beaufort urchins appeared to have stouter spines than urchins from other regions. Table 3-3 compares length and width of aboral and ambital spines among the different regions. Beaufort spines differ to a great extend compared to the other regions. Beaufort urchins have shorter (Kruskal-Wallis χ^2 = 122, df = 3, $P < 0.0001$), stouter (Kruskal-Wallis χ^2 = 296, df = 3, $P < 0.0001$) aboral spines and longer (Kruskal-Wallis χ^2 = 74, df = 3, $P < 0.0001$), stouter (ANOVA F = 137, $P <$ 0.0001) ambital spines. In all measures except for aboral spine length, each region is significantly different (Fig. 3-9). Keys and Panama have equally long aboral spines.

Table 3-3: Mean values and standard deviations of spine length and width ratios. Numbers in bold indicate the greatest value for that character. Spine length and width measured as the fraction of the test diameter. Aboral spine length (ABSL), ambital spine length (AMSL), aboral spine width (ABSW), ambital spine width (AMSW). Results of Kruskal-Wallis (χ^2) comparisons of means: *** = $P < 0.0001$.

		Beaufort n=112		Gulf n=113		Keys n=104		Panama n=45		Brazil n=43			
	Characters	mean	*st. dev.*	mean	*st. dev.*	mean	*st. dev.*	mean	*st. dev.*	mean	*st. dev.*		χ^2
Spine	ABSL	0.123	*0.018*	0.135	*0.019*	**0.156**	*0.024*	**0.151**	*0.021*			***	122
	AMSL	**0.293**	*0.048*	0.273	*0.037*	0.254	*0.034*	0.230	*0.043*			***	74
	ABSW	**0.016**	*0.002*	0.015	*0.002*	0.013	*0.002*	0.011	*0.002*			***	175
	AMSW	**0.024**	*0.003*	0.020	*0.002*	0.017	*0.002*	0.013	*0.003*			***	262

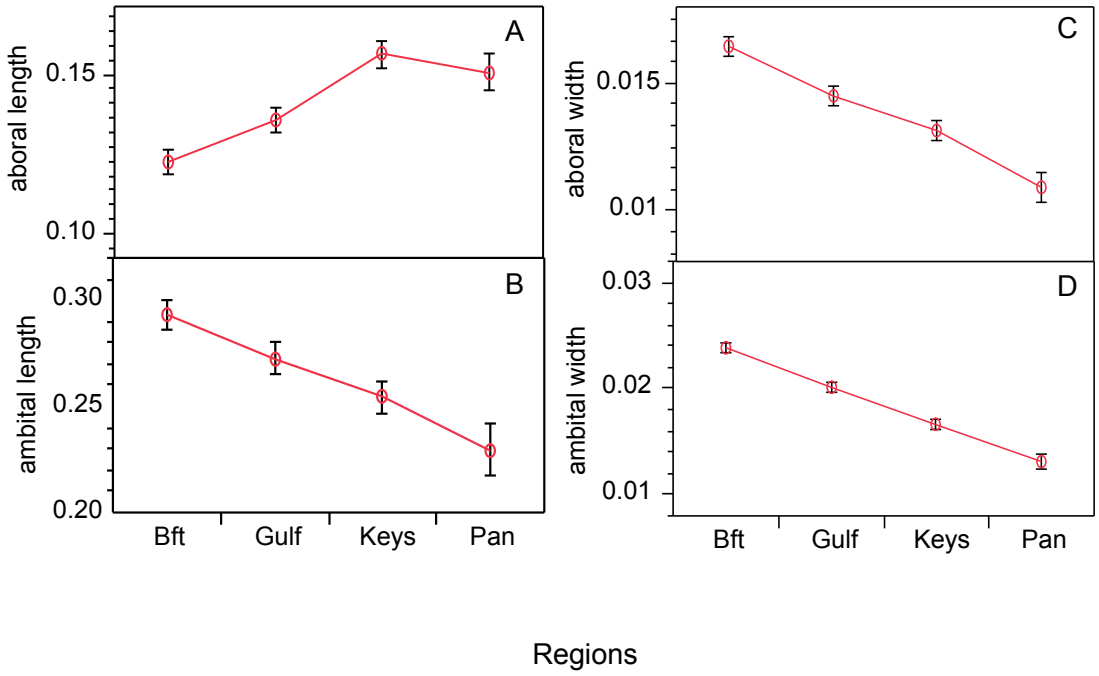

Figure 3-9: Mean values (± SE) for spine length and width ratios. Graphs A and B show that Beaufort has shorter aboral spines but longer ambital spines in relation to test diameter. Keys and Panama have the longest aboral spines (Beaufort ≠ Gulf ≠ Keys = Pan) but shorter ambital spines in relation to test diameter (Beaufort ≠ Gulf ≠ Keys ≠ Pan). Graphs C and D show that Beaufort spines are the stoutest of all regions, whereas, Panama spines are the most slender. Beaufort ≠ Gulf ≠ Keys ≠ Pan for both comparisons.

Because the morphological traits are not independent of each other, a multivariate approach that factors in the correlation between the traits is an effective way to analyze the data. Results of multivariate analysis of variance (MANOVA) confirms that regions have significantly different morphologies (Wilks' Lambda = 0.019, $F \sim 75$, $P < 0.0001$). MANOVA (Fig. 3-10) shows the extent of divergence between the

regions. The biplot rays show the direction and magnitude of the 10 ratio variables from the grand mean. Six of the variables are the most discriminatory. Four of these—aboral and ambital spine width, ambital spine length and lantern weight ratios—separate the regions on the 1st canonical plane. On this plane all regions are well differentiated with Beaufort urchins diverging most from the other regions by having thicker spines, longer ambital spines and heavier lanterns. Aboral spines in Beaufort urchins are 30% thicker and ambital spines are almost 50% thicker and 20% longer than in Panama urchins (Table 3-3). Lanterns are also twice as heavy in Beaufort as in Gulf or Panama urchins (Table 3-2).

In the second canonical plane the difference in H/D ratio separates Gulf urchins from the other regions. Ambital spine thickness and test thickness also factor in. However, the biplot rays indicate that for all six variables their influence to varying degrees is in both canonical planes.

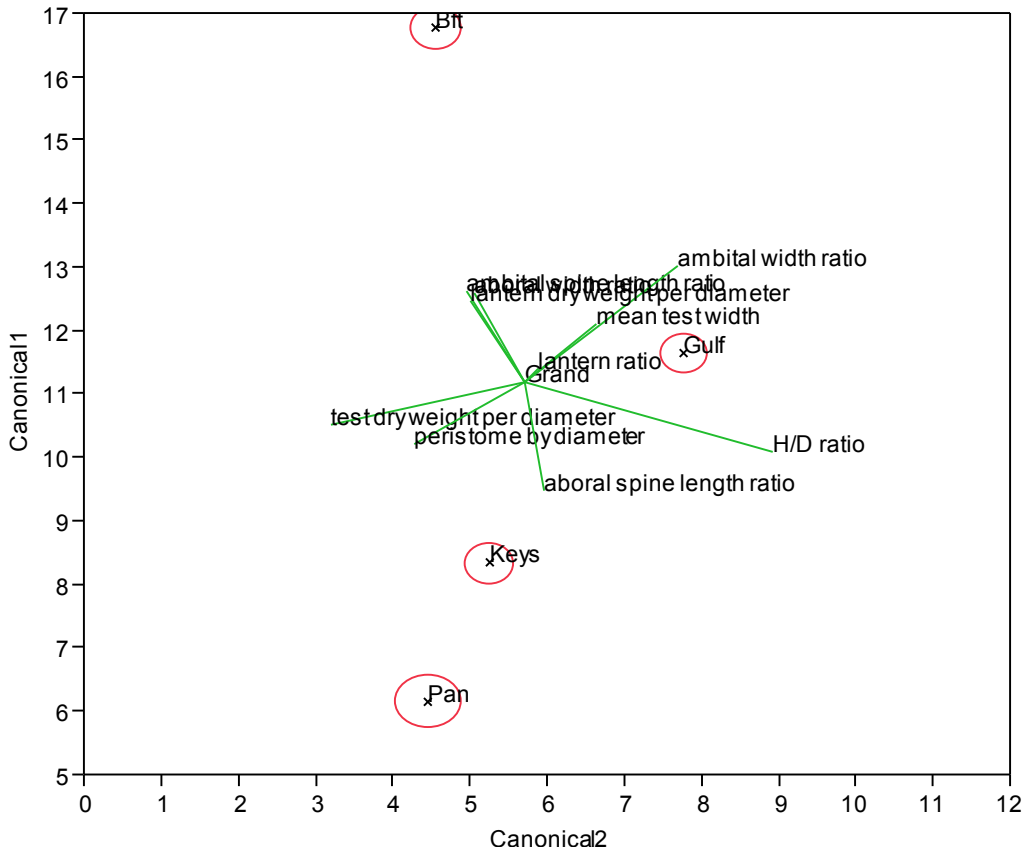

Figure 3-10: Multivariate analysis of variance canonical plot of test, spine and lantern ratios between the 4 regions. In canonical plane 1 ambital spine length, aboral spine width and lantern weight have the greatest influence in distinguishing the regions. In canonical plane 2 test shaped (H/D), test wall thickness and ambital spine width have the greatest influence in distinguishing the regions.

The full measure of geographic diversity is captured in the 3D discriminant plot (Fig. 3-11) which shows the extent of divergence between the regions. Discriminant analysis separates the regions identically to MANOVA, although it does so by grouping based on similarity. The morphological features that are most distinguishing and separate the groups are the same ones that classify them into homogeneous groups by region. The most parsimonious plot (fewest characters providing the greatest separation

between groups) classified the urchins by region of origin and included 7 characters (H/D ratio, mean test width, aboral and ambital length and width ratios and either lantern L/W ratio or lantern weight ratio). This provided the best fit with the fewest misclassified urchins and lowest error rate (21 misclassified, ~7% error, Wilks' Lambda = 0.026, F ~ 55, $P < 0.0001$).

The colored spheres in the discriminant plot correspond to 50% of the values for each region and from this we can see that each region is very clearly differentiated from others. The remaining 50% is contained in the cloud of points and here too the separation is clearly defined. The 1st canonical plane is where the bulk of the differentiation occurs and encompasses 90% of the variation. It includes the same 4 variables as the MANOVA plot. The next level of distinction, which includes an additional 9% of the differentiation, is in the 2nd canonical plane which separates Gulf urchins from the other regions. The bulk of the differentiation is carried by H/D ratio which is greatest in Gulf urchins as well as test mean thickness. The 3rd canonical plane explains the remaining 1% and as seen in bottom graph the regions are more closely aligned with the most separation between Keys and Panama urchins. In both tests, spine and lantern morphology as well as test shape factor prominently in regional differentiation.

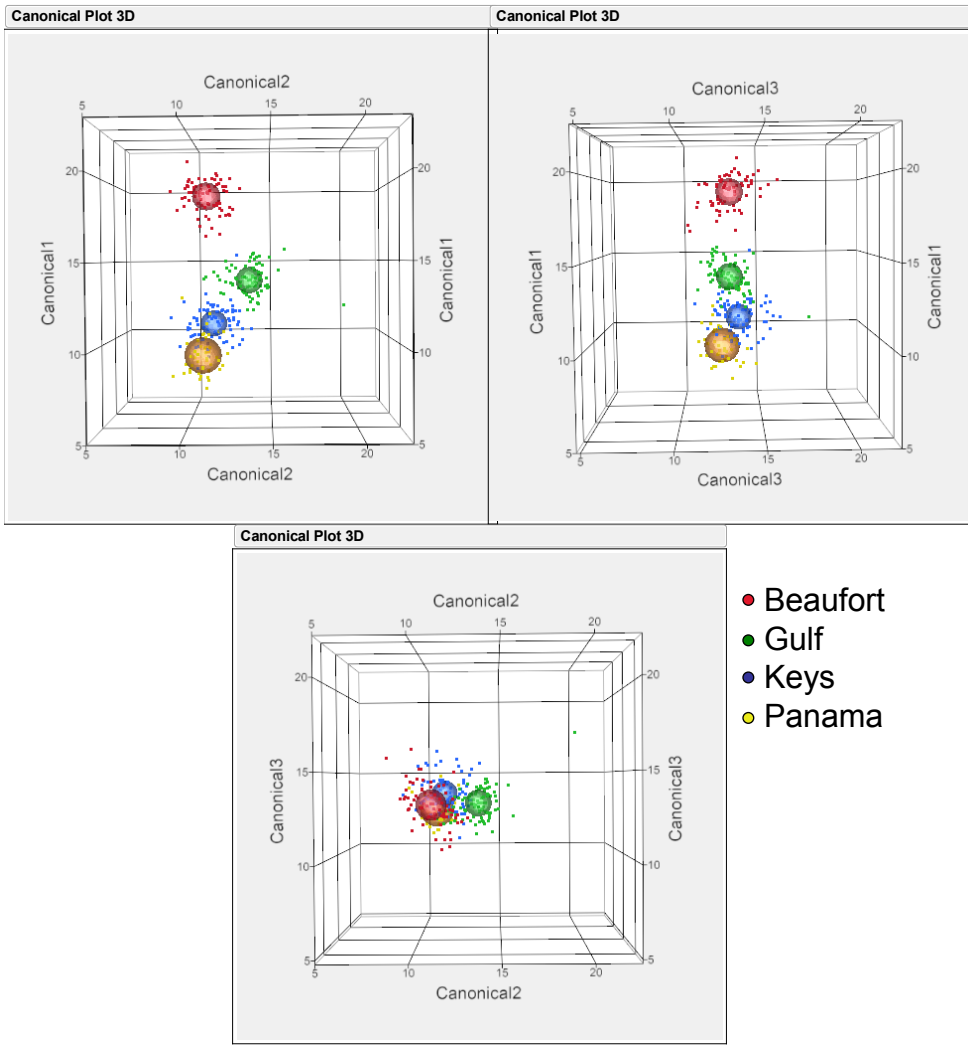

Figure 3-11: 3 views of the discriminant analysis planes that separate samples from the 4 regions into their respective groupings. The most parsimonious plot included 7 morphological characters (H/D ratio, test thickness, aboral and ambital spine length and width ratios, and lantern weight ratio) providing the best fit and fewest errors between Beaufort (red), Gulf (green), Keys (blue) and Panama (gold). Overall, 21 urchins were misclassified at a 7.2% error rate. Keys urchins (16) were the most misclassified, usually as Panama (13) or Gulf (3). Panama had 5 misclassified as Keys. Beaufort and Gulf were not misclassified. Colored spheres enclose 50% of the values for each region.

3.3.2 Morphology after removal of spines and epidermis

Morphological measurements taken on 498 tests from Beaufort (358) and Tavernier Key (140) showed the same relationship between the two regions as in the previous section (Table 3-4). Beaufort tests were heavier (mean weight 4.91 ± 2.67 g) than Keys (3.74 ± 1.19 g) tests (*t*-test assuming unequal variances = 5.60, $P < 0.0001$). Beaufort tests had thicker walls (mean thickness 0.86 ± 0.14 mm) than Keys (0.62 ± 0.08 mm) tests (*t*-test assuming unequal variances = 24.77, $P < 0.0001$). The shape of denuded tests is the same as in the previous section—Keys tests have a larger H/D ratio. Beaufort tests are flatter (0.53 ± 0.047) compared to Keys tests (0.60 ± 0.029) (*t*-test assuming unequal variances = 18.68, $P < 0.0001$). Weight expressed as a ratio of grams of dry weight per mm of diameter shows that Beaufort tests are significantly heavier relative to their size, weighing on average 0.10 ± 0.313 g/mm versus 0.07± 0.232 g/mm for Keys tests (*t*-test assuming unequal variances = 12.48, $P < 0.0001$).

Test characters for the Beaufort sample had a larger variance than the Keys sample. Beaufort data was a composite: tests were collected from 4 sites—Cape Lookout (CL), Oscar Shoal (OS), Turning Basin (TB) and Offshore (Off). A look at the range of test sizes reveals that much of the variability in the Beaufort data set was likely due to 2 sites: Turning Basin and Offshore. Test diameters for TB encompassed a larger range than at the other 3 sites and the diameters for the offshore site were larger than the 3 inshore sites (Fig. 3-12). However, the frequency distributions of all characters except for weight

were normally distributed for each site. Table 3-4 lists the ranges, means and standard deviations for all test characters at each of the Beaufort sites.

Table 3-4: Lists the ranges, means and standard deviations of all morphological characters measured on denuded tests from each of the Beaufort sites. Differences between the four sites for all test characters were significantly different. Results of one-way ANOVA (F) and Kruskal-Wallis (χ^2) comparisons of means: *** = $P < 0.0001$.

Character		Cape Lookout n = 91	Oscar Shoal n = 130	Turning Basin n = 84	Offshore n = 52		χ2	F
diameter (mm)	range	33.27–48.67	35.35–50.14	26.39–61.20	48.09–71.31	***	136	
	mean	41.69	42.58	41.26	58.56			
	st dev.	3.30	2.71	6.50	5.80			
height (mm)	range	16.07–25.46	19.92–30.12	10.90–32.56	26.58–40.76	***	191	
	mean	21.06	23.68	20.65	33.61			
	st dev.	1.93	1.89	4.31	3.90			
aboral (mm)	range	0.47–0.98	0.58–1.22	0.48–1.12	0.69–1.28	***		45
	mean	0.67	0.75	0.70	0.89			
	st dev.	0.11	0.11	0.12	0.12			
ambital (mm)	range	0.60–1.38	0.74–1.61	0.54–1.82	084–1.38	***	45	
	mean	0.93	1.00	0.95	1.11			
	st dev.	0.18	0.12	0.20	0.13			
mean (mm)	range	0.56–1.10	0.66–1.32	0.57–1.38	0.76–1.33	***	74	
	mean	0.80	0.88	0.82	1.00			
	st dev.	0.13	0.10	0.15	0.11			
periproct (mm)	range	6.18–9.51	6.47–9.08	2.95–12.42	5.35–12.67	***	116	
	mean	7.48	7.73	7.37	10.09			
	st dev.	0.61	0.58	1.46	1.33			
peristome (mm)	range	12.48–16.58	13.17–17.32	11.03–21.14	17.44–22.15	***	155	
	mean	14.74	15.28	15.96	20.03			
	st dev.	0.94	0.82	1.83	1.20			
dry weight (g)	range	1.58–6.90	2.60–6.50	0.80–13.76	4.80–16.83	***	140	
	mean	3.90	4.33	3.89	9.75			
	st dev.	1.05	0.79	2.36	3.00			
IA	range	18–26	19–30	13–25	21–30	***	164	
	mean	22	22	20	26			
	st dev.	1.60	1.55	2.02	1.91			
AMB	range	25–37	20–36	16–34	30–42	***	156	
	mean	31	31	28	36			
	st dev.	2.45	2.05	3.17	2.87			

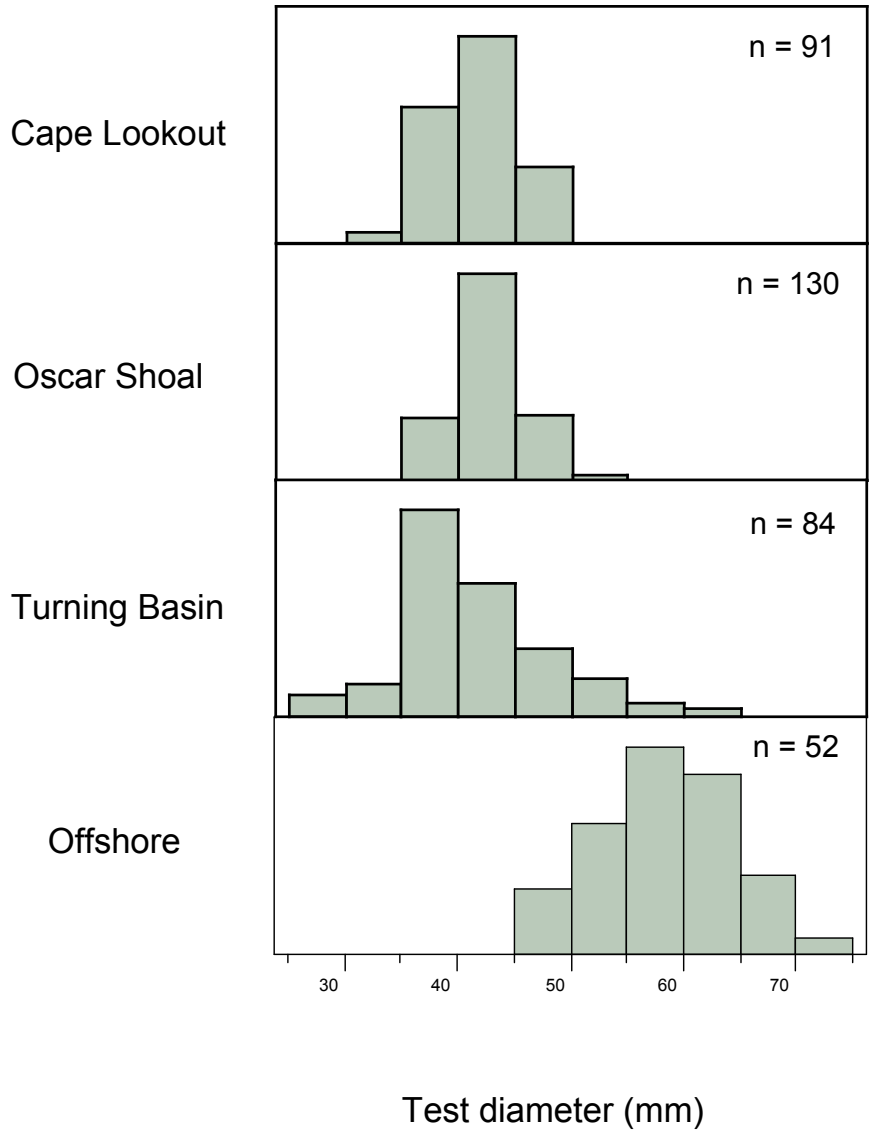

Figure 3-12: Histograms showing the range of test diameters for the four Beaufort sites. Cape Lookout and Oscar Shoal have tests of equal size, whereas, Turning Basin has a greater range of sizes and Offshore tests are larger than those inshore.

The mean values for all test characters were largest for the offshore site. One-way ANOVA and the nonparametric Kruskal-Wallis test showed mean differences for all characters to be highly significantly different ($P < 0.0001$) (Table 3-4).

The mean test diameter was 41–42 mm for the three inshore sites but jumped to 58 mm for the offshore site (Kruskal-Wallis $\chi^2 = 136$, df = 3, $P < 0.0001$). Mean test thickness was also greatest for the offshore site (Kruskal-Wallis $\chi^2 = 74$, df = 3, $P < 0.0001$). This pattern—larger mean values for the offshore site—was repeated for all the remaining characters. Comparing mean values for just the inshore sites, significant differences persist, indicating morphological variability at the local scale. Oscar Shoal tests had the greatest mean value for all characters except peristome (Kruskal-Wallis, df = 2 for all comparisons, $P < 0.05$ for diameter, $P < 0.0003$ for periproct, $P < 0.0001$ for height, peristome, IA, AMB and mean test thickness). Turning Basin had the largest peristomial opening.

As in the section on spine, test and lantern characters, several measures were converted to ratios to eliminate the effect of size, for a more accurate comparison between sites, given that offshore tests are generally larger than inshore tests. The shape the test expressed as the height-diameter ratio, the dry weight per test diameter and the size of both the periproct and peristome expressed as a fraction of test diameter are given in Table 3-5. Graphs of the ratios are given in Figure 3-13.

Table 3-5: Mean values and standard deviations of H/D ratio, weight ratio, periproct and peristome ratios for each of the Beaufort sites. Numbers in bold indicate the greatest value. Weight ratio was measured as test dry weight per diameter. Periproct and peristome ratios measured as fractions of the test diameter. Results of Kruskal-Wallis (χ^2) comparisons of means: df = 3, *** = $P < 0.0001$.

	Cape Lookout n = 91		Oscar Shoal n = 130		Turning Basin n = 84		Offshore n = 52			
	mean	*st. dev.*	mean	*st. dev.*	mean	*st. dev.*	mean	*st. dev.*		χ^2
H/D ratio	0.51	*0.044*	0.56	*0.026*	0.50	*0.043*	**0.57**	*0.029*	***	157
weight ratio g/mm)	0.092	*0.019*	0.101	*0.013*	0.090	*0.036*	**0.164**	*0.037*	***	135
Periproct ratio	0.178	*0.012*	0.182	*0.011*	0.180	*0.021*	0.172	*0.016*	***	23
Peristome ratio	0.353	*0.012*	0.359	*0.012*	**0.390**	*0.021*	0.344	*0.018*	***	151

H/D, weight and periproct ratios deviated from a normal distribution for the TB sample. I transformed the ratios into a natural logarithm scale for all sites. This approximately normalized the data. As variances were not homogeneous and the sample sizes differed I compared means using the nonparametric Kruskal-Wallis test of means. Offshore tests maintained the largest values for 2 of the 4 ratios: H/D and weight. For periproct and peristome ratios Offshore tests had the smallest means.

The shape of the test (H/D ratio) was significantly different across all sites (Kruskal-Wallis $\chi^2 = 157$, df = 3, $P < 0.0001$). The offshore site had the largest ratio (0.57 ± 0.029) followed by Oscar Shoal (0.56 ± 0.026) but Cape Lookout (0.51 ± 0.044) and Turning Basin (0.50 ± 0.043) did not differ. Both Off and OS tests are more dome-shaped than CL and TB but all Beaufort sites have flatter tests than urchins in the central portion of the range (see Table 3-2). The weight of tests relative to size was also greatest for Offshore (0.164 ± 0.037 g/mm) followed by Oscar Shoal (0.101 ± 0.013 g/mm) while not differing between Cape Lookout (0.92 ± 0.019 g/mm) and Turning Basin (0.90 ± 0.036

g/mm) (Kruskal-Wallis $\chi^2 = 135$, $P < 0.0001$). Inshore urchin tests weigh approximately half as much (0.9 g/mm) as their offshore counterparts (0.16 g/mm). In contrast, the peristomial opening is smallest for Offshore tests (0.344 ± 0.018) and largest at Turning Basin (0.390 ± 0.021) with Oscar Shoal (0.359 ± 0.012) and Cape Lookout (0.353 ± 0.012) at intermediate values (Kruskal-Wallis $\chi^2 = 151$, $P < 0.0001$). The size of the periproct is also smallest for offshore tests (0.172 ± 0.016) but does not differ among the inshore sites (CL 0.178 ± 0.012, OS 0.182 ± 0.011, TB 0.180 ± 0.021) (Kruskal-Wallis $\chi^2 = 23$, $P < 0.0001$).

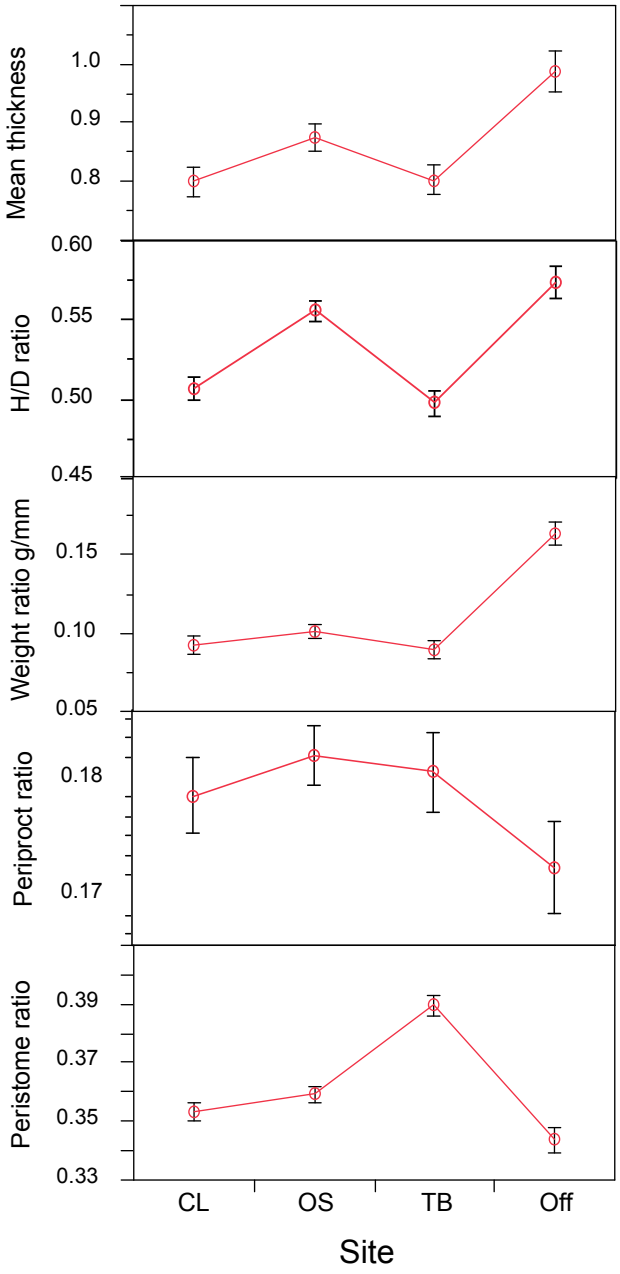

Figure 3-13: Mean values (± SE) for ratios of test height-diameter, weight, test thickness, periproct and peristome across each of the four Beaufort sites. Offshore tests are more dome-shaped, heavier with thicker test walls than inshore tests. Tests from TB have the largest peristomial openings and offshore tests the smallest. All inshore tests have a larger periproct than the offshore tests.

3.4 Discussion

The data presented in this chapter illustrate the range in variation for the 16 traits measured in *L. variegatus*. Urchins from each region are morphologically distinct and concord with Serafy's [1973] conclusion that morphology is not a reliable indicator of subspecific distinction. Variability in all the characters made subspecies distinctions problematic. Morphological differences between regions are likely driven by local environmental differences.

The pattern of divergence in morphology between regions is similar to the pattern seen in the color phenotype data (Ch. 2): urchins in Beaufort and Brazil are significantly different morphologically from urchins in the central portion of the range (Tables 3-1 and 3-2). Beaufort urchins have the thickest test walls (Fig. 3-3) and consequently the heaviest tests (Fig. 3-8 A). Spine width is greatest for Beaufort urchins (Fig. 3-9 C, D) as is ambital spine length (Fig. 3-9 B). Brazil has the longest and heaviest lanterns (Fig. 3-7) as well as having generally larger urchins (Fig. 3-2). Each of the traits measured differs throughout the geographic region. Urchins in the central portion of the range generally have intermediate values except for test shape (H/D ratio) which is greatest in the Gulf and the length of aboral spines, which are longest in the Keys and Panama. MANOVA and Discriminant analysis (Figs. 3-10 and 3-11) both show that the correlation between traits highlights the morphological divergence of each region.

Analysis of morphological variation in *L. variegatus* by Serafy [1973] concluded that subspecies distinctions were not guaranteed using morphological characters.

However spine thickness was greater for the subspecies *carolinus* than for *atlanticus* or *variegatus*. From his analysis *atlanticus* was closer to *carolinus* in overall morphology. Samples from Bermuda were not included in this study so comparisons cannot be made. However, given the diversity in morphology between the regions sampled, differences in morphology for Bermuda with regards to the other regions would be expected. The partial data collected on Brazil urchins also precludes full comparisons. However, the traits that are available indicate that morphologically they most resemble Beaufort urchins. Lanterns are longer and heavier and tests are flatter much as they are in Beaufort (Fig. 3-7). Data on spine morphology is lacking but visual inspection of photographs taken of Brazil urchins indicates that overall the spines are robust and long at the ambitus much like in Beaufort urchins. If this were the case and Brazil and Beaufort urchins shared similar morphological characteristics then the morphological data would concur with the color data in singling out the two extremes of the range as having the greatest differences.

The difference in phenotype between the regions signals the level of genetic variability within the species. The underlying mechanism responsible for the difference is unknown since the data document field populations and the differences may be due to habitat-driven selection on phenotypes or plastic responses to environmental cues. In both cases the differences could be induced through either biotic (predation, food resources) or abiotic factors (seawater temperature, hydrodynamic forces, etc.) or potentially some combination of both.

How much of the difference in morphology between regions is due to divergent selection on heritable genotypes is not known. Data on the differential survival of individuals of differing genotypes in response to agents of selection are lacking. Predation pressure on *L. variegatus* is largely a matter of speculation as evidence is limited [Keller, 1983; Rivera, 1978]. Known predators are fish, crabs, a gastropod and birds [Watts, et al., 2007]. Temperature and salinity are important abiotic factors in the distribution and abundance of *L. variegatus* [Moore, et al., 1963; Watts, et al., 2007]. Sudden increases and decreases in temperature and salinity can cause mass mortality [Beddingfield, et al., 1994; Boettger, et al., 2002; Moore, et al., 1963; Rivera, 1978]. However, information of their impact on survival of differing genotypes is absent. The effect of these stressors on phenotypic plasticity is also unknown in juvenile and adult stages.

Differences in spine and lantern sizes play a major role in morphological differentiation between regions. Aboral and ambital spine width, ambital spine length and lantern weight featured prominently in the 1st canonical plane for both MANOVA and Discriminant analysis (Figs. 3-10 and 3-11). Spine morphology plays an important role in structural support, locomotion, protection from both predators and waterborne projectiles and lanterns are a vital food gathering structure [Strathmann, 1981]. In both cases, changes in morphology in response to local environmental conditions would be advantageous and increase the relative fitness of those individuals.

The mechanism responsible for the morphological differences in post-metamorphic individuals of *L. variegatus* across the geographic range is impossible to

assign in the absence of data linking changes in morphology with changes in specific habitat-level parameters. However, data from other echinoid species indicates that environmental conditions may factor largely in intrapopulation differentiation. Hydrodynamic regime, water temperature and food resources, appear to be the primary factors in microhabitat differentiation within populations of both tropical and temperate urchins [Black, et al., 1982; Dix, 1970; Guidetti, et al., 2005; Lewis, et al., 1984]. Increased spine and test thickness was found in areas of increased wave exposure [Dix, 1970] as well as in areas of decreased water temperatures. Conversely, decreases in test height were correlated to habitats of increased wave exposure [Dix, 1970; Guidetti, et al., 2005; Lewis, et al., 1984]. Lantern size was negatively correlated with food abundance [Black, et al., 1982; Levitan, 1991]. These correlations do not prove causation since multiple factors may be responsible for the observed patterns. However, they point to possible sources that can be experimentally tested.

Water temperature directly affects spine chemistry and structure influencing regeneration rates [Davies, et al., 1972; DuBois, et al., 1989; Magdans, et al., 2004]. Cold water enhances deposition of calcium ions, increasing the concentration in urchins spines [Davies, et al., 1972], whereas, warmer water enhances deposition of magnesium ions [Davies, et al., 1972; Magdans, et al., 2004]. This may directly impact spine morphology in urchins from the different regions. Urchins in Beaufort, the northern Gulf of Mexico and Brazil inhabit areas of considerable thermal variability compared to the Caribbean basin. Mean seasonal water temperatures have a much broader range in Beaufort (11-30° C, Peter Crumley, NOAA Beaufort), Gulf (16-30° C, NOAA National

Data Buoy Center) and Brazil (low teens to high 20's C, [Junqueira, et al., 1997; Netto, et al., 2005; Ventura, et al., 1995] than in Panama (24-33° [Kaufmann, et al., 2005]) or the Keys (23-30° C, NOAA National Data Buoy Center). Moreover, water temperatures in Beaufort and the Gulf reach lower values and remain low for extended periods during winter months and the area of Cabo Frio is characterized by seasonal upwelling events bringing in colder South Atlantic Central Water [Junqueira, et al., 1997; Netto, et al., 2005; Ventura, et al., 1995]. Water temperatures in the Keys and Panama are relatively constant throughout the year. Colder waters in winter plus the warmer waters of summer in Beaufort and Gulf may enhance the deposition of both calcium and magnesium explaining the more robust nature of their spines compared to urchins in the Keys and Panama.

Temperature likely contributes to the thicker test walls of urchins in Beaufort and the Gulf compared to those from the Keys and Panama. A similar correlation of lower water temperatures and increased test thickness was found the sand dollar *Dendraster excentricus* [Raup, 1958] and the sea urchin *Evechinus chloroticus* [Dix, 1970].

Test thickness may also be influenced by hydrodynamic conditions, with thicker tests providing greater structural support in high energy habitats. Inshore Beaufort urchins in Bogue Sound inhabit a channel basin with strong (0.4-0.8 m/s) semidiurnal tidal flows [Carr, et al., 2005] where areas of shelter are absent. Such constant exposure may necessitate various morphological modifications to counter the physical forces acting on the individual, especially in the absence of sheltering structures such as crevices and seagrass beds. Urchins from relatively sheltered habitats have thinner test

walls than those from high energy habitats [Dix, 1970; Lewis, et al., 1984]. This holds true for *L. variegatus*, as well, with urchins in seagrass beds (Keys and Panama) having thinner test walls than those from Beaufort on exposed substrates.

Test shape, expressed as H/D ratio may be under similar hydrodynamic stress. Flatter tests (smaller H/D ratio) may benefit urchins by lowering the center of gravity and presenting a smaller surface area to the current, thus reducing the pressure drag on the individual. Comparisons of test shape indicate that urchins in areas of increased wave action versus sheltered areas have flatter tests [Dix, 1970; Guidetti, et al., 2005; Lewis, et al., 1984]. Urchins of similar diameter (ignoring the spines) but differing heights have different frontal areas. Increases in the frontal area increase the drag force on objects [Vogel, 1994]. Flatter tests may be an effective strategy to minimize frontal area thus minimizing hydrodynamic drag to prevent the urchins from being swept away in the current [Vogel, 1994]. Figure 3-6 demonstrates the difference in the cross-sectional area of Beaufort versus Gulf urchins of the same diameter. The increase in height increases the frontal area increasing the size of the urchin. Similar changes in profile height have been documented in gastropod snails inhabiting intertidal shores [Janson, 1982; Johannesson, 1986; Trussell, 1996]. Profile height can be experimentally altered in urchins. *S. purpuratus* grown in pits under constant current had more rounded tests than those grown on the flat surface [Hernandez, et al., 2010]. This indicates a certain amount of plasticity in test morphology. How plastic the response is to environmental changes is genetically determined and may differ across populations [Pigliucci, 2001a].

L. variegatus from the seagrass beds have rounder tests than urchins from open substrates. How much of this difference is due to plasticity is unknown. Also unknown is the magnitude of the alteration in test shape given the underlying genotype. Data from crosses in Chapter 4 show that H/D ratio differs in Beaufort versus Keys crosses mirroring the field population. This indicates that the genotypic range for this trait differs in the two populations. Beaufort urchins may be genetically at the lower end of the H/D range compared to the other regions. This difference highlights the influence of environmental parameters shaping morphological characters since Beaufort and Gulf urchins are of the same subspecies but have very different test shapes. The reduction in water flow inside seagrass canopies [Fonseca, et al., 1982; 1983; Gambi, et al., 1990] may function in the same manner as the pits in the experimental study above allowing the test to assume a more rounded aspect compared to urchins inhabiting open substrates. This same pattern was documented in another urchin species: *Psammechinus miliaris*. Urchins with more rounded tests were more common in seagrass beds than those dredged from exposed deeper sites [Lindhal, et al., 1929].

The phenotypic range and possible plastic nature of test shape in Beaufort urchins is evident in the data for denuded tests. The offshore urchins have a more rounded test than inshore urchins but it is still lower than Gulf, Keys or Panama urchins. The difference in test shape for inshore versus offshore urchins may reflect differential recruitment and survival. Post-metamorphic juveniles with lower H/D ratios may be at a selective advantage in inshore waters where the greater tidal current velocity may impose greater stresses resulting in greater survival of flatter urchins. The difference in

H/D ratio for inshore urchins at Oscar Shoal compared to Cape Lookout and Turning Basin is unclear but small-scale substrate topography inshore may moderate tidal flow and current speed [Carr, et al., 2005] resulting in smaller scale spatial variability allowing rounder urchins to recruit and settle.

Having shorter aboral spines may also help reduce drag. Drag increases not only with increased frontal area but also with the roughness of the object (e.g. protuberances such as spines, eyes, antennae, tentacles etc., [Vogel, 1994]). Shorter aboral spines would maintain the lower height profile without drastically increasing the overall diameter thus potentially reducing drag due to roughness. Experimentally measured drag forces were larger for long spined *S. nudus* versus short spined *S. intermedius* urchins of the same diameter [Yamasaki, et al., 1993]. The much shorter aboral spines on Beaufort urchins compared to their conspecifics in the seagrass beds likely helps in reducing the overall drag force. Conversely, the longer ambital spines could help anchor the urchin to the substrate to more effectively counter the hydrodynamic forces. Behavioral mechanisms such as covering with shells could further reduce drag by providing a smoother surface over which water flows and also by increasing the weight of the animal [Lees, et al., 1972]. In the most extreme case of spine reduction, the urchin *Colobocentrotus atratus* dispensed with spines altogether, replacing them with plates on the aboral side. The reduction in the drag coefficient (drag value due to shape) compared with the sympatric *Echinometra mathaei* is dramatic: 1.09 for *E. mathaei* versus 0.42 for *C. atratus* [Denny, 1994]. The latter typically inhabits very dynamic, high energy,

wave swept areas and the increase in water acceleration is too great for sympatric spined urchins.

The differences in test and spine robustness between regions could also be due to natural selection on genotypes conferring increased robustness. Survival of post-metamorphic juveniles in Beaufort may select for individuals at the higher range of spine and test thickness and lower range of H/D ratio. Such selection eliminates unfit individuals and drives the population mean toward higher or lower values of specific traits [Falconer, et al., 1996]. Experimental crosses of Beaufort and Keys urchins grown in a common garden design demonstrate that differences in morphology of F_1 juveniles largely mirror the differences found in the parental population (Ch. 4). This indicates genotypic differences in trait means between the regions. The magnitude of the difference between regions may be augmented by the amount of plasticity in the genotypes. To partition the contribution of genes and environment on the mean trait value would require an experimental design of crosses grown in multiple environments.

Both phenotypic plasticity and heritability factor into the difference in lantern size between the regions. The same experimental crosses of Beaufort and Keys urchins revealed no difference in the weight of the lanterns but lantern size in F_1 juveniles mirrored the differences found in the parental population (Ch 4). The ambiguity in the two responses suggests that the lantern plasticity may be more complicated than has been reported. In general lantern size in echinoids increases under conditions of food limitation, with both tropical (*Diadema antillarum*, *D. setosum*, *Echinometra mathaei*) and temperate urchins (*S. purpuratus* and *Evechinus chloroticus*) responding in similar

manner, as shown experimentally [Black, et al., 1984; Ebert, 1980; Edwards, et al., 1991; Levitan, 1991]. The assumption is that urchins with larger lanterns are better able to capture what little food is available by grazing larger areas than urchins with smaller lanterns [Black, et al., 1984]. This compensatory strategy during periods of food scarcity is also evident in the field in habitats of contrasting resource availability [McShane, et al., 1997] and under conditions of high urchin density [Black, et al., 1982].

However, with respect to *L. variegatus*, food resources may be substantively and nutritionally different across the regions and influence lantern growth and size differently. *L. variegatus* is an opportunistic feeder and will graze on whatever is available [Beddingfield, 1997; Beddingfield, et al., 1998; Watts, et al., 2007]. Food resources in Beaufort and Brazil appear more limited compared to the ready availability of the seagrass blades and associated epibionts in seagrass meadows. Grazing on seagrass and its epiphytes as well as sessile and infaunal organisms within the seagrass bed [Beddingfield, 1997] likely provides a high quality diet for relatively little effort. Stomach content of urchins from the Gulf, Keys and Panama showed a preponderance of green plant material compared to the stomach contents of urchins from Beaufort and Brazil. However the similarity in lantern length of Beaufort urchins with those of the Gulf, Keys and Panama may indicate that food resources in the Beaufort sand, shell-hash substrate are more abundant than is assumed. Gut contents of Beaufort urchins revealed quite a lot of unidentified tiny crustaceans and other digested organic material within the chalky calcareous mix (personal obs). The organic material likely increased the nutritional value of the diet. The larger lanterns in Brazil compared to Beaufort, Gulf,

Keys and Panama may reflect the difficulty of grazing on a rocky substrate with little or no plant material. This probably increases the effort and cost of food acquisition.

Compensatory mechanisms, such as increasing the size of the lantern in habitats of low or uneven resource availability (e.g. patchy distribution, seasonal differences in food resources and/or high urchin density), would help maximize energy intake [Boggs, 1992] and thus be highly advantageous. Experimental evidence demonstrates that *L. variegatus* when confronted with low quality food will ingest a greater amount of if than of high quality food [Hammer, et al., 2004; Valentine, et al., 2001]. The greater weight of Beaufort and Brazil lanterns compared to those from the other three regions may be related to the greater ingestion and incorporation of calcium carbonate rather than the quantity of food ingested.

Sustained differences in feeding preference can lead to specialization in feeding strategy and concomitant changes in the feeding apparatus. If the differences become an adaptation this can lead to population differentiation and ultimately to speciation. Differences in feeding preferences and the specialization of consumption on hard-shelled *Mytilus edulis* by *S. pallidus* likely led to speciation from sympatric *S. droebachiensis* which feeds on macroalgae [Hagen, 2008]. If this were to occur in *L. variegatus*, differentiation would likely occur at the extremes of the range leading to speciation of Beaufort and Brazil urchins.

As with color variability, morphological variability in *L. variegatus* does not show concordance with subspecies distinctions. The morphological characters showing the greatest differentiation varied between regions thus crossing subspecies boundaries.

Spine, test and lantern characteristics between Beaufort and Brazil populations were more similar to each other indicating potential habitat similarity. Conditions at the edges of the geographic range may impose various physical stresses that could necessitate similar morphological modifications. Likewise, the difference in test shape, and lantern size between Gulf and Beaufort urchins argues for local environmental influences on their morphology rather than shared heritage. The distinctiveness of each region is evident in both the MANOVA and discriminant plots which argue against subspecies similarities in morphology and points to local influences.

Differences in morphology are both ecologically and evolutionarily interesting. In the marine environment, as on land, environmental heterogeneity encompassing both spatial and temporal parameters has allowed us to understand the interplay between natural selection and phenotypic plasticity in creating the observed phenotypes. Whereas the contribution of genes versus environment in the creation of phenotypes has been elucidated in many organisms, in others it remains obscure. Such is the case with *L. variegatus*. The differences in spine, test and lantern morphology present an opportunity to explore and better understand the mechanisms underlying the differences. Whether they represent true adaptations will require empirical data.

4. Genetic crosses

4.1 Introduction

Color variation is one of the most striking and obvious features of many species and has been the focus of considerable attention by scientists throughout the ages. Much of what is known about color variability and the genetic mechanisms underlying pigment synthesis has been studied in floral and mammalian model systems. The genetics and biochemistry of anthocyanin and melanin pigments have been identified and well characterized [Hearing, et al., 1991; Holton, et al., 1995], as has the identity of the structural genes involved in pigment synthesis [Harker, et al., 1990]. The biosynthesis of floral pigments is generally conserved although there are important differences between species in the types of anthocyanins produced [Holton, et al., 1995]. Mutations in the structural and regulatory genes governing pigment production create the variations in color and patterning that distinguish species and varieties within species [Dooner, et al., 1991; Grotewold, 2006]. Similarly, changes in the melanin biosynthetic pathway create the elaborate variability in pigmentation in mammals [Hearing, et al., 1991].

In marine environments the pattern of color inheritance has been studied in a wide variety of fish and invertebrates. The diversity of colors and color patterns has

generated interest in uncovering the biological mechanisms for variability, especially in the richly diverse mollusks. Current interest in aquaculture has spawned numerous studies on the heritability of important traits in fish and mollusks such as bivalves [Nell, 2001]. Consumer preferences for certain qualitatively heritable traits such as flesh pigmentation in fish and shell pigmentation in edible marine bivalves has contributed to the growing number of breeding experiments examining the genetic and environmental effects on color phenotype [Nell, 2001]. While the patterns of inheritance of color phenotypes have been analyzed, the structural and regulatory genes governing pigmentation in marine invertebrates has not been elaborated as in floral and mammalian systems.

The best studied invertebrates are mollusks. Numerous studies have examined the genetic factors affecting shell color in polymorphic bivalve species of mussels, clams and oysters, as well as gastropods such as abalone and numerous snails [Adamkewicz, et al., 1988; Brake, et al., 2004; Innes, et al., 1977; Kobayashi, et al., 2004; Luttikhuizen, et al., 2008; Newkirk, 1980; Palmer, 1984]. Differences in shell color have been attributed to environmental and genetic effects. Genetic factors play a major role in the color of bivalve shells. Variations in shell coloration follow simple Mendelian patterns, inherited as discreet color morphs controlled by one or two loci [Adamkewicz, et al., 1988; Innes, et al., 1977; Newkirk, 1980; Winkler, et al., 2001] and conform to expected Mendelian phenotypic ratios (e.g. 1:1 or 3:1). The bay scallop *Argopecten irradians,* the Chilean scallop *Argopecten purpuratus*, the tellinid *Macoma baltica* and the mussel *Mytilus edulis* show discrete shell colors that are determined by one or two loci [Adamkewicz, et al.,

1988; Innes, et al., 1977; Luttikhuizen, et al., 2008; Winkler, et al., 2001]. Controlled mating experiments in gastropods such as the Pacific abalone *Haliotis discus hannai* have shown Mendelian segregation at a single locus [Kobayashi, et al., 2004; Liu, et al., 2009]. In other mollusks, color can be a continuously distributed quantitative trait under polygenic control. Bivalves such as the Pacific oyster *Crassostrea gigas* show a continuum of shell pigmentation [Brake, et al., 2004; Evans, et al., 2009; Hedgecock, et al., 2006].

Echinoderms have a wide variety of color patterns. Studies investigating the inheritance of color patterns in echinoderms are not as extensive as in bivalves. Asteroids such as *Pisaster ochraceus* and *Linkia laevigata* are conspicuous components of the benthic habitat in which they are found and have several discrete color morphs. *Pisaster* can be purple, orange and brown and *Linkia* can be a striking royal blue or orange. In both cases, as in other asteroids as well, the pigmentation is due to carotenoproteins in the calcareous integument [Fox, et al., 1966]. The pigments are acquired through the diet. The functional significance of the colors and color variations is poorly understood.

Echinoids have a wide range of color phenotypes. Color variation in the external calcareous parts is due to naphthoquinones [Anderson, et al., 1969; Thomson, 1971]. The origin of these pigments remains poorly understood. In some species color variation is thought to be related to differences in habitat [Growns, et al., 1994] or behavior [Tsuchiya, et al., 1985]. Genetic factors influence the color phenotype of at least two species of echinoids: *Paracentrotus lividus* [Louise, et al., 1993, 1995] and *Lytechinus*

variegatus [Pawson, et al., 1982]. Environmental factors influencing color variability in echinoids are not well studied.

As a species, *L. variegatus* has the broadest range in color of western Atlantic urchins. While color variability across its geographic range and within some sites may be broad, many sites are more homogeneous (See Ch. 2). Color phenotype is usually assessed by referencing the color of the spines as these give the overall color. White, green, purple, and pink are the most common spine colors. Many urchins show dual spine coloration with purple-green and red-green and white-green being the most common. The underlying test is also pigmented but is not readily visible. In many cases the most that can be said is that it is light or dark, although certain colors such as dark red, pink, beige and green seem to predominate. Urchins from the Florida Keys display a feature not at all common in other areas: patterning. Patterning encompasses the differential coloration of the test, and sometimes spines. In the most common form, the ambulacral wedges are markedly darker in color than the interambulacral wedges. In other cases, the pattern may be more random and cut across the ambulacral or interambulacral sections giving a mottled appearance.

In this chapter I evaluate the genetic component to color phenotype by creating a series of crosses within and between color morphs. All the parental urchins are wild caught individuals and therefore their genotype is unknown. I hypothesize that color phenotype is inherited in simple Mendelian fashion for a one-locus trait for spine color, test color and patterning.

I will also evaluate the morphological differences in offspring of crosses created from Beaufort and Tavernier Key urchins, as well as hybrid crosses made from urchins of both sites. I hypothesize that a common-garden experimental design will produce offspring of similar morphology.

4.2 Materials and Methods

4.2.1 Urchin collection

Urchins from Beaufort NC (Beaufort) and Tavernier Key (Tavernier) in the Florida Keys were used to make genetic crosses. Adult sized Beaufort urchins (>30 mm horizontal diameter) were collected from in the shallows of Bogue banks near the Morehead City port by dredging. They were then brought to the Duke Marine Lab and kept in flow-through seawater tanks at ambient temperature (22-27°) for up to a week until use in experiments. Adult sized Tavernier urchins (>30 mm horizontal diameter) were collected by Ken Nedimeyer of Tavernier Key and shipped to the McClay lab at Duke University. From the McClay lab urchins used in the genetic crosses and the morphological study were collected and transported in large 20 L buckets in an air conditioned car to DUML. Tavernier urchins were not kept in the flow-through seawater tanks (to avoid possible release of gametes into the local population) but in 20 L buckets at ambient seawater temperature (22-25°) and aerated. Water was changed daily. All urchins were fed local sea grass (*Zostera marina* and *Halodule wrightii*) or macro algae

(*Codium* spp., *Ulva lactuca, Gracilaria* sp., and *Dictyota* sp.) depending on availability ad libitum until use in experiments. Once used in the crosses the urchins were sacrificed and used in the study of morphological and color variability (Chapter 2).

4.2.2 Larval culture

The protocol for obtaining gametes and fertilizing eggs laid out by Foltz et al. [2004] and culturing larvae to metamorphosis described by Wray et al.[2004] was followed with minor modifications. Natural sea water was used. Seawater was filtered through inline cartridge filters at 10 micron (Culligan level 3 CW-F Polypropylene wound) and allowed to sit in non-aerated vats to acclimate to room temperature (22-25°) for 24-48 hrs before use in experiments.

Gametes from male and female urchins were obtained by injection of 1-2 ml of 0.55 M KCl into the coelomic cavity through the peristomial membrane. Sperm was collected "dry" (i.e., pipetted off the urchin and put into a 1.5 ml eppendorf tube) and put on ice until use. Female urchins were inverted over a small glass beaker and the eggs were shed into 200 ml of filtered sea water. They were washed 3-4 times. Fertilizations were conducted in clean 25 ml glass finger bowls. Approximately 5 ml of well-mixed egg concentrations from each female was added to 20 ml of filtered seawater in the finger bowls according to the cross design (no attempt was made to determine the exact egg concentration (#eggs ml^{-1}) from each spawning female). In most cases egg concentration appeared visually to be about equal. However, Tavernier females in

crosses 16-19 (PkG, RG) and 37, 38 (GW), spawned few eggs and two females of similar phenotype were combined. A dilute sperm suspension was made by adding 20-40 μl of "dry" sperm to 50 ml of filtered sea water. 1-2 ml of the sperm suspension from each male was then added to each bowl according to the cross design. Fertilization was deemed successful if approximately 90-95% of the eggs developed a fertilization envelope after approximately 1 min. 1-2 ml more sperm was added if the percent fertilization was lower. All crosses except 9-11 and 26-27 (Tavernier within-site) were made at the Duke Marine Lab. Fertilizations for these five crosses were made in the McClay lab on Duke Main Campus in Durham and developing early-stage embryos were brought down by car to the marine lab in 50 ml vials. Once at the marine lab the embryos (at the blastula stage) were transferred to clean 25 ml glass finger bowls.

Embryos developed in the glass finger bowls until they reached the prism stage (approximately 18 hrs post fertilization). At the prism or early pluteus stage 1-5 larvae ml^{1} were transferred to and raised in 4 L glass jars filled ¾ full with filtered seawater at 23-25° C, at a salinity of 31-34 ppt. A 1 ml pipette connected to a pump gently bubbled air into each jar for aeration and to provide water movement. Three replicate jars per cross were maintained. When the larvae reached the feeding stage, 2 days post fertilization, approximately 25 ml of the green algae *Dunaliella tertiolecta* (2-4 x 10^{6} cells/ml) was added to each jar. Water in the jars was changed every 3-4 days by vacuuming out most of the water through a mesh strainer as outlined in Wray et al. [2004]. Clean filtered seawater was added and 25 ml of *Dunaliella* was again added to each jar.

When the larvae became competent to metamorphose i.e., they had a well developed ciliary band (epaulets), the rudiment was visible and pedicellaria were exposed at the base of the larvae [Hinegardner, 1969; Mazur, et al., 1971], they were placed in large glass finger bowls (1600 ml) filled approximately ¾ full with filtered seawater. The finger bowls had been allowed to soak for several days in seawater tanks containing adult *L. variegatus* to accumulate a biofilm. Initiation of metamorphosis generally occurred rather quickly, usually within an hour or so for the faster growing larvae. Slower growing larvae could take several days to complete metamorphosis. When most of the larvae had completed metamorphosis the juvenile urchins were transferred to 4 L jars filled ¾ full with filtered seawater. Again, three replicate jars per cross were maintained. The jars had been filled several days earlier to allow the water to acclimate to room temperature and approximately 100 ml of *Dunaliella* was added and allowed to settle and accumulate as a thin biofilm inside the walls of the jar. Light was provided by goose-neck table lamps on a 24H light cycle to hasten algal growth. A tall drinking glass (500 ml) was inserted in all jars to increase the surface area for algal growth. Once the juveniles were transferred, more *Dunaliella* was added and the urchins were allowed to grow. Water was changed every 10-14 days by gently pouring out the water through a mesh strainer to catch dislodged juveniles i.e. overflow method as described by Wray et al. [2004] and refilled with filtered seawater.

Changes were made to culture conditions starting with crosses 12-19 (first set of among-site crosses). Prism or early pluteus stage larvae were placed in 2 L glass jars and placed on a Lab-Line Incubator-Shaker set at 22° C and shaking at approx. 50 rpm. The

gentle shaking facilitated water movement and kept larvae suspended in the water column. In these latter crosses, the water was changed infrequently or not at all throughout larval development. 10 ml of *Dunaliella* was added every 2-3 days as it was consumed. Water quality was monitored and changed when algal concentration did not diminish. Competent larvae were not transferred to glass finger bowls but remained in the original jar throughout development. To induce metamorphosis, I inserted plastic Petri dishes that had been allowed to soak for several days in seawater tanks containing adult *L. variegatus* to accumulate a biofilm. The time of larval development decreased from ≥30 days in the first four crosses to about 14 days in later crosses.

4.2.3 Juvenile cultures

Juvenile urchins were kept in the 4 L or 2 L glass jars until most reached a size of approximately 2-3 mm in horizontal diameter. They were then transferred to 40 L glass aquarium tanks (76 mm x 31 mm x 30 mm) in the water wing. The tanks were primed for the transfer at least 3-4 weeks prior: they were filled with filtered seawater and 200+ ml of *Dunaliella* was added as well as F2 medium to induce a copious growth of algae. Air was supplied through an airstone and 3-4 tall drinking glasses (500 ml) were added to each tank to increase the surface area for algal growth. Crosses with many juveniles (≥20) were divided into 2 or more tanks. Juvenile urchins fed on the algal growth until they reached a size (≥5 mm) that allowed for feeding on macro algae (*Codium* spp., *Ulva*

lactuca, *Gracilaria* sp., and *Dictyota* sp.) and sea grass (*Zostera marina* and *Halodule wrightii*) depending on availability. Water in the tanks was changed every 7-10 days by emptying them out and refilling with filtered seawater collected a day or two prior and stored in 2 large 400 L non-aerated vats to acclimate to ambient temperature (22-27° C).

4.2.4 Crosses

Crosses were made to examine the mode of inheritance of color phenotype. F_1 offspring from 38 crosses were grown to late juvenile/early adult size to assess maternal and paternal color patterns on their phenotype. Of the 38 crosses, 26 paired males and females from the same location (i.e. within-site Beaufort-Beaufort and Tavernier-Tavernier) and 12 paired across location (among-site Beaufort-Tavernier). Table 4.1 lists the within-site crosses and the maternal and paternal phenotypes. Table 4.2 lists the among-site crosses and the maternal and paternal phenotypes.

Table 4-1: Within-site crosses. Crosses are grouped by date and only males and females listed under the same date were crossed together.

Cross	Date of crosses	Maternal phenotype (phenotype ID)	Paternal phenotype (phenotype ID)	Cross ID	# ♀ : # ♂
1	June 2006	Beaufort white (WL)	Beaufort white (WL)	WLxWL	4 : 4
2		Beaufort white (WD)	Beaufort white (WD)	WDxWD	
3		Beaufort purple (P)	Beaufort purple (P)	PxP	
4		Beaufort green (G)	Beaufort green (G)	GxG	
5	Oct 2006	Beaufort pink (Pk)	Beaufort purple (P)	PkxP	2 : 2
6		Beaufort pink (Pk)	Beaufort white (WL)	PkxWL	
7		Beaufort purple (P)	Beaufort purple (P)	PxP	
8		Beaufort purple (P)	Beaufort white (WL)	PxWL	
9	Mar 2007	Tavernier white (W)	Tavernier purple-red (P)	WxP	3 : 1
10		Tavernier purple (P)	Tavernier purple-red (P)	PxP	
11		Tavernier green (G)	Tavernier purple-red (P)	GxP	
12	Nov 2007	Beaufort white (WL)	Beaufort pink (Pk)	WLxPk	2 : 2
13		Beaufort white (WL)	Beaufort light purple (LP)	WLxLP	
14		Beaufort purple (P)	Beaufort pink (Pk)	PxPk	
15		Beaufort purple (P)	Beaufort light purple (LP)	PxLP	
20	May 2008	Beaufort pink (Pk)	Beaufort white (WL)	PkxWL	2 : 3
21		Beaufort pink (Pk)	Beaufort white-green (WG)	PkxWG	
22		Beaufort pink (Pk)	Beaufort purple (P)	PkxP	
23		Beaufort white (WR)	Beaufort white (WL)	WRxWL	
24		Beaufort white (WR)	Beaufort white-green (WG)	WRxWG	
25		Beaufort white (WR)	Beaufort purple (P)	WRxP	
26	Jan 2009	Tavernier green-white (GW)	Tavernier green-white (GW)	GWxGW	1 : 2
27		Tavernier green-white (GW)	Tavernier pink-green (PkG)	GWxPkG	
34	Oct 2009	Beaufort white (WL)	Beaufort white (WL)	WLxWL	1 : 1
35	Nov 2009	Beaufort purple (P)	Beaufort white (WL)	PxWL	2 : 2
38		Tavernier green-white (GW)[a]	Tavernier green-white (GW)	GWxGW	

Table 4-2: Between-site crosses. Crosses are grouped by date and only males and females listed under the same date were crossed together.

Cross	Date of crosses	Maternal phenotype (phenotype ID)	Paternal phenotype (phenotype ID)	Cross ID	# ♀ : # ♂
16	Nov 2007	Tavernier pink-green (PkG)[a]	Beaufort pink (Pk)	PkGxPk	2 : 2
17		Tavernier pink-green (PkG)[a]	Beaufort light purple (LP)	PkGxLP	
18		Tavernier red-green (RG)[a]	Beaufort pink (Pk)	RGxPk	
19		Tavernier red-green (RG)[a]	Beaufort light purple (LP)	RGxLP	
28	Aug 2009	Beaufort white (WL)	Tavernier pink (Pk)	WLxPk	2 : 3
29		Beaufort white (WL)	Tavernier white-green (WG)	WLxWG	
30		Beaufort white (WL)	Tavernier green-purple (GP)	WLxGP	
31		Beaufort green-purple (GP)	Tavernier pink (Pk)	GPxPk	
32		Beaufort green-purple (GP)	Tavernier white-green (WG)	GPxWG	
33		Beaufort green-purple (GP)	Tavernier green-purple (GP)	GPxGP	
36	Nov 2009	Beaufort purple (P)	Tavernier green-white (GW)	PxGW	2 : 2
37		Tavernier green-white (GW)[a]	Beaufort white (WL)	GWxWL	

4.3.5 F_2 and F_3 generations

14 F_1 full sibling urchin crosses spawning spontaneously *in situ* produced F_2 generations. The larvae from each cross were collected from the parental tank by a mesh strainer (80μm), put into three 2 L jars and placed on the Lab-Line Incubator-Shaker set at 22° C and shaking at approx. 50 rpm. The larvae were reared as for the previous F_1 generation. Post-settlement juveniles reaching a size ≥ 2-3 mm in horizontal diameter

were then put into larger 40 L tanks and allowed to grow to late juvenile/early adult size.

Three of the F_2 crosses in turn produced a new F_3 generation. The larvae were again collected from the parental tank by a mesh strainer (80μm), put into three 2 L jars and placed on the Lab-Line Incubator-Shaker set at 22° C and shaking at approx. 50 rpm and reared to late juvenile/early adult size as in the previous F_1 and F_2 generations.

4.3.6 Morphological and color variability

The same morphological measurements taken on adult wild-caught urchins were taken on F_1 and F_2 urchins greater than ca. 12 mm in diameter. F_1 offspring from 30 crosses and F_2 offspring from 14 F_1 crosses were assessed for color phenotype, color variability of test and spines and physical measures of tests, spines and lanterns. Of the 30 F_1 crosses, 15 were within-site Beaufort, 5 were within-site Tavernier Key, 10 were among-site Beaufort-Tavernier hybrids. The mean value for each character within a cross was derived by averaging the offspring values for that cross.

As many of the spine, test and lantern character frequency distributions deviated from a normal distribution and all had heteroscedastic variances that did not improve with transformations of the data, I compared the means using the nonparametric Kruskal-Wallis test of means in JMP ver. 8.

Color phenotype was assessed for juveniles as for *in situ* field survey color scoring: the color was based on the most obvious color on the primary spines and

assigned to one the 14 color morphs. Spine and test color of each offspring were visually matched to standard color paint cards from Lowe's Home Improvement Store and coded as described in Chapter 2.

Inheritance of color phenotype was tested in juvenile F_1 generations from several single color crosses using the simple Mendelian model of a single-locus, two-allele trait for spine color, test color and patterning (i.e. 0:1, 1:1 or 3:1 phenotypic ratios for each of the three traits). Observed frequencies were tested against the expected frequencies based on the model. Deviations of the observed color ratios were tested with a chi-square test. From these ratios, parental P_1 genotypes were deduced.

Since the urchins serving as parents in the crosses were all wild-caught there was no foreknowledge of their genotype. Therefore, assuming simple Mendelian inheritance was the simplest approach and provided an easily falsifiable null hypothesis. Of the 30 crosses that produced juveniles, 19 were examined for observed phenotypic frequencies of spine color, test color and patterning.

Juvenile F_2 and F_3 generations were not tested against the expected frequencies because the identity of the individual F_1 urchins contributing the gametes was unknown and therefore precluded me from being able to assume a direct parent-offspring relationship. The phenotypes were, however, compared against parental and grandparental phenotypes to denote potential deviations or novel combinations.

4.3 Results

4.3.1 Cross color phenotypes

A total of 30 crosses produced F_1 offspring. No difference in pigmentation was evident in post-settlement juveniles (approximately 0.5-1 mm in horizontal diameter). All juvenile urchins at this stage were very similar in phenotype—the test a translucent white with red pigment granules on the aboral surface and a light purple band midway down the spines. Differences in phenotype between individuals in all crosses became apparent as the urchins grew. Pigmentation patterns diverged and became increasingly distinctive starting at approximately 4 mm horizontal test diameter. For urchins developing into the white phenotype the purple band around the spines slowly lightened until finally disappearing. For urchins developing into other color phenotypes the appearance of darker colors intensified slowly over time. At approximately 10-12 mm horizontal diameter the final phenotype was apparent but became more distinctive the larger the urchin grew. Patterning, like color, became more apparent as the urchins grew. The juveniles were raised to ≥ 12 mm horizontal diameter which allowed their phenotype to be determined and morphological measurements to be taken.

4.3.2 Cross F_1 color morphs

A total of 745 F_1 offspring from the 30 crosses were counted and scored for color phenotype as for in situ field surveys. 15 within-site Beaufort crosses produced 322 offspring, 5 within-site Tavernier crosses produced 88 offspring and 10 among-site Beaufort-Tavernier hybrid crosses produced 335 offspring. The final tally of color morphs was 9, a subset of the 14 found in the field surveys: green, white, pink, purple, red, pink-green, purple-green, red-green and white/green (Table 4.3). No triple color morphs were produced in any of the crosses. All single color morphs were found in the F_1 juveniles but green and red were in very small numbers.

Table 4-3: Color morphs of F_1 offspring. Numbers indicate the number per color morph for within-site Beaufort, within-site Tavernier and Beaufort-Tavernier hybrid crosses. Dual color morphs with bicolor spines are listed with a hyphen, whereas, dual color morphs in which the spines are uniform in color but the color differs on different areas of the test are listed with a slash. Triple color morphs are also listed with a slash. No triple color morphs were produced in any of the crosses.

Color phenotype	Beaufort	Tavernier	Hybrid	Row total
green	0	0	1	1
white	179	5	200	222
pink	48	0	39	87
purple	93	0	15	108
red	0	0	5	5
pink-green	0	11	20	31
purple-green	2	7	51	60
red-green	0	15	2	17
red-purple	0	0	0	0
white/green	0	50	2	52
white/pink	0	0	0	0
white/purple	0	0	0	0
green/white/other	0	0	0	0
green/white/purple	0	0	0	0
Total	322	88	335	745

In Beaufort the predominant color morphs are white (81%), pink (7.4%) and purple (11.4%) and for the within-site Beaufort crosses these same color morphs predominate, although the proportions differ (56.6%, 15%, and 29% respectively) they are in the same relative rank. The color morphs of the Tavernier Key offspring do not represent the totality of color morphs found in the native parental region, with only 5 of 14. However, the color morphs that are present in the crosses (5) and parents (7) fall

within the range of the color morphs found in the region of origin (Fig. 4.1). The offspring color morphs resemble those of the parents but are dominated by the white/green morph (56.8%). The color morphs of the among-site Beaufort-Tavernier hybrid offspring are a mix of both sets of color morphs with white taking up the largest share (59.7%), as in the Beaufort data.

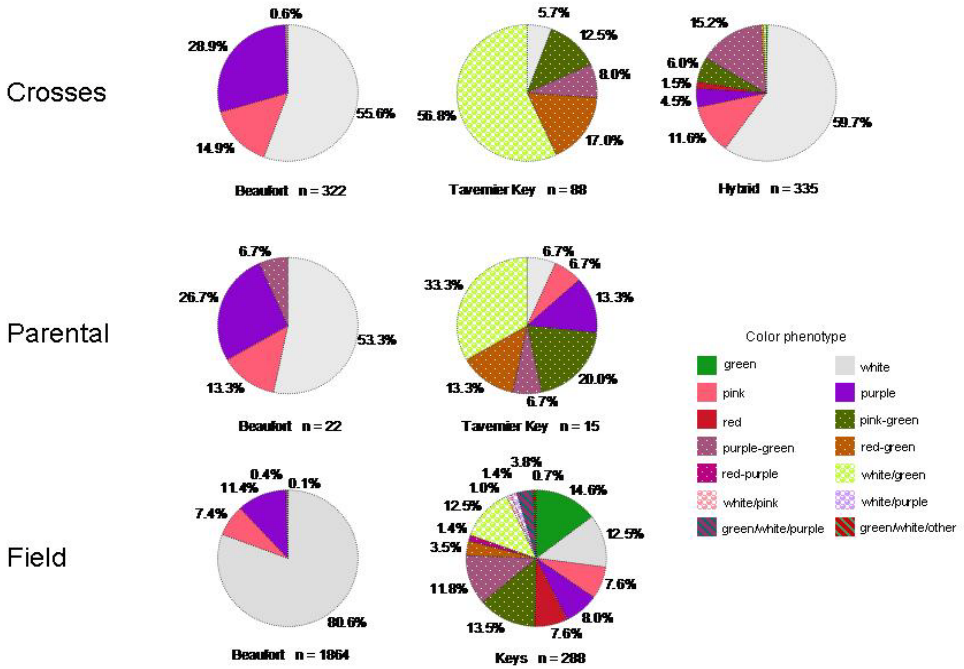

Figure 4-1: Top panel: color morphs of F_1 offspring of all Beaufort, Tavernier and hybrid crosses (n = total number of offspring for each cross type). Color morphs and number of the parentals and field sample given for comparison. Tavernier cross and parental color morphs are a subset of the full Keys palette. Hybrid color morphs are a mix of Beaufort and Keys morphs.

A similar pattern emerges for color variability of spines and tests. The combined spine and test color data reveal that the color categories in the cross juveniles are the same as those of the field data (Fig. 4.2). For the Tavernier crosses the relative proportion of the categories is strikingly consistent from juveniles to parents to field data, with green being the largest category for all three. For the Beaufort crosses, the pink/red and purple/lavender categories take up a larger share of the total than for the field data (45.7% and 31.1% for crosses versus 24.6% and 26.2% for the field sample). The relative proportions of color categories for the hybrid crosses are more homogeneous than either of the 2 within-site crosses. Pink/red, purple/lavender and brown categories are represented in equal proportions.

The color categories that comprise distal spine color are consistent with the field data. Purple/lavender is the most abundant category for all three types of crosses (Beaufort 59.2%, Tavernier 60.7% and Hybrid 49%) (Fig. 4.3). The relative proportion of categories for Tavernier crosses is strikingly consistent with the field data (Fig. 2.5), whereas for Beaufort crosses, purple/lavender and pink/red increases in proportion relative to white and brown. The composition of hybrid distal categories more closely resembles Beaufort crosses.

Green dominates the proximal ends of Tavernier crosses (91.8%) but Beaufort proximal spines are more diverse with pink/red (40.8%), white (26.6%) and green (22.1%) making up 89.5% of the total (Fig. 4.3). Hybrid crosses are a composite like Beaufort crosses with green (44.1%), white (32.9%) and pink/red (21%) totaling 98%.

Comparing the distribution with field populations (Fig. 2.5), shows that the difference is more pronounced than for the distal colors. Proximal spine color for Tavernier crosses is almost exclusively green (91.8%) whereas the in the field samples green makes up 63.6% and white 22.7% of the total. For Beaufort crosses the difference is less extreme. Proximal spine color is a composite of three categories—white (26.6%), pink/red (40.8%) and green (22.1%). The field sample is skewed to white (53.8%) and pink/red at 26.9%.

Beaufort crosses and Tavernier crosses have very different test IA colors (Fig. 4.3). Pink/red colors comprise the majority (64.8%) of test color in Beaufort crosses and white (68.9%) in Tavernier crosses. Tavernier test color encompasses all 6 categories (although the frequencies of 4 of the categories are < 7%), whereas, Beaufort test color is more homogeneous, being made up of 3 categories—pink/red (64.8%), purple/lavender (27%) and to a smaller extent brown (7.9%). The hybrid crosses like Beaufort crosses are more homogeneous having predominantly pink/red (39.2%) or brown (42.7%) tests.

The difference between cross test color and field test color (Fig. 2.7) is very pronounced. Beaufort field test color is composed of essentially three categories—brown (43.6%), purple/lavender (28.2%) and pink/red (23.1%). Cross test color is skewed heavily to pink/red (64.8%) with some purple/lavender (27%). A similar situation is present for the Keys field test color which is divided roughly between brown (35.2%), green (35.1%) and white (25%), whereas, cross test color is dominated by white (68.9%).

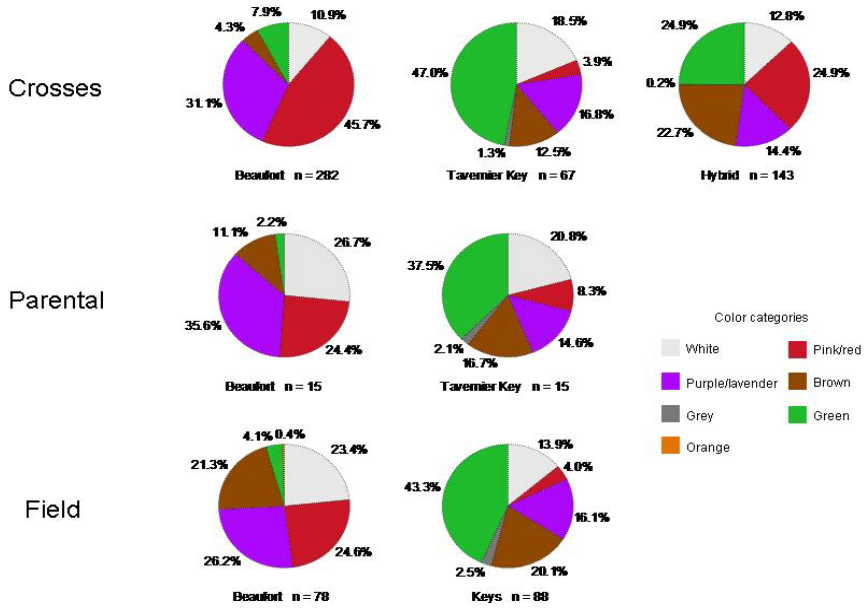

Figure 4-2: Color categories for spine and test traits combined for F_1 offspring of all Beaufort, Tavernier and hybrid crosses (n = number of offspring scored for color. The number differs from the total number of offspring produced since some crosses had high late-stage mortality). Color categories for the parentals and for field data given for comparison. Tavernier cross color categories are remarkably consistent with field data.

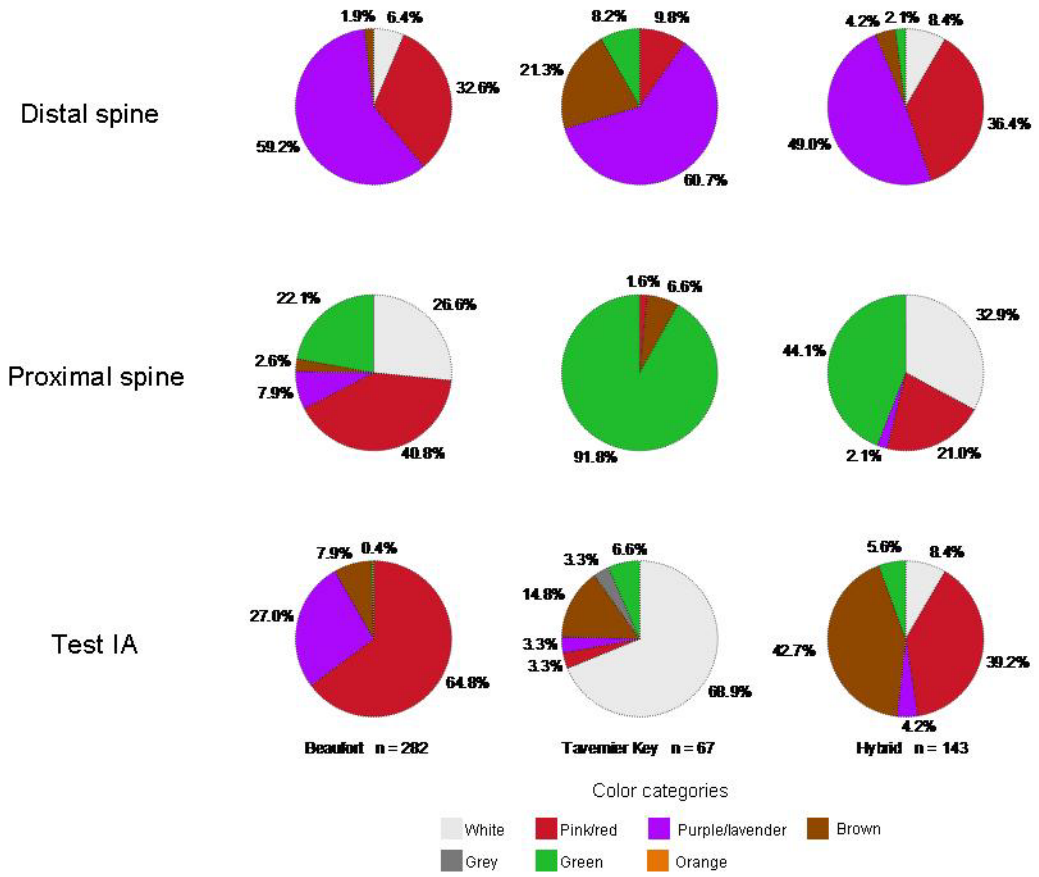

Figure 4-3: Color categories for individual spine and test traits for F_1 offspring of all Beaufort, Tavernier and hybrid crosses. Top panel: distal spine colors. Middle panel: proximal spine colors. Bottom panel: test interambulacral (IA) colors.

Partitioning the categories into individual colors allows for greater discrimination of color within crosses. Figure 4.4 highlights the variability within the categories for each cross type. From the graph we see that there is broad agreement with the colors for Beaufort and Keys field urchins (Fig. 2.8). Also evident is the difference in composition of the color categories between Beaufort and Tavernier crosses. Specifically, the purple/lavender color of distal spines is comprised almost exclusively of purple in Tavernier crosses whereas both purple and lavender colors feature prominently in Beaufort and hybrid crosses. The pink/red category comprising a sizeable portion of test IA and proximal and distal spine color in Beaufort crosses is more evenly split between pink and red than for the Beaufort field population where red is wholly absent.

Of course, inferences about the differences in color between field samples and crosses cannot be made since the individual urchins assessed in the crosses are not independent samples. Siblings are more likely to share similar characteristics than unrelated individuals so the data has a heavy bias. Nevertheless, the data does indicate that color morphs and color variability are broadly consistent within regions.

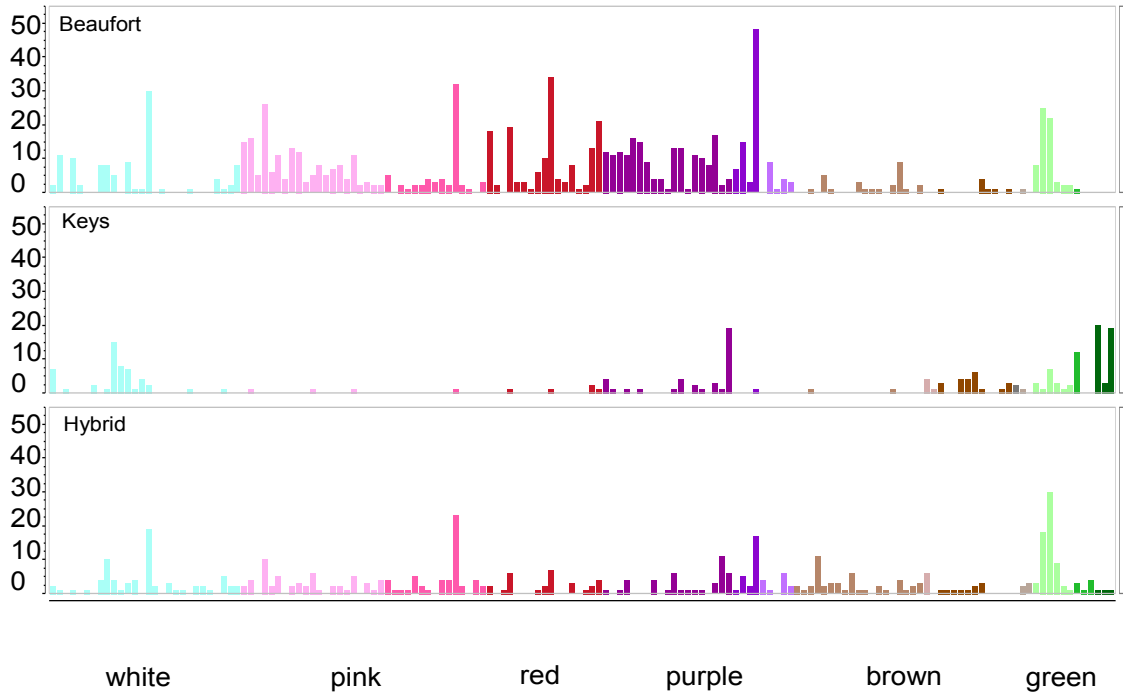

Figure 4-4: Histogram showing the frequency of 156 colors for all three traits combined. Colors are partitioned into 15 categories. Colors with categories range from light to dark. Crosses from top to bottom: Beaufort, Tavernier, hybrid.

Table 4.4 lists the number and general phenotype of surviving F_1 offspring for each of the 30 crosses. The eventual number of surviving juveniles decreased in all cases from initial settlement numbers. Crosses 2, 5-8, 32 advanced past the post-metamorphosis stage but were lost before the juveniles reached a size adequate to assess the phenotype. Crosses 1, 3, 4, 12, 29, 31, 33, 34 produced < 4 juveniles and inferences about phenotypic variability from these crosses cannot be made. Crosses 9, 11, 13-19, 20-28, 35-38 produced sufficient juveniles per cross to examine color and morphological variability. Crosses 13, 14 and 18 lost a sizable number of juvenile urchins prior to harvesting. For crosses 13 and 14 this still left a substantial number of juveniles (57 and 64 respectively) but for cross 18 the final number diminished to just four individuals. The cause of the mortality is unknown but was most likely due to bacterial contamination in the tanks. The dead juveniles were placed in seawater for several days to allow for the removal of the spines and the epidermis. Once the tests were cleaned they were rinsed in fresh water and allowed to air dry for several days. The tests were then scored for color and morphological measurements taken.

Table 4-4: Number and color phenotype of all surviving F_1 offspring from all 38 crosses.

Cross #	P_1 Maternal Phenotype	P_1 Paternal Phenotype	Cross ID	Post-metamorphic F_1 offspring N =	Surviving F_1 offspring N =	F_1 offspring phenotypes N =
1	White	White	WL-WL	3	1	1 White
2	White	White	WD-WD	6	0	N/A
3	Purple	Purple	P-P	7	2	2 Purple
4	Light green	Light green	G-G	69	4	2 Purple 1 Purple-green 1 White
5	Pink	Purple	Pk-P	11	0	N/A
6	Pink	White	Pk-WL	47	0	N/A
7	Purple	Purple	P-P	39	0	N/A
8	Purple	White	P-W	6	0	N/A
9	White	Red-purple	W-P	21	10	5 White 5 Red-green
10	Purple	Red-purple	P-P	0	0	N/A
11	Green	Red-purple	G-P	20	10	4 Red-green 6 Dark red-green
12	White	Pink	WL-Pk	3	4	1 Purple 1 White 1 Light purple 1 Pink
13	White	Light purple	WL-LP	>100	57	37 White 11 Light purple 4 Purple 4 Pink 1 Dark pink
14	Purple	Pink	P-Pk	>100	58	14 Purple 13 White 11 Light purple 8 Pink 8 Dark pink 3 Lavender 1 Purple-green

Cross #	P_1 Maternal Phenotype	P_1 Paternal Phenotype	Cross ID	Post-metamorphic F_1 offspring N =	Surviving F_1 offspring N =	F_1 offspring phenotypes‡ N =
15	Purple	Light purple	P-LP	65	31	9 Purple 8 Pink 5 Dark pink 5 Lavender 4 Light purple
16	Pink-green	Pink	PkG-Pk	10	9	3 White 3 Pink 2 Light purple 1 Purple-green
17	Pink-green	Light purple	PkG-LP	70	66	18 Pink 12 Light purple 11 Pink-green 7 Light purple-green 7 Light pink-green 3 Dark pink 3 White 2 Green-white 2 Lavender-green 1 Green
18	Red-green	Pink	RG-Pk	18	*4	5 Dark Pink 5 Pink 4 Red 2 White 1 Red-green 1 Purple-green
19	Red-green	Light purple	RG-LP	7	8	3 Pink 2 Dark pink 1 Purple 1 Red 1 Red-green
20	Pink	White	Pk-WL	36	5	5 White
21	Pink	White-green	Pk-WG	>100	29	28 White 1 Pink
22	Pink	Purple	Pk-P	47	19	7 White 7 Purple 4 Pink 1 Light purple

Cross #	P_1 Maternal Phenotype	P_1 Paternal Phenotype	Cross ID	Post-metamorphic F_1 offspring N =	Surviving F_1 offspring N =	F_1 offspring phenotypes‡ N =
23	White	White	WR-WL	>100	14	10 White 4 Pink
24	White	White-green	WR-WG	>100	15	11 White 4 Light purple
25	White	Purple	WR-P	77	6	2 White 2 Purple 1 Light purple 1 Pink
26	Light green-white	Green-white	GW-GW	>100	7	5 Green-white 2 Light green-white
27	Light green-white	Pink-green	GW-PkG	>100	18	11 Pink-green 7 Purple-green
28	White	Pink	WL-Pk	32	6	6 White
29	White	White-green	WL-WG	7	4	4 White
30	White	Green-purple	WL-GP	0	0	N/A
31	Green-purple	Pink	GP-Pk	1	1	1 Pink-green
32	Green-purple	White-green	GP-WG	1	0	N/A
33	Green-purple	Green-purple	GP-GP	2	2	1 Light pink-light green 1 Purple-green
34	White	White	WL-WL	5	3	3 White
35	Purple	White	P-WL	90	55	55 White
36	Purple	Green-white	P-GW	60	40	40 Purple-green
37	Green-white	White	GW-WL	>200	170	170 White
38	Green-white	Green-white	GW-GW	>100	46	46 Green-white

‡Phenotypes listed are based on first impression color. A more detailed breakdown of spine and test phenotypes is given later in the text.
*Cross RG-Pk produced 18 surviving juveniles. The death of 14 occurred just prior to harvesting but phenotypes are listed in the table since they were previously photographed and are known.

4.3.3 Mendelian ratios

Table 4.5 lists the observed patterns of segregation for spine color, the observed and expected phenotypic ratios and the deduced genotype of the parents for 5 crosses in which the parental phenotypes differed and one parent was white and the other purple. In 4 of the 5 crosses the observed color segregation of white to purple offspring fell into the expected Mendelian ratio 1:1, indicating that one of the parental phenotypes was dominant and the individual was heterozygous. The fifth cross differed, all offspring were of the white phenotype (1:0) indicating that the white parent was homozygous and white is dominant over purple.

Table 4-5: Observed phenotypic ratios for F_1 offspring of white and purple urchins. Urchins were used in more than one cross as indicated by the color scheme. White encompasses spines that are entirely white or light pink as well as spines that have pink, light purple or light pink tips. Purple refers to spines that are fully purple and various shades thereof, including red, dark pink, lavender and light purple. χ^2 tests are not significantly different from expected Mendelian ratios.

Cross #	♀ P_1	♂ P_1	Observed color segregation W	P	Observed ratio W:P	Deduced P_1 genotype ♀	♂	Expected ratio W:P	X^2 df = 1
25	White	Purple	3	3	1:1	w/p	p/p	1:1	
22	White	Purple	11	7	1.5:1	w/p	p/p	1:1	0.88 0.5 > *p* > 0.1
13	White	Purple	30	27	1:1	w/p	p/p	1:1	0.158 0.9 > *p* > 0.5
14	Purple	White	22	36	1:1.6	p/p	w/p	1:1	2.4 0.1 > *p* > 0.05
35	Purple	White	55	0	1:0	p/p	w/w	1:0	

Table 4.6 lists the observed patterns of segregation for spine color, the observed and expected phenotypic ratios and the deduced genotype of the parents for 5 crosses in which the parental phenotypes are identical—both parents white or purple. In 4 of the crosses both parents were white and in 2 of them the observed color segregation of white to purple offspring fell neatly into one of the expected Mendelian ratios (1:0), indicating that at least one of the parents was homozygous for the dominant allele. In a

3rd white cross the observed phenotypic ratio of offspring conformed to the predicted 3:1 pattern indicating that both parents were heterozygous. One of the white crosses (21) resulted in offspring phenotypes that did not conform to expected Mendelian ratios, as indicated by the significant result of the chi-square test ($\chi 2 = 8.05$, $P < 0.01$). The single purple cross produced only purple offspring, indicating that both parents were homozygous. This in concert with the other crosses indicates that purple was very likely the recessive phenotype.

Table 4-6: Observed phenotypic ratios of spine color for F_1 offspring of white and purple Beaufort urchins. Cross 21 did not conform to the expected Mendelian ratio of 3:1 based on genotype assigned to the parents ($\chi 2 = 8.05$, $P < 0.01$).

Cross #	♀ P_1	♂ P_1	Observed color segregation W P	Observed ratio W:P	Deduced P_1 genotype ♀ ♂	Expected ratio W:P	X^2 df = 1
15	Purple	Purple	0 31	0:1	p/p p/p	0:1	
24	White	White	11 4	3:1	w/p w/p	3:1	0.022 0.9 > *p* > 0.5
23	White	White	14 0	1:0	w/p w/w	1:0	
20	White	White	5 0	1:0	w/p w/w	1:0	
21	White	White	28 0	1:0	w/p w/p	3:1	8.05** *p* < 0.01

From these crosses we see that white urchins can produce both white and purple offspring, whereas, purple urchins only produced purple urchins. This strongly suggests that white is the dominant phenotype and purple is recessive.

Crosses were also made with urchins of another color phenotype: green. Green is a common phenotype in the Keys amounting to 14.6% of the total (Fig. 4.1). Crossing green urchins with white and purple urchins presented a slightly different picture (Table 4.7). Crossing white with green did not change the hierarchy of dominance/recessiveness with respect to white. White was dominant over green as shown in cross 37 where all 170 offspring were white. When crossed with purple, both colors were expressed in the offspring, resulting in the dual colored purple-green phenotype. The co-expression of green and purple appears to be explicit with respect to the spatial positioning of the colors. Green is always at the proximal end and purple at the distal end. Crosses in which both parents were green produced only green offspring.

Table 4-7: Observed phenotypic ratios of spine color for F_1 offspring of white, green and purple urchins. Green crosses (26 & 38) produced only green offspring. Green-purple crosses (11& 36) produced the dual colored phenotype purple-green indicating that neither allele is dominant over the other. The green-white cross (37) produced white urchins, confirming white as the dominant allele.

Cross #	♀ P_1	♂ P_1	Observed color segregation F_1 W G P	Observed ratio W:G: P	Deduced genotype ♀ ♂	Expected ratio W:G: P
26	Green	Green	0 7 0	0:1:0	g/g g/g	0:1:0
38	Green	Green	0 43 0	0:1:0	g/g g/g	0:1:0
11	Green	Purple	0 8/8 8/8	0:1:1	g/g p/p	0:1:1
36	Purple	Green	0 40/40 40/40	0:1:1	p/p g/g	0:1:1
37	Green	White	170 0 0	1:0:0	g/g w/w	1:0:0

Test phenotypic ratios were examined in a similar fashion (Table 4.8). Test phenotypes were assessed as either light or dark. The phenotypic ratios for this trait fell within expected Mendelian ratios for 9 of the 11 crosses examined but the fit was more equivocal than for the spine data. Two crosses produced offspring test color that deviated significantly from the expected 1:1 ratio. The discrepancy for cross 20 may be due to sampling error given that the results are based on a small sample of 5 individuals.

Generally high mortality resulting in few offspring may have biased the outcome. The probability of getting 5 offspring with a dark test as opposed to the expected even split is quite possible given the stochastic nature of post-metamorphic survival. Cross 13 in which there were 57 offspring is more problematic since this number of offspring should have generated a closer fit to the expected 1:1 ratio. The significant deviation may signal that the categories used to identify the phenotype were not appropriate and need to be adjusted to reflect the true measure.

Table 4-8: Observed phenotypic ratios of test color for F_1 offspring. Tests were scored as either dark or light. Dark colored tests can range from dark red, purple, brown or dark green, whereas light colored tests may be light red, pink or beige. All but 2 of the 11 crosses did not deviate substantially from expected Mendelian ratios assuming dominance/recessiveness of dark versus light. However, the fit was not as unequivocal as it was for the spine color data. This discrepancy may signal that the categories need to be refined to a more accurate measure.

Cross #	♀ P_1	♂ P_1	Observed color segregation D	L	Observed ratio D:L	Deduced P_1 genotype ♀	♂	Expected ratio D:L	X^2 df = 1
15	Dark	Dark	24	7	3.4:1	D/d	D/d	3:1	1.13 $0.5 > p > 0.1$
14	Dark	Dark	47	11	4.3:1	D/d	D/d	3:1	1.13 $0.5 > p > 0.1$
9	Dark	Light	3	5	1.7:1	D/d	d/d	1:1	0.5 $0.5 > p > 0.1$
11	Dark	Light	3	5	1.7:1	D/d	d/d	1:1	0.5 $0.5 > p > 0.1$
22	Dark	Dark	12	6	2:1	D/d	D/d	3:1	0.33 $0.5 > p > 0.1$
21	Dark	Light	18	10	1.8:1	D/d	d/d	1:1	2.3 $0.1 > p > 0.05$
23	Dark	Light	9	5	1.8:1	D/d	d/d	1:1	1.14 $0.5 > p > 0.1$
24	Dark	Light	8	7	1:1	D/d	d/d	1:1	0.07 $0.5 > p > 0.1$
25	Dark	Dark	4	2	2:1	D/d	D/d	3:1	0.22 $0.9 > p > 0.5$
20	Dark	Light	5	0	1:0	D/d	d/d	1:1	5.0** $p < 0.01$
13	Light	Dark	44	13	3.4:1	d/d	D/d	1:1	16.86** $p < 0.01$

The final character assessed under Mendelian patterns of inheritance was patterning. This trait, little described in the literature, was observed on Keys urchins. Crosses were made in which both urchins were patterned and where only one urchin was patterned. The results were consistent and unequivocal in all cases. For all crosses

the offspring were patterned (Table 4.9). This trait was inherited in dominant fashion for all 7 crosses.

Table 4-9: Observed phenotypic ratios for F_1 offspring of patterned (Tavernier) and non-patterned (Beaufort) urchins. Patterned urchins in crosses 36-38 are F_1 offspring of cross 26.

Cross #	♀ P_1	♂ P_1	Observed segregation P NP	Observed ratio P:NP	Deduced genotype	Expected ratio P:NP
26	Patterned	Patterned	7 0	1:0	p+/p+ p+/p+	1:0
27	Patterned	Patterned	18 0	1:0	p+/p+ p+/p+	1:0
28	Non-patterned	Patterned	6 0	1:0	p-/p- p+/p+	1:0
29	Non-patterned	Patterned	4 0	1:0	p-/p- p+/p+	1:0
36	Non-patterned	Patterned	40 0	1:0	p-/p- p+/p+	1:0
37	Patterned	Non-patterned	170 0	1:0	p+/p+ p-/p-	1:0
38	Patterned	Patterned	43 0	1:0	p+/p+ p+/p+	1:0

The patterns of segregation for the crosses give the overall phenotype. However, as outlined in figures 4.3 and 4.4 the phenotypes are more complex with distal and proximal spine, as well as, test colors differing. To better understand the details of inheritance Table 4.10 lists the colors observed in both the parents and the offspring in 5

single color cross types. The data encompass multiple crosses per cross type (n = the number of crosses, the total number of offspring coded) and demonstrate that despite the high number of offsping the number of colors inherited is rather modest. The variability arises in the combination of distal, proximal and test colors that are observed and listed at the bottom of each cross type. Not every possible combination was seen.

Table 4-10: Parental and offspring spine and test colors observed in 5 single color cross types. The data encompass multiple crosses per cross type. P_1 = parental colors, F_1 = offspring colors. n = the number of crosses, the total number of offspring coded for combined spine and test traits. The greatest number of observed color combinations in the offspring is listed at the bottom of each cross type.

white x white n = 4, 63

	distal	proximal	test IA
P1	white	white	purple
	light pink	light pink	brown
			light brown
			light green
F1	purple	lavender	purple
	lavender	beige	red
	beige	light pink	pink
	light lavender	light green	beige
	light pink	white	
	white		

color combinations = 13

white x purple n = 5, 166

	distal	proximal	test IA
P1	purple	lavender	purple
	lavender	light pink	brown
	pink	white	lavender
	light pink		light pink
F1	purple	purple	purple
	lavender	lavender	brown
	brown	brown	red
	red	green	pink
	pink	pink	beige
	beige	beige	light green
	light lavender	light lavender	
	light pink	light pink	
	white	light green	
		white	

color combinations = 36

white x green n = 1, 22

	distal	proximal	test IA
P1	purple	dark green	light pink
	pink	light pink	white
F1	beige	light lavender	beige/tan
	light lavender	light pink	light green
	light pink	white	white
	white		

color combinations = 13

purple x purple n = 1, 31

	distal	proximal	test IA
P1	purple	lavender	lavender
		white	
F1	purple	purple	purple
	lavender	lavender	red
	red	pink	pink
	pink	light lavender	
	light lavender	light pink	
		white	

color combination = 19

purple x green n = 2, 28

	distal	proximal	test IA
P1	purple	purple	purple
	brown	dark green	brown
		light green	white
		white	
F1	purple	purple	brown
	brown	dark green	dark green
	lavender	green	red
		light green	grey
		light brown	pink
			beige/tan

color combinations = 12

4.3.4 F_2 and F_3 offspring

Table 4.11 lists the number of surviving F_2 juveniles, their phenotype and the phenotype of the parental F_1 cross as well as the phenotype of the grandparental P_1 urchins. The color phenotype was assessed on these juveniles as previously described.

Phenotypes in the F_2 generation are a composite that include identical phenotypes as in the preceding F_1 parental generation and the P_1 generation. There are also novel phenotypes that demonstrate independent segregation of the alleles coding for spine color. Phenotypes in the Beaufort crosses overwhelmingly encompassed white (34.2%), pink (18.3%) and purple (45.8%) as in the F_1 crosses and field samples. Only one novel phenotype, pink-green, appeared in the F_2 offsping of Beaufort cross 15. Pink-green is a phenotype associated with Keys and Gulf urchins (Fig, 2.2) but is absent in Beaufort. Phenotypes in the Tavernier F_2 crosses reflected the previous generation and field samples. Representation of the white/green phenotype was increased to 77.4% from 56.8% in the F_1 generation.

Hybrid F_2 crosses increased the proportion of green, purple pink-green and red-green phenotypes while decreasing white. The only phenotype not documented previously, an albino, occurred in the F_2 offsping of cross 16. If we examine the color morphs between the crosses we see that they are consistent in type from generation to generation but differing in the proportions (Fig. 4.5).

Table 4-11: Number and color phenotype of all surviving F_2 offspring. Also given is the phenotype of the parental F_1 cross and the P_1 cross for comparison across generations. Phenotypes assessed as for field caught urchins.

P_1 cross phenotypes ♀ ♂ Cross ID Cross #	F_1 cross phenotypes	Post-metamorphosis F_2 juveniles N =	Surviving F_2 juveniles N =	Surviving F_2 juvenile phenotypes N =
White Red-purple W-P Cross 9	5 White 3 Green	27	7	4 White 1 Red 1 Light green 1 Light pink-green
Green Red-purple G-R Cross 11	5 Red-green 3 Dark red-green	69	12	4 Light pink 2 Red 2 Red-green 1 Red-purple 1 Green 1 Light green 1 Dark pink-green
White Light purple WL-LP Cross 13	37 White 23 Light purple 5 Purple 4 Pink 1 Dark pink	48	21	11 White 5 Light purple 3 Lavender 2 Purple
Purple Pink P-Pk Cross 14	14 Purple 13 White 11 Light purple 8 Pink 8 Dark pink 3 Lavender 1 Purple-green	>100	52	16 Purple 13 Pink 7 Lavender 7 Light purple 5 Dark pink 3 White 1 Purple-green*
Purple Light purple P-LP Cross 15	9 Purple 8 Pink 5 Dark pink 5 Lavender 4 Light purple	29	15	5 Lavender 4 Purple 2 Dark pink 2 Pink 1 Light purple 1 Pink-green
Pink-green Pink PkG-Pk Cross 16	3 White 3 Pink 2 Light purple 1 Purple-green	>100	15	3 White 7 Pink 2 Light purple 1 Purple 1 Pink-green 1 Albino*

P_1 cross phenotypes ♀ ♂ Cross ID Cross #	F_1 cross phenotypes	Post-metamorphosis F_2 juveniles N =	Surviving F_2 juveniles N =	Surviving F_2 juvenile phenotypes N =
Pink-green Light purple PkG-LP Cross 17	18 Pink 12 Light purple 11 Pink-green 7 Light purple-green 7 Light pink-green 3 Dark pink 3 White 2 White-green 2 Lavender-green 1 Green	16	15	6 Pink-green 4 Light purple 2 Green-purple 1 Lavender 1 Red 1 Dark pink
*Red-green Pink RG-Pk Cross 18	5 Dark Pink 5 Pink 4 Red 2 White 1 Red-green 1 Purple-green	13	13	4 Red-green 4 White 2 Dark pink 1 Pink-green 1 Light purple 1 Pink
Red-green Light purple RG-LP Cross 19	3 Pink 2 Dark pink 1 Purple 1 Red 1 Red-green	19	15	4 Pink-green 4 Light green 3 Green 3 Light pink 2 Lavender
Grn-wht Grn-wht GW-GW Cross 26	5 Green-white 2 White-green	>75	20	20 Green-white
Purple White P-WL Cross 35	56 White	>90	31	26 White 5 Purple
Purple Grn-wht P-GW Cross 36	40 Purple-green	60	25	7 Purple-green 6 Purple 12 Green
Grn-wht White GW-W Cross 37	170 White	>100	51	27 White 11 Green 3 Purple
Grn-wht Grn-wht GW-GW Cross 26	5 Green-white 2 White-green	>60	45	45 Green-white

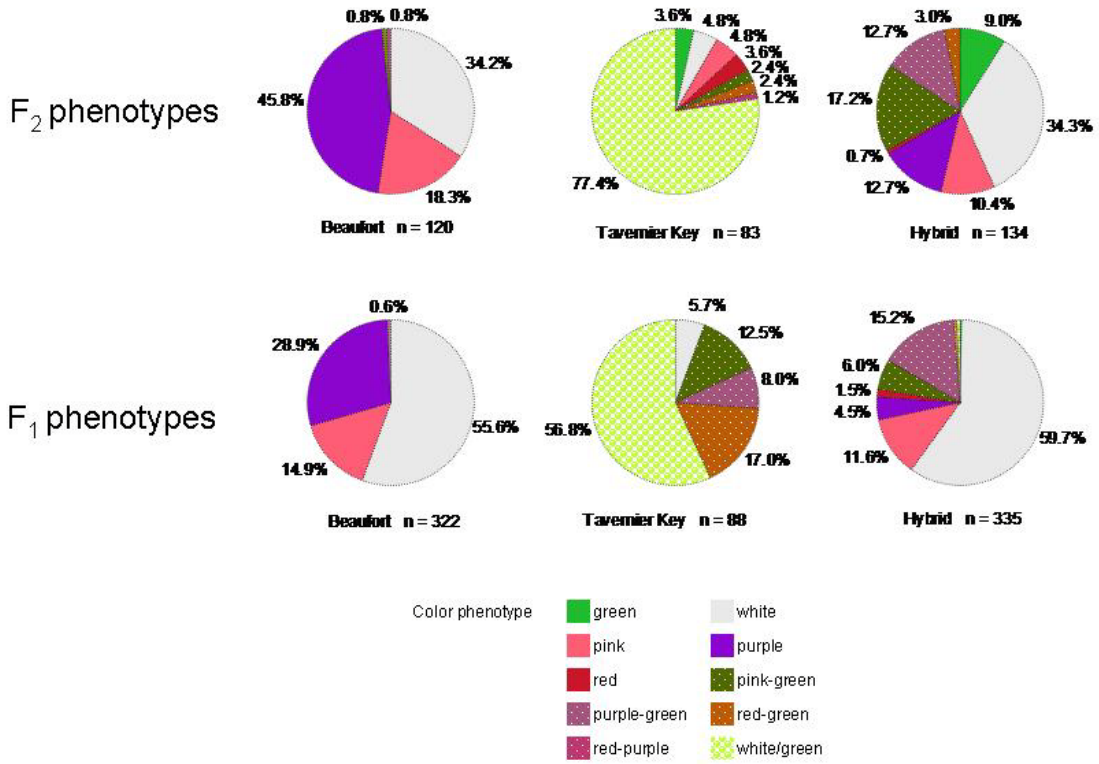

Figure 4-5: Phenotypes for F_1 and F_2 juveniles.

Color categories for all combined traits in the F_2 generation were similar to the F_1 generation (Fig. 4.6), differing in the proportions. Purple/lavender increased substantially in total share for both Beaufort (30.6%) and Tavernier Key (14.8%) crosses. Pink/red increased by 12.5% for hybrid crosses. In contrast, white decreased by 10% for Beaufort and 13.4% for Tavernier Key. Categories grey and orange were not present in the F_2 generation.

Three of the F_2 crosses (1 Tavernier cross and 2 hybrid crosses) produced an F_3 generation. The total number of offspring was 28 encompassing 6 color morphs. The Tavernier cross had 4 color morphs: white, pink, pink-green and red-green. The color morphs for the hybrid crosses were white, pink, pink-green, green and purple.

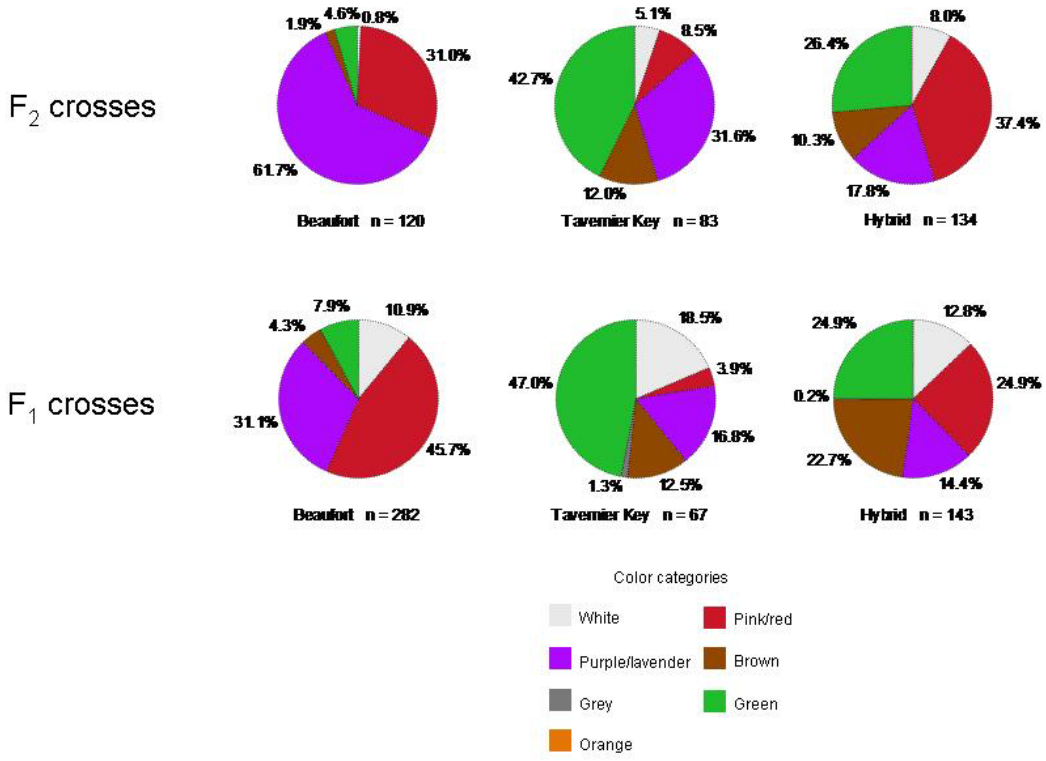

Figure 4-6: Color categories for all traits combined for F_1 and F_2 juveniles.

4.3.5 Cross morphology

Of the 16 characters listed in Table 4.12, only ambital spine length (Kruskal-Wallis $\chi^2 = 7.59$, df = 2, $P < 0.023$) and width (Kruskal-Wallis $\chi^2 = 9.54$, df = 2, $P < 0.0085$) and aboral spine width (Kruskal-Wallis $\chi^2 = 9.87$, df = 2, $P < 0.0072$) differed between crosses. Spine width was greatest for Beaufort crosses and spine length was greatest for Tavernier crosses.

The remaining 15 characters did not differ significantly between the three crosses but 10 of the characters had the largest mean value for the Beaufort crosses and 5 characters had the largest mean value for the Tavernier crosses. Wet and dry test weight, test wall thickness, lantern weight, both wet and dry, lantern height and IA and AMB plates were larger in Beaufort crosses. Test diameter, height, peristome size, aboral spine length and lantern height were larger in Tavernier crosses. Hybrid crosses had intermediate values for all characters (Table 4.12).

The wide range in mean values for many of the characters in each of the crosses reflects the difference in the number of juveniles between crosses. 9 crosses had > 20 juveniles, 6 crosses had 10-20 juveniles, 6 crosses had 5-10 juveniles and 9 crosses had < 5 juveniles. Size differences between full siblings within a cross could vary greatly as well. In all crosses there were marked differences in individual growth rate. In general crosses with < 6 siblings had the narrowest range in diameter size (2.27-6.52 mm difference between the largest and smallest urchin). The size range increased with > 8 siblings per

cross. The largest difference was 15.29 mm in cross 15 with 31 full sibling urchins. The smallest range (2.27 mm) was in cross 20 with 5 full sibs.

Table 4-12: Lists the ranges, means and standard deviations of all morphological characters measured on Beaufort, Tavernier and hybrid F_1 crosses. Results of Kruskal-Wallis (χ^2) comparisons of means: * = $P < 0.05$, ** = $P < 0.01$, NS = $P > 0.05$.

Character			Beaufort n = 15	Tavernier n = 5	Hybrid n = 10		χ^2
Test	diameter (mm)	range	17.29–35.72	19.71–32.90	20.39–28.80	NS	0.57
		mean	24.28	25.32	23.41		
		st dev.	5.93	6.03	3.23		
	height (mm)	range	9.98–18.54	11.81–19.45	11.72–17.11	NS	1.30
		mean	13.67	14.97	13.60		
		st dev.	3.03	3.38	1.77		
	aboral	range	0.29–0.79	0.25–0.38	0.26–0.45	NS	3.83
	thickness (mm)	mean	0.41	0.32	0.34		
		st dev.	0.13	0.05	0.06		
	ambital	range	0.31–1.18	0.23–0.42	0.25–0.49	NS	4.11
	thickness (mm)	mean	0.47	0.34	0.38		
		st dev.	0.21	0.08	0.08		
	mean	range	0.30–0.99	0.24–0.40	0.26–0.47	NS	3.93
	thickness (mm)	mean	0.44	0.33	0.36		
		st dev.	0.17	0.07	0.07		
	dry weight (g)	range	0.50–3.45	0.47–1.78	0.63–1.69	NS	0.65
		mean	1.31	0.98	0.99		
		st dev.	0.93	0.57	0.38		
	peristome (mm)	range	8.42–13.88	8.79–13.49	9.24–12.05	NS	0.34
		mean	10.61	10.73	10.18		
		st dev.	1.77	2.06	1.08		
	IA	range	13–19	13–18	13–17	NS	1.81
		mean	16	15	15.00		
		st dev.	1.62	2.34	1.43		
	AMB	range	14–26	15–23	14–23	NS	1.78
		mean	20	18	18.00		
		st dev.	3.38	3.43	2.88		
Lantern	length (mm)	range	6.99–12.18	7.41–11.24	6.01–10.91	NS	2.13
		mean	9.34	8.90	8.43		
		st dev.	1.72	1.75	1.64		
	width (mm)	range	6.63–11.54	7.30–11.88	6.18–10.41	NS	0.44
		mean	8.72	9.11	8.31		
		st dev.	1.71	2.08	1.33		
	wet weight (g)	range	0.15–0.66	0.14–0.57	0.09–0.44	NS	0.37
		mean	0.33	0.32	0.26		
		st dev.	0.18	0.20	0.11		
Spines	ABSL (mm)	range	3.71–5.45	4.74–5.59	3.65–5.90	NS	2.66
		mean	4.53	5.17	4.70		
		st dev.	0.63	0.36	0.80		
	AMSL (mm)	range	8.75–12.06	10.51–12.25	9.67–12.11	*	7.59
		mean	9.94	11.64	10.86		
		st dev.	1.06	0.77	0.85		
	ABSW (mm)	range	0.39–0.63	0.26–0.43	0.33–0.50	*	9.87
		mean	0.49	0.36	0.42		
		st dev.	0.07	0.07	0.06		
	AMSW (mm)	range	0.55–0.92	0.47–0.54	0.47–0.64	*	9.54
		mean	0.66	0.51	0.59		
		st dev.	0.10	0.03	0.06		

To eliminate the effect of size, nine measures were converted to ratios. The ratios were the same as those in the morphological data of field caught urchins: test height-diameter (H/D), dry weight per diameter, peristome per diameter, lantern length-width, wet weight per diameter and spine length and width per diameter ratios (Table 4.13).

Kruskal-Wallis tests indicate that 4 of the 9 ratios are significantly different between the crosses. Lantern length-width is significantly different at the $P < 0.01$ level (Kruskal-Wallis $\chi^2 = 14.93$, df = 2). Test height-diameter (H/D) ratio (Kruskal-Wallis $\chi^2 = 8.57$, df = 2) and aboral and ambital spine length (Kruskal-Wallis $\chi^2 = 5.99$, $\chi^2 = 7.39$, df = 2, respectively) are significantly different at the $P \leq 0.05$ level. 5 of the 9 ratios are not significantly different.

Table 4-13: Mean values and standard deviations of test, spine and lantern ratios for F_1 crosses. Numbers in bold indicate the greatest significant values for the character. Ratios include test height-diameter (H/D), test dry weight per diameter, peristome diameter per test diameter, lantern length-width and lantern wet weight per test diameter. Spine length and width measured as the fraction of the test diameter. Aboral spine length (ABSL), ambital spine length (AMSL), aboral spine width (ABSW), ambital spine width (AMSW). Results of Kruskal-Wallis (χ^2) comparisons of means: * = $P \leq 0.05$, ** = $P < 0.01$, NS = $P > 0.05$.

		Beaufort n=15		Tavernier n = 5		Hybrid n = 10			
Characters		mean	*st. dev.*	mean	*st. dev.*	mean	*st. dev.*		χ^2
Test	H/D ratio	0.57	*0.024*	0.59	*0.015*	0.60	*0.029*	*	8.57
	Test ratio (g/mm)	0.049	*0.022*	0.036	*0.013*	0.041	*0.010*	NS	2.54
	Peristome ratio	0.45	*0.035*	0.43	*0.021*	0.44	*0.025*	NS	1.51
Lantern	Lantern L/W ratio	**1.07**	*0.035*	0.98	*0.042*	1.01	*0.045*	**	14.93
	Lantern weight ratio (g/mm)	0.013	*0.004*	0.012	*0.004*	0.011	*0.003*	NS	0.92
Spine	ABSL	0.19	*0.028*	**0.24**	*0.028*	0.20	*0.030*	*	5.99
	AMSL	0.42	*0.086*	**0.54**	*0.024*	0.47	*0.081*	*	7.39
	ABSW	0.021	*0.003*	0.017	*0.004*	0.018	*0.003*	NS	5.92
	AMSW	0.027	*0.004*	0.024	*0.003*	0.025	*0.003*	NS	2.97

Of the three test ratios, only H/D ratio was significantly different between Beaufort, Tavernier Key and hybrid crosses. It differed between Beaufort (0.57 ± 0.024) and hybrid (0.60 ± 0.029) crosses but neither differed from Tavernier Key (0.59 ± 0.015) crosses (Fig. 4.7 A). The weight of tests relative to size did not differ between crosses and neither did the size of the peristome in relation to test diameter (Fig. 4.7 C & D respectively). Figure 4.7 shows that the means for all three test ratios differed the most

between Beaufort and Tavernier Key. The high variability within each of the crosses precluded any differentiation between the means.

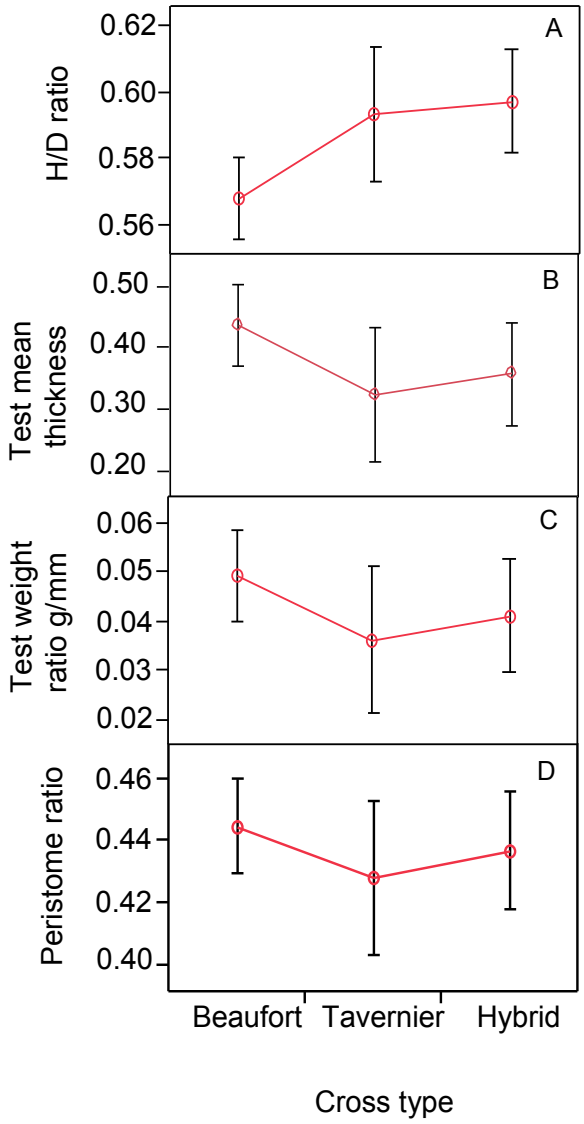

Figure 4-7: Mean values (± SE) for test height-diameter ratio (H/D), mean test thickness, test dry weight per test diameter and peristome ratio for F_1 crosses. H/D ratio was significantly different between crosses. Beaufort = Tavernier Key = hybrid ≠ Beaufort. Mean test thickness, test weight per diameter and peristome ratio did not differ between crosses.

Lantern length-width ratio was significantly different between crosses (Kruskal-Wallis $\chi^2 = 14.93$, df = 2, $P < 0.01$). Beaufort lanterns were longer (1.07 ± 0.035) than those of Tavernier Key (0.98 ± 0.042) and hybrid (1.01 ± 0.045) crosses, which did not differ (Fig 4.8 A) but they were not heavier (Kruskal-Wallis $\chi^2 = 0.92$, df = 2, $P > 0.05$). Lanterns from all crosses were similar in weight (Fig. 4.8 B).

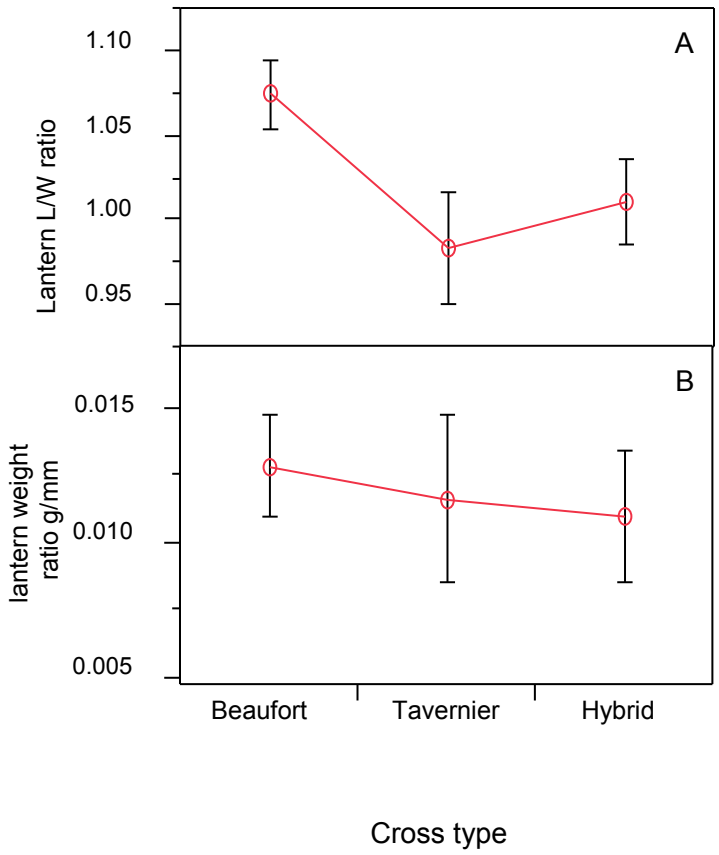

Figure 4-8: Mean values (± SE) for lantern length-width ratio and lantern wet weight per test diameter ratio. Lanterns of Beaufort crosses were longer than those of Tavernier Key and hybrid crosses but they were no heavier.

Spine length was significantly greater in Tavernier Key crosses than in Beaufort crosses for both aboral (Kruskal-Wallis $\chi^2 = 5.99$, df = 2, $P \leq 0.05$) and ambital (Kruskal-Wallis $\chi^2 = 7.39$, df = 2, $P \leq 0.05$) spines. In contrast, spine width did not differ between

crosses either aborally (Kruskal-Wallis $\chi^2 = 5.92$, df = 2, $P > 0.05$) or ambitally (Kruskal-Wallis $\chi^2 = 2.97$, df = 2, $P > 0.05$) (Fig. 4.9).

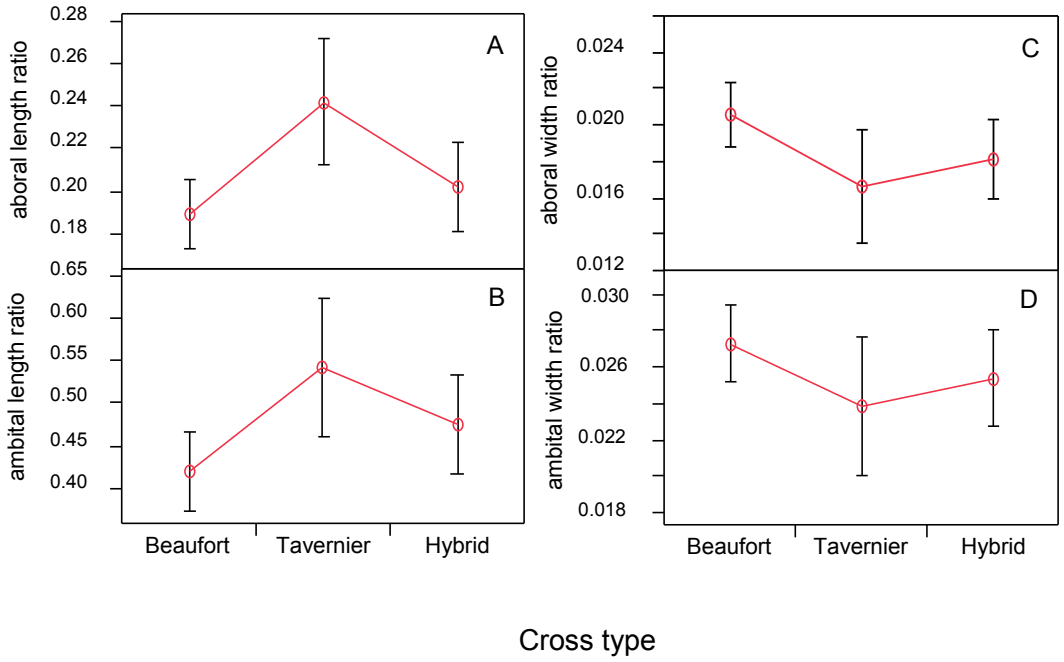

Figure 4-9: Mean values (± SE) for spine length and width ratios. Graphs A and B show that Tavernier Key crosses have longer aboral and ambital spines than Beaufort crosses. Hybrid spine length does not differ from either. Graphs C and D show that mean spine width for Beaufort crosses is greater than Tavernier Key and hybrid crosses but not statistically significant.

Comparing the relative difference in mean trait values for the ratios between Beaufort and Tavernier crosses with the same relative difference in Beaufort and Keys field urchins shows that the magnitude of the difference for many of the traits is similar.

Table 4.14 lists the percent difference between the ratio means of field urchins and crosses. In all cases except one (ambital spine length, AMSL), the polarity of the difference is identical (i.e., H/D ratio and aboral spine length for Keys greater than Beaufort but for all other comparisons Beaufort > Keys). Ambital spines are longer in Beaufort field urchins but in the crosses, Tavernier juveniles have longer ambital spines. The magnitude of the difference in percentage for all traits except lantern weight and ambital spine width is well below 10. This demonstrates that genetically the Beaufort and Keys populations are morphologically different as the relative difference is maintained in the crosses.

Table 4-14: Relative difference in trait means for Beaufort and Keys field populations and Beaufort and Tavernier crosses. Positive difference indicates that field urchins had a larger difference in trait means. The magnitude of the difference between field populations and crosses is identical in aboral spine length and width and very similar in all other traits except lantern weight and ambital spine width. For ambital spine length* the polarity of the difference is changed. Tavernier crosses have longer ambital spines than Beaufort crosses.

		Field	Crosses	difference
Test	H/D ratio§	10%	3%	+7
	Test thickness	21%	25%	-4
	Test ratio (g/mm)	32%	27%	+5
	Peristome ratio	5%	4%	+1
Lantern	Lantern L/W ratio	4%	8%	-4
	Lantern weight ratio (g/mm)	37%	8%	*+29*
Spine	ABSL	21%	21%	0
	AMSL*	13%	22%	-9
	ABSW	19%	19%	0
	AMSW	29%	11%	*+18*

Because the frequency distributions of spine and test characters deviated from a normal distribution and all had heteroscedastic variances, multivariate analysis of variance could not be done on the F_1 crosses. Since comparisons of MANOVA and discriminant analysis in the field data gave almost identical results, I employed discriminant analysis to compare the morphological differences of crosses. The same 7 variables were used (H/D ratio, aboral and ambital spine length and width ratios, mean test thickness, lantern weight ratio) plus test weight ratio which provided the most

accurate classification of the crosses (Fig. 4.10). It provided the best fit with the lowest error rate (1 misclassified, a 4% error rate, Wilks' Lambda = 0.069, F ~ 5.22, $P < 0.0001$). 1 hybrid cross was classified as a Tavernier cross. No Beaufort or Tavernier crosses were misclassified. Despite the non-significant difference in means between crosses for many of the traits, if taken together the separation between crosses is readily apparent.

Again, the greatest differentiation is in the 1st canonical plane and encompasses 98% of the variation. The traits most responsible for the separation between crosses are aboral and ambital spine width and lantern weight ratio. The 2nd canonical plane adds the remaining 2% and includes H/D ratio.

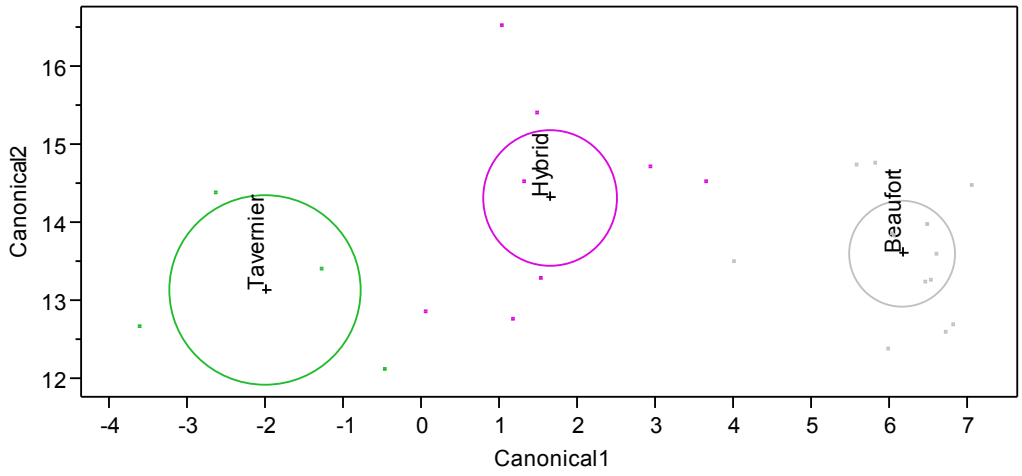

Figure 4-10: Discriminant analysis of F_1 crosses based on 7 test spine and lantern characters. A 95% confidence ellipse surrounds the multivariate mean of each cross. 1 hybrid cross was misclassified as Tavernier. No Beaufort or Tavernier crosses were misclassified.

337 F_2 offspring from 10 F_1 crosses were produced. 184 were weighed and 16 morphological characters were measured: 87 offspring from 3 Beaufort crosses, 39 offspring from 3 Tavernier crosses and 58 offspring from 4 Beaufort-Tavernier crosses. The range, mean values and standard deviations of all characters measured for each of the crosses is listed in Table 4.15.

Table 4-15: Lists the ranges, means and standard deviations of all morphological characters measured on Beaufort, Tavernier and hybrid F_2 crosses.

Character			Beaufort n = 3	Tavernier n = 3	Hybrid n = 4
Test	diameter (mm)	range	16.71–20.93	18.20–23.26	15.28–21.88
		mean	19.12	20.57	18.73
		st dev.	2.17	2.55	3.27
	height (mm)	range	10.47–12.15	11.96–13.46	8.72–13.18
		mean	11.22	12.92	10.89
		st dev.	0.853	0.834	2.47
	aboral	range	0.26–0.33	0.27–0.32	0.26–0.35
	thickness (mm)	mean	0.31	0.30	0.29
		st dev.	0.040	0.029	0.041
	ambital	range	0.28–0.36	0.28–0.35	0.29–0.33
	thickness (mm)	mean	0.33	0.31	0.31
		st dev.	0.044	0.036	0.017
	mean	range	0.27–0.35	0.28–0.33	0.28–0.34
	thickness (mm)	mean	0.32	0.3	0.30
		st dev.	0.044	0.025	0.029
	wet weight (g)	range	0.89–1.75	0.85–1.62	0.54–1.62
		mean	1.36	1.25	1.06
		st dev.	0.436	0.386	0.522
	dry weight (g)	range	0.44–0.86	0.39–0.73	0.28–0.78
		mean	0.66	0.56	0.50
		st dev.	0.211	0.17	0.231
	peristome (mm)	range	8.31–9.65	9.06–9.74	7.80–10.08
		mean	9.02	9.42	8.97
		st dev.	0.674	0.342	0.997
	IA	range	13–15	13–14	13–15
		mean	14	13	14
		st dev.	0.744	0.471	1.31
	AMB	range	15–16	14–16	16–19
		mean	15	15	17
		st dev.	0.605	0.981	1.278
Lantern	length (mm)	range	6.96–8.20	7.16–7.95	6.56–8.73
		mean	7.68	7.58	7.49
		st dev.	0.644	0.397	1.086
	width (mm)	range	6.84–8.05	7.36–8.33	6.18–8.50
		mean	7.45	7.93	7.33
		st dev.	0.605	0.507	1.088
	wet weight (g)	range	0.15–0.26	0.14–0.22	0.10–0.25
		mean	0.21	0.18	0.18
		st dev.	0.055	0.040	0.076
	dry weight (g)	range	0.11–0.16	0.10–0.13	0.06–0.18
		mean	0.13	0.12	0.11
		st dev.	0.025	0.017	0.057
Spines	ABSL (mm)	range	3.54–3.99	4.36–5.46	3.89–4.43
		mean	3.83	4.85	4.17
		st dev.	0.254	0.559	0.271
	AMSL (mm)	range	7.58–10.17	8.85–13.34	8.61–11.41
		mean	9.16	10.43	9.63
		st dev.	1.39	2.52	1.55
	ABSW (mm)	range	0.38–0.47	0.37–0.42	0.34–0.38
		mean	0.43	0.39	0.36
		st dev.	0.045	0.025	0.021
	AMSW (mm)	range	0.47–0.61	0.47–0.54	0.44–0.51
		mean	0.56	0.51	0.48
		st dev.	0.076	0.036	0.038

Due to the very low number of samples per cross statistical analyses between crosses could not be made. Discriminant analysis correctly classified the crosses but was not significant (Fig. 4.11).

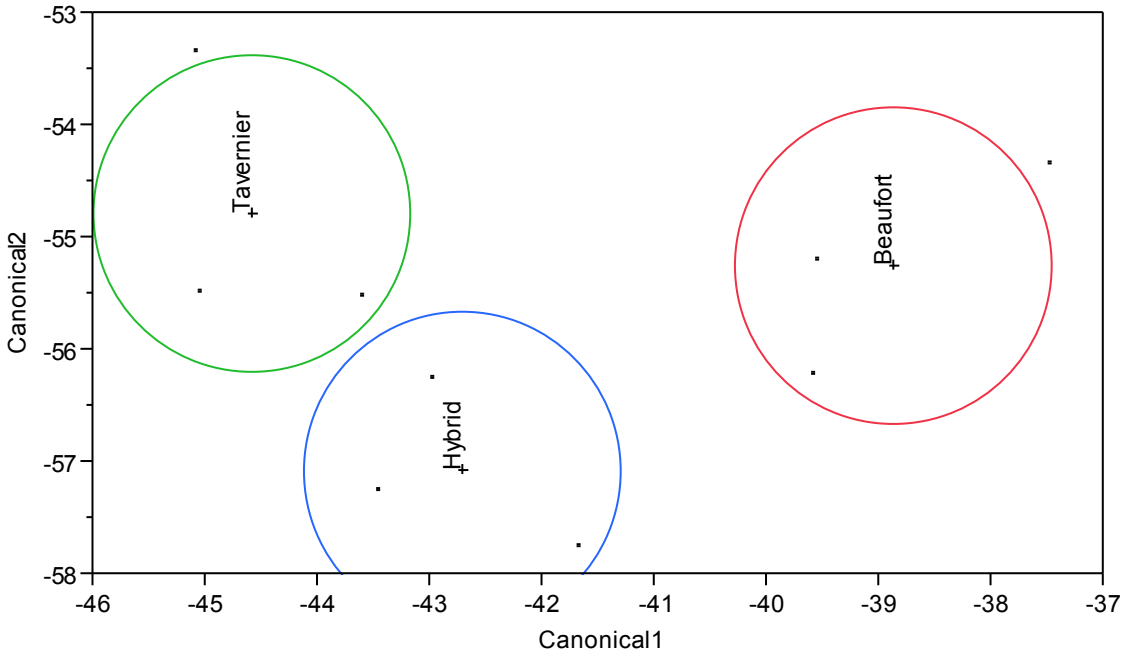

Figure 4-11: Discriminant analysis of F_2 crosses based on the same 7 characters as previous analyses. No crosses were misclassified.

4.4 Discussion

The data presented in this chapter highlight the similarity of color phenotype and morphology of the crosses to their respective field populations. The color phenotypes of F_1 and F_2 offspring of Beaufort, Tavernier and Hybrid crosses resembles the parental phenotypes. Cross morphology displays similar divergences with respect to the differences seen in the field populations. The information from the crosses indicates that there is a strong genetic component to the variability of color phenotype, patterning and morphology in *L. variegatus* across the geographic range.

4.4.1 Cross color phenotypes

The phenotypes listed in Table 4.1 and shown graphically in Figure 4.1 for both Beaufort and Tavernier crosses demonstrate that they fall within the norm of their respective field populations. The small sample size precludes a complete representation of the Keys phenotypes.

The most frequent color morph for the Beaufort crosses, as for the field caught urchins, is white (55.6%). In the Tavernier crosses the more limited phenotypic range reflects the restricted parental pool but the color morphs are representative of the Keys phenotypes. The hybrid Beaufort-Tavernier crosses have a mixed palette. Similarly, the data for combined spine and test color categories (Fig. 4.2) show that offspring colors fall

within the range of field colors. For Tavernier crosses the proportions of the categories is strikingly consistent across generations. The hybrid crosses have intermediate values.

The heritability of color phenotypes suggests that genes rather than environment have a major role in the frequency distribution of the color morphs. The common garden environment of the crosses allows for the genotypic variance within the two regional gene pools to be seen without the potential influence of environmental variables.

As discussed in Ch. 2 the ecological relevance of color variation in *L. variegatus* remains elusive. The dual pressures of predation and UV exposure may impose a selection gradient on the phenotypes that are found in a given region. White is overabundant in Beaufort compared to other regions and may be selected for given the shelterless, monochromatic habitat of the North Carolina coastal region. Predation pressure resulting in greater survival of the white phenotype compared to colored phenotypes would increase the frequency of the alleles for this trait with respect to the other alleles. The alleles for rare colors are not completely eliminated. This is evident in the Beaufort population where green and red are expressed in a certain number of offspring. Figure 4.2 demonstrates the increased frequency of the pink/red and green categories compared to the field population. The frequency histogram in Figure 4.4 reveals that red and green have a much greater presence in the crosses than in the field population (Fig. 2.8) where red is completely absent. This suggests that individuals carrying these alleles are being selected out of the population. Likewise, the rarity of the white morph from other regions suggests selection against it.

The crosses establish that in *L. variegatus* the white phenotype is a dominant autosomal trait. Observed patterns of segregation for crosses with white, purple and green urchins indicate a dominance/recessive pattern for the three colors: white dominant to both purple and green and green and purple are co-expressed. Tables 4.5 and 4.6 show that offspring phenotypes conform to expected Mendelian ratios (3:1, 1:1) for matings involving dominant heterozygous whites and a recessive purple. Moreover, two crosses, 35 and 37, strongly support the homozygous dominant state of the white parent over the purple and green parent respectively. All F_1 offspring of both crosses are white. In the subsequent F_2 generation for cross 35, the purple phenotype returns with a 26:5 ratio of white to purple offspring. The ratio does not deviate significantly from the expected Mendelian ratio of 3:1 ($\chi 2 = 1.58$, df = 1, NS with Yates' correction) confirming the dominance of white over purple (Table 4.10). The F_2 generation for cross 37 reveals 27:11 white/green offspring ratio again conforming to the expected 3:1 ratio ($\chi 2 = 0.59$, df= 1, NS with Yates' correction), for white dominant to green. In the one full purple cross all offspring (31) were the recessive purple color. An F_2 generation also produced all purple offspring (15) supporting the classification of purple as a recessive color.

These crosses demonstrate that the recessive purple and green phenotypes should appear in small frequencies in Beaufort. Figure 2.2 illustrates that purple and green occur (11.4% and 0.4% respectively) but in smaller frequencies than would be expected, especially for green. The indication is that selection against these phenotypes is strong in Beaufort.

Conversely, the diminished frequency of the white phenotype in regions other than Beaufort, despite its dominant heritability over green and purple, suggests that selection against this morph is occurring. The frequency of the white allele in the Gulf, Keys and Panama is low. The absence of the white phenotype in Brazil suggests that the population is either fixed for alleles other than white or that if white urchins are produced they are very quickly eliminated.

A similar pattern is seen in *Paracentrotus lividus*. In this urchin purple and green are the most common color phenotypes. White is very rarely found but like *L. variegatus* it is an autosomal dominant trait over purple and green [Louise, et al., 1993]. The factors responsible for its rarity in the population are not discussed but the authors attribute the dominance of the white phenotype to an epistatic relationship between the genes responsible for coloration although the number of genes is not specified. In their analysis the allele for the white phenotype acts to suppress pigment production. This is a reasonable conclusion given the fact that this occurs in many systems [Ennos, et al., 1983; Frost, et al., 1979; Haase, et al., 2009].

In flowers and well as animals, the white phenotype is indicative of the loss of pigment production through a variety of changes within the pigment biosynthetic pathway [Clegg, et al., 2000; Dooner, et al., 1991; Frost, et al., 1979; Haase, et al., 2009; Johansson, et al., 1992; Marklund, et al., 1998]. In flowers and some animals it is a recessive trait. For flowers such as the common morning glory *Ipomea purpurea*, the snapdragon *Antirrhinum majus* and legumes such as Mendel's pea plant the white

phenotype is a result of a mutation involving transposable elements that alter the DNA sequence of the enzymes at various stages of the anthocyanin synthesis pathway thus blocking pigment production [Clegg, et al., 2000; Hellens, et al., 2010]. The white phenotype for the Mexican axolotl is produced in a different manner: pigment in the chromatophores is produced normally but the recessive variant of the pigment deposition gene blocks its deposition in the skin resulting in a white (leucistic) phenotype [Frost, et al., 1979]. The animals are not albino since they produce the pigment which is seen in the dark eyes and in a small number of pigment-containing melanophores in the head region.

For other animals such as horses, mice, and pigs the white phenotype is due to a dominant autosomal gene that prevents pigment production to few or most areas of the body [Haase, et al., 2007; 2009; Johansson, et al., 1992; Marklund, et al., 1998]. This differs from albinism which is a recessive trait and is the complete absence of pigment production. The types of mutations that result in the absence of color differ between taxa (i.e. deletions, point mutations, frameshift mutations, chromosomal inversions) but involve disruptions in the transcription of the pigment coding gene [Haase, et al., 2007; Haase, et al., 2009; Marklund, et al., 1998]. The gene (KIT) has several alleles that code for the different mutations that give rise to the variety of phenotypes seen. In pigs the phenotype ranges from the dark wild type to white with a range of black spots or splotches [Johansson, et al., 1992]. Similarly, in horses coat color can range from fully

pigmented (black or chestnut with small areas of no pigment) to piebald to fully white [Haase, et al., 2009] depending on the mutation encoded in the alleles of the KIT gene.

Thus, the crosses in *L. variegatus* demonstrate that the white phenotype is a dominant autosomal trait as in the horse and pig example rather than the recessive trait common to flowers and the axolotl. As such, its production could involve similar mechanisms of disruption to the pigment production gene in the spines. The precise mechanism is impossible to identify because information on the biochemical synthesis of naphthoquinones (echinochrome A and various spinochromes) in urchins is unknown. However, if we assume that pigment production for naphthoquinones functions in a similar manner as for anthocyanins or melanins then a mutation causing the disruption of synthesis is plausible. A small number of alleles could encode for the different patterns—from fully white spines to mostly white with pigmented tips. Again, this would differ from the albino phenotype since white urchins can have very pigmented tests (e.g. white urchins in the Gulf).

The same mechanism may function in the tests and explain the occurrence of the patterning trait common in the Keys urchins. This is a dominant autosomal trait and separate from the spine pigment gene (Table 4.9) and may be an example of the spatial disruption of pigment production in the tests. This gene could function in a manner analogous to the (KIT) gene in horses whereby it has an unknown number of alleles that code for the different mutations giving rise to a fully pigmented or partially pigmented

test. Patterned tests have more than one variation (personal observation) so a series of mutations may help explain their formation.

Intimation of the mechanism responsible for the absence of pigment may come from urchin larvae. The biosynthesis of echinochrome pigment in urchin larval tissues is dependent on several pigment cell specific genes: transcription factor glial cells missing (gcm), the polyketide synthase gene cluster (pks-gc), three members of the flavin-containing monooxygenases multigene family (fmo) and a sulfotransferase (sult) [Calestani, et al., 2003]. In *S. purpuratus* larvae the expression of SpPks, SpSult and SpFmo in pigment cell precursors is positively regulated by SpGcm and both SpPks and SpFmo1 are required in the biosynthesis of echinochrome pigment [Calestani, et al., 2003]. Embryos, up to 72 hours post-fertilization, depleted of SpPKS and SpFMO1 enzymes were morphologically normal but lacked echinochrome in the pigment cells. Whether pigment production was permanently affected or could have been reconstituted as the embryo progressed into the larval phase is unknown. If these genes also operate in post-metamorphic urchins then disruption to one or more of them could potentially alter pigment expression in the spines and test rendering them pigment-less.

The dual coloration seen in many *L. variegatus* suggests that purple and green are co-dominant alleles. The field data for color phenotypes for Gulf and Brazil urchins, in particular, highlights the ubiquity of bicolored spines: pink-green, purple-green and red-green. The co-dominance of the alleles appears to have a strict spatial component: green is always proximal and pink, purple or red always distal. This suggests that when both

alleles are present they are expressed differently along the length of the spine. This could occur if green is generally not expressed distally unless homozygous. When heterozygous the second allele is expressed. This might explain why green is never distal to other colors. F_1 offspring in crosses 11 and 36 all have bicolored red-green and purple-green spines respectively, demonstrating the heritability of green, purple and red colors from their single colored parents. The crosses also demonstrate the strict spatial deposition of color. In all offspring green is proximal to purple and red. F_2 offspring for cross 36 reveal the return of single colored purple and green phenotypes although the expected 1:2:1 purple/purple-green/green ratio is not observed. Similarly, F_2 offspring for cross 11 have single colored red and green urchins. In *P. lividus* purple is dominant to green [Louise, et al., 1995]. Bicolored spines are not a factor and this may be the key difference in the co-expression of the alleles in *L. variegatus*.

The mechanism leading to the production of the different colors is obscure. The biochemical synthesis of naphthoquinones has not been categorized and therefore the mechanism underlying the production of green, purple or red colors is a matter of speculation. However, the synthesis pathway for anthocyanin and melanin pigments is well characterized and the identities of the structural and regulatory genes influencing pigment production are known and may provide some insight [Dooner, et al., 1991; Grotewold, 2006; Hearing, et al., 1991; Holton, et al., 1995]. In flowers different color phenotypes are the result of changes in the anthocyanin biosynthetic pathway. Purple and pink flowers in the morning glory are produced via a single mutation in the

anthocyanin biosynthetic pathway [Zufall, et al., 2003]. The mutation alters the gene that encodes one of the enzymes resulting in the failure to produce the required substrate in the purple cyanidin-based pathway. Instead, the substrate produced leads to the pink pelargonidin-based pathway [Zufall, et al., 2003]. If we assume that the difference between colors in urchins is due to a similar alteration in the pigment production pathway, then different mutations, encoded in the alleles for green, purple, red etc. could determine the pathway leading to the final product.

The variability in color phenotypes for *L. variegatus* seen in the crosses and the color distribution figure argues against a simple one-locus, two allele Mendelian model associated with the traditional pea plant example. It is likely that the system is similar to flowers such as the common morning glory *Ipomea purpurea* or the snapdragon *Antirrhinum majus* where color phenotype is composed of a few/several loci (e.g. loci for pigmentation, patterning, and intensity of color) each with a certain number of alleles that contribute to the final phenotype. In this respect, each trait behaves in a simple Mendelian fashion as seen in the crosses with pigmentation and patterning. In this fashion many phenotypes are produced but each is created from discrete entities. Colors can have a large range but they are discrete—purple doesn't grade into green or vice versa and they are spatially distinct. Patterning is a discrete feature—it's there or it's not. Spine and test colors are separate entities. Each of these features produces a set of individual color phenotypes as can be seen in Table 4.4. listing the color phenotypes of F_1 offspring for each of the crosses. The phenotypes are composed of separate spine and

test color as listed in 5 cross types in Table 4.10. The data demonstrate the variability within a cross type but also the discrete nature of the phenotypes.

Discrete color phenotypes are common in many marine invertebrates. Simple genetic models for color and pattern variability have been shown in tellinids, mussels, abalone, oysters and scallop [Adamkewicz, et al., 1988; Evans, et al., 2009; Innes, et al., 1977; Kobayashi, et al., 2004; Liu, et al., 2009; Luttikhuizen, et al., 2008]. The interaction of a few loci determining the phenotype has been proposed in marine invertebrates such as the intertidal gastropod Nucella emarginata [Palmer, 1984]. Shell color and shell banding pattern are heritable traits segregating independently. Banding appears to result from suppressed pigmentation in the shell with overall intensity of color due to a separate intensity gene [Palmer, 1985b]. In the bivalves *Macoma balthica* and *Argopecten irradians* color variability in is controlled by 4 alleles which display a linear hierarchy (e.g. red > orange > yellow > white in *M. baltica*) [Adamkewicz, et al., 1988; Luttikhuizen, et al., 2008; Winkler, et al., 2001]. Intensity of coloration is also present and segregates independently of color [Luttikhuizen, et al., 2008]. The structural and regulatory genes influencing pigment production leading to the different colors have not been elaborated.

The adaptive value of the various color morphs and banding patterns is attributed to ecological parameters, usually predation pressure and/or physiological tolerance [Berger, et al., 1997; Ekendahl, 1998; Palmer, 1985a]. Differential predation by crabs and birds in many species of intertidal and terrestrial snails maintain the color polymorphisms [Cain, et al., 1954; Ekendahl, 1998; Hoagland, 1977; Johannesson, et al.,

2002]. Physiological stress due to insolation and desiccation [Phifer-Rixey, et al., 2008], salinity [Berger, et al., 1997; Sokolova, et al., 2000] and wave exposure [Palmer, 1985b] are also correlated with differential survival of color morphs. However, in many instances the significance of color polymorphisms and banding patterns remains uncertain. The evolutionary significance of the types of phenotypic diversity seen in the marine intertidal is largely unknown. Given enough time divergence of phenotypes may portend the separation of populations. If separation proceeds long enough then the population can accrue enough genetic dissimilarity to eventually become different species.

4.4.2 Cross morphology

Data from cross morphology presents a more complex picture than for the color phenotypes. Mean values for 15 of the 18 traits listed in Table 4.12 are not significantly different among the 3 cross types. Only spine width and ambital spine length differ significantly. However, when the data is converted to ratios (Table 4.13) differences in test shape (H/D), lantern shape (L/W) and spine length in relation to test diameter are evident among the crosses.

The non-significance of many of the comparisons in mean trait values is attributable to the large variance within each of the crosses. This is seen clearly in Figures 4.7-4.9. The small number of crosses, especially for Tavernier (n = 5), makes

comparisons less robust. However, the figures clearly indicate that there are differences between the crosses, especially between Beaufort and Tavernier. For most traits, hybrid crosses fall somewhere in-between the two within site crosses. If we compare the relative difference between trait means for the crosses and the field samples the results are clear (Table 4.14). The data demonstrate that the differences are within the same order of magnitude for most of the trait ratios. This indicates that populations in Beaufort and the Keys are genetically different for these traits. Figures 4.7-4.9 demonstrate that despite the high variance within each of the crosses the difference in means is evident and the polarity of the differences in all traits except ambital spine length is identical to the field population. In most cases Beaufort has the largest values except for H/D ratio and spine length which are greater in Keys urchins.

Juvenile ambital spines in Tavernier crosses are longer than in Beaufort crosses which is contrary to that in the adult urchins. In this instance the direction of difference is opposite that of the field population. The reason for the discrepancy is not immediately obvious given that aboral spine length differences are identical in the field and cross comparison. But the greater girth of ambital spines in Beaufort field urchins suggests that ambital spine growth between the regions may differ. Having longer, more robust ambital spines may be important in anchoring Beaufort urchins to the substrate. Growth may need to proceed on both axes simultaneously such that length doesn't compromise spine strength. Tavernier crosses had more brittle spines, especially at the tips, than Beaufort crosses suggesting faster, less robust growth.

Similar heritable differences in spine morphology were observed in crosses of *A. punctulata* from Woods Hole versus the Gulf of Mexico [Marcus, 1980]. The longer spines of pure-bred Gulf crosses versus Woods Hole crosses indicated genetic differentiation of the populations. Hybrid *A. punctulata* crosses had intermediate spine lengths as do the hybrid crosses for *L. variegatus*.

Lantern weight as a function of test diameter differed considerably between field and cross comparison. The discrepancy may be related to lantern plasticity. The constant grazing of Beaufort field urchins on shells and sand may induce strengthening of the lantern skeletal matrix via ingestion and incorporation of a great quantity of calcium carbonate [Stevenson, et al., 1966; Weber, 1973]. The magnitude of the difference in lantern weight could reflect the difference in diet between the field populations and the crosses which were fed exclusively macroalgae (*Codium* spp., *Ulva lactuca*, *Gracilaria* sp., and *Dictyota* sp.). The effects of diet were not observed as differences in lantern size as has been shown in other urchin species [Black, et al., 1984; Edwards, et al., 1991; Levitan, 1991], as a result, lantern size in both Beaufort and Tavernier juveniles mirrored field populations. The difference in size between the two regions may reflect divergence in genetic variability.

The discriminant plot (Figure 4.10) shows the extent of the differentiation between crosses. Each cross type clusters together reinforcing similarity based on geographic origin. Comparison of Figure 4.10 with the top left panel of Figure 2.22 (canonical 1 on Y axis in this 3D representation) confirms the similarity of cross and field

morphology. The same traits used in the analysis of field populations differentiated the crosses, with only 1 hybrid cross misclassified. This gives the clearest indication that morphological differences are genetically based.

Genetic differences in spatially disjunct populations are maintained either through natural selection or stochastic processes such as genetic drift. The factors responsible for the differentiation in *L. variegatus* are not clear but could involve one or both processes. Natural selection on genotypes could involve similar factors outlined in Chapter 3. Hydrodynamic regime, water temperature and food resources could play a role in maintaining the regional differences by imposing a selection gradient that weeds out individuals outside the population mean. For example, in Beaufort the hydrodynamic conditions of Bogue Sound may select for individuals genetically predisposed to having flatter, thicker tests and more robust spines. This selection regime over generations will change the genotype frequencies such that these characteristics will predominate [Falconer, et al., 1996].

Although Keys and Panama were more similar to each other than to Beaufort or Gulf the field data on morphology do not support the previous subspecies classification since urchins in all 4 regions were distinct. This could be due to similarity of habitat in the Keys and Panama over the other regions. The heritability of the traits, as seen in crosses from 2 of the regions, upholds the differentiation of the regions.

In other marine invertebrates, geographic variation in morphology is shown to correspond to habitat level differences. For the intertidal snail *L. saxatilis* morphological

variability often occurs on a microgeographic scale (within meters) and reflects differential selection [Johannesson, et al., 1993]. Similar ecotypes are found in disjunct populations. Differences in shell morphology are also heritable as shown in common garden laboratory growth experiments. Similarities in form reflect geographic ancestry and not growth environment [Conde-Padín, et al., 2009] indicating a steep selection gradient from low to high intertidal. The causal mechanisms have been identified as predation from crabs and desiccation in the high intertidal and increased wave action in the lower intertidal. Analysis of polymorphic enzyme loci indicate greater differentiation between shell forms within sites than the same form across sites [Johannesson, et al., 1993]. So in the case of the intertidal snail *L. saxatilis* morphological variability corresponds to natural selection due to similar environmental pressures.

For the bay scallop *Argopecten irradians*, geographic variability was indicative of subspecies status. F_1 offspring of crosses from disjunct populations from Massachusetts to Texas demonstrated similar clustering according to geographic ancestry [Wilbur, et al., 1997]. The authors suggest the heritable differentiation as an indicator of population divergence in light of genetic data showing significant difference in RFLP analysis of mitochondrial genes. The genetic data suggested restricted gene flow among scallop populations from four sites from Massachusetts to Texas [Wilbur, et al., 1997]. The functional significance of the differentiation was not clear and the authors acknowledge that divergence may have arisen through isolation and drift rather than selection.

The underlying question regarding geographic variation in animals is whether the variation is ecologically relevant. In some plants and animals this is known (i.e. flower color and pollinator preference, shell size and predation). The question for *L. variegatus* is whether the phenotypic and morphological differences represent ecologically relevant traits. If they do then are the changes adaptations? If yes, could these adaptations become defining characteristics leading to population divergence and potentially to speciation? These are questions that remain unresolved.

5. Genetic versus phenotypic variability

5.1 Introduction

The exchange of genes between populations (gene flow), genetic drift and natural selection can have a large influence on genetic and phenotypic differentiation at the population level [Endler, 1988; Falconer, et al., 1996; Hartl, et al., 2007; Schluter, 2000]. Gene flow has a homogenizing effect on allele frequencies and will eliminate genetic differentiation between populations. In contrast, genetic drift and natural selection will increase differentiation even if there are no obvious extrinsic barriers to gene flow. Drift occurs through stochastic fluctuations in gene frequencies whereas, selection proceeds through the differential survival and reproduction of individuals within the population. The large geographic ranges and high dispersal capabilities of many marine organisms have been interpreted as allowing extensive gene flow that links populations separated by hundreds or thousands of kilometers into one large genetic population. This is especially true for marine invertebrates with planktonic larval phases.

However, Knowlton's seminal papers highlighted the ubiquitous nature of sibling species among all major marine groups [Knowlton, 1993, 2000]. Knowlton [1993] contended that the scientific community had accepted without question the view that the broad geographic ranges of many species were the result of widespread dispersal in

the vast, borderless expanse of the ocean. What were thought to be widely dispersed and often morphologically similar populations of a single species have been shown to be sibling or cryptic species [Bavestrello, et al., 1992; Palumbi, et al., 1991]. The defining features of sibling species is their lack of morphological differentiation and their broad habitat and geographic distributions [Knowlton, 1993, 2000].

The view of vast panmictic populations with little genetic structure across hundreds of kilometers was examined in several marine invertebrate taxa with a planktonic larval phase. Some species in the Indo-Pacific such as giant clams [Benzie & Williams, 1992] and sea stars [Benzie & Stoddart, 1992] showed little genetic differentiation over thousands of kilometers confirming the assumptions of low levels of spatial variation among populations spread over large distances. Likewise, different echinoderms showed little genetic structure over hundreds of kilometers along the US Pacific coast [Addison, et al., 2004; Harley, et al., 2006; Moberg, et al., 2000; Palumbi, et al., 1990]. The lack of genetic structure was thought to be a consequence of a lack of obvious barriers to larval dispersal thus allowing the larvae to disperse widely connecting disjunct populations into a single large gene pool [Hartl, et al., 2007].

However, with the advent of powerful molecular techniques there is growing evidence that genetic structure is common in the marine environment, even in species with extensive geographic ranges and high dispersal ability. In the Indo-Pacific, echinoderm species such as *Linkia laevigata*, *Centrostephanus rodgersii*, *Echinometra spp.* and *Acanthaster planci* have more structure than was previously believed [Banks, et al., 2007; Benzie, 1999; Palumbi, et al., 1997; Williams, 2000; Williams, et al., 1997]. In both *L.*

laevigata and *A. planci* there was a marked genetic discontinuity in populations between the Indian and Pacific oceans [Benzie, 1999; Williams, et al., 1997] and genetic divergence between the two ocean basins suggested isolation by distance. For the sea urchin *C. rodgersii*, genetic differentiation between Australia and New Zealand was low but along the southeastern Australian coast fine-scale genetic structure was detected [Banks, et al., 2007]. Sea surface temperatures and the East Australia Current were associated with the fine-scale structure observed along the southeastern Australian coast.

Similar patterns of genetic differentiation have been noted in echinoderm species common to the Mediterranean and eastern Atlantic coast. Both the sea urchin *Paracentrotus lividus* and the brittle star *Amphipholis squamata* show a sharp break between populations in the Atlantic and the Mediterranean basin [Calderón, et al., 2008; Duran, et al., 2004; Le Gac, et al., 2004]. However, *P. lividus* shows a lack of genetic differentiation between populations within each ocean basin [Calderón, et al., 2008], whereas, for *A. squamata* genetic structure is present [Boissin, et al., 2008; Le Gac, et al., 2004]. This is likely due to the difference in dispersal capabilities. *P. lividus* has planktotrophic larvae whereas, *A. squamata* broods its offspring [Boudouresque, et al., 2001; Deheyn, et al., 1999].

The relationship between genetic diversity and phenotypic diversity, particularly using color polymorphisms has been examined. In some cases no concordance between color variability and genetic differentiation was found [Sponer, et al., 2001]. However, for some species color variability was associated with genetic differentiation. In the colonial ascidian *Pseudodistoma crucigaster* genetic divergence between 2 clades

corresponded to separate color phenotypes [Tarjuelo, et al., 2004]. Significant differences in allelic frequencies were observed between the color morphs of the sea urchin *Paracentrotus gaimiardi*, prompting the authors to suggest that some sort of assortative mating between the color morphs was occurring [Calderon, et al., 2010]. Often, the underlying ecological function of the color polymorphism is unknown making it difficult to reconcile genetic and phenotypic discrepancies.

Recent genetic analysis by Zigler and Lessios [2004] of the mitochondrial COI gene within the genus *Lytechinus* examined the genetic diversity between the species. Their analysis separated the Pacific species from the Atlantic species as well as separating the Atlantic *L. variegatus* into two separate clades: *L. variegatus variegatus* from the Florida Keys, Caribbean and Bermuda, and *L. variegatus carolinus* from North Carolina and the Gulf of Mexico. However, their analysis did not examine the relationship of color phenotype in *L. variegatus* to the mitochondrial genealogy except to note that a few individuals thought to be phenotypically one subspecies were genetically related to the other subspecies: 6 urchins collected near Miami with *L. v. variegatus* phenotype fell within the *L. v. carolinus* clade, conversely one individual with a *L. v. carolinus* phenotype collected in Tampa had a haplotype that clustered with *L. v. variegatus*. The classification used by Zigler and Lessios [2004] of color phenotype taken from Serafy [1973] is not comprehensive and as demonstrated in Chapter 2, may offer an incomplete association of haplotype, phenotype and subspecies.

In this chapter I examine the genetic variability of *L. variegatus* across 3 regions and the relationship to color phenotype. I recreate the phylogeny presented by Zigler

and Lessios [2004] based on the mitochondrial COI gene and include data on the color phenotype of individuals sampled from Beaufort NC, the northern Gulf of Mexico and the Florida Keys. The aim is to assess the degree of genetic divergence between and within the regions and whether there is any congruence between the phylogenetic COI data and color phenotypes.

5.2 Materials and Methods

A total of 109 *Lytechinus variegatus* individuals were sampled from three regions: Beaufort, North Carolina (Bf), the northern Gulf of Mexico (Gf) and the Florida Keys (Keys). Samples were collected in relatively shallow waters (1-5 m depth) by snorkeling, diving or dredging (Bf). All but 32 samples (16 Bf and 16 Keys) were photographed immediately after collection to establish and record the color phenotype. For 8 Bf and 8 Keys urchins, color phenotype was not recorded and cannot be determined post hoc. Twelve spines were taken from each individual at the ambital section, taking care to remove the spine from the test at the base to preserve the tissue-rich collar, and preserved in 95% ethanol.

Tissue was taken from the spines by cutting off approximately 2-3 mm from the proximal end to only include the collar. DNA was isolated using the DNAeasy Blood and Tissue Kit from Qiagen according to the manufacturer's instructions. The mitochondrial COI gene was amplified using the Polymerase Chain Reaction (PCR) with the primer COIa (5'-AGTATAAGCGTCTGGGTAGTC-3') and COIf (5'-

CCTGCACGGAGGAGGAGAYCC-3') as described by Zigler and Lessios [2004]. Sequencing was performed using an ABI 3730xL DNA analyzer at the Duke Marine Lab. The sequences were edited using the program CodonCode Aligner. Sequence data was obtained on a 536-bp fragment of COI for 102 specimens of *L. variegatus* (38 individuals from Beaufort, 39 from Florida and 25 from the Gulf). 7 specimens failed to amplify and were excluded from the analyses. Previously published mitochondrial COI sequences of congeneric *Lytechinus* species were used as comparisons (Pacific *L. pictus* and Atlantic *L. williamsi*; GenBank accession numbers: AY183283, AY183280. *L. anamesus* was not included since it was indistinguishable from *L. pictus*, or *L. semituberculatus* and *L. panamensis* as they are Pacific species). Sequences of outgroups (*Toxopneustes roseus*, and *Tripneustes ventricosus;* GenBank accession numbers: AY183177, AY205524) were used as outgroups to root the tree. Sequences from *L. variegatus* and *L. williamsi* comprised the dataset used for the phylogenetic analyses and to construct a haplotype network. The sequences were run in DNAsp [Rozas, et al., 2003] to identify the number of unique and shared haplotypes. Shared haplotypes were collapsed and represented by one individual for phylogenetic analyses.

Phylogenetic analyses were conducted with maximum parsimony (MP), maximum likelihood (ML) and neighbor joining (NJ) methods. MEGA version 5 [Tamura, et al., 2011] was used for MP heuristic searches and NJ analyses and maximum likelihood (ML) heuristic searches were performed in both MEGA version 5 and in GARLI 0.96 [Zwickl, 2006]. Bootstrap replicates of 500 were performed for MP and NJ and 100 replicates for ML. For ML and NJ analyses, the best model of nucleotide

substitution was selected using jModelTest 0.1.1 [Posada, et al., 1998]. The best fit Akaike Information Criterion (AIC) model was TrN + I (–lnL = 2073.9872) with different equilibrium base frequencies (A = 0.2898, C = 0.2494, G = 0.1647, T = 0.2962). The proportion of invariable sites was 0.6474. GARLI ML trees were analyzed with input form the AIC model. TCS software v. [Clement, et al., 2000] with a 95% connection limit between haplotypes was used to build an unrooted parsimony network of haplotypes to visualize the relationship of haplotype to region of origin and to color phenotype for the 102 samples of *L. variegatus* plus *L. williamsi*.

Population structure analyses to examine genetic diversity within and between clades were conducted in Arlequin version 3.5.1.2 [Excoffier, et al., 2010]. Haplotype and nucleotide diversity were calculated as was Tajima's D to test for selection. Analysis of molecular variance (AMOVA) was performed to identify significant genetic differentiation between clades and populations within clades. F-statistics were calculated and F_{ST} values were determined for the clades (Clade 1 and Clade 2) identified in the phylogenetic analysis and within populations (regions) of Clade 2. Samples from Clade 1 were restricted to 1 site (Tavernier Key) and therefore within-clade F_{ST} values could not be determined.

5.3 Results

56 different haplotypes were identified from the COI gene among 102 *L. variegatus* sequences plus 1 *L. williamsi*. Haplotypes 1-14 were found exclusively in individuals collected from the Florida Keys (Keys: Clade 1), haplotypes 15-56 were

found in urchins from all 3 regions (Bf-Gf-Keys: Clade 2). *L. williamsi* clustered in Clade 1. Most haplotypes were unique and found in a single urchin, however, 13 haplotypes were shared, 10 of which were shared among regions (Fig. 5.1). Two haplotypes (7 and 54) were shared by 13 individuals each. Haplotype 7 in Clade 1 was restricted to Keys urchins but haplotype 54 in Clade 2 was shared by urchins from all 3 regions—8 Beaufort, 4 Gulf and 1 Keys (Fig. 5.1).

Haplotype diversity (Hd) indicated high diversity within regions of Clade 2 thus diminishing any genetic differences between Beaufort, Gulf and Keys urchins (Table 5.1). Low nucleotide diversity (π) means that most of the haplotypes were closely related, differing by one nucleotide change. This is demonstrated by the star-shaped pattern of having few very common haplotypes with many related haplotypes differing by one or few nucleotides radiating from them (Fig. 5.2). Tajima's D values were negative for all populations but only significant ($P < 0.05$) for Clade 1 and the Beaufort population in Clade 2 (Table 5.1).

Table 5-1: COI summary statistics from Clade 1 (Keys) and Clade 2 (Beaufort, Gulf and Keys). N = number of individuals, H = number of haplotypes, Hd = haplotype diversity (standard deviation), π = nucleotide diversity (standard deviation), D = Tajima's D with significance $P < 0.05$ indicated in bold.

		N	H	Hd	π	D
Clade 1	Keys	26	13	0.76 (0.091)	0.004 (0.001)	**-2.218**
Clade 2	Beaufort	38	23	0.94 (0.026)	0.007 (0.001)	**-1.989**
	Gulf	25	19	0.97 (0.024)	0.007 (0.001)	-1.143
	Keys	13	12	0.99 (0.035)	0.008 (0.001)	-1.697

The pairwise F_{ST} value comparing Clade 1 and Clade 2 (0.89, $P < 0.001$) was highly significant showing strong genetic differentiation between *L. v. variegatus* and *L. v. carolinus*. Pairwise F_{ST} values within Clade 2 were very low (Table 5.2) indicating that urchins within the 3 regions in this clade were genetically indistinct. The analyses indicated two distinct mitochondrial lineages consistent with the phylogenetic analysis—one comprising the *L. v. variegatus* subspecies and another comprising the *L. v. carolinus* subspecies with the 13 individuals phenotypically resembling *L. v. variegatus*. Analysis of molecular variance (AMOVA) within Clade 2 indicated no variation within this mitochondrial lineage (Table 5.3) either by designated subspecies (Beaufort/Gulf *carolinus* versus Keys *variegatus*, 0.85%) or between populations within the *carolinus* subspecies (Beaufort versus Gulf, -0.73%, $P = 0.53$). Most of the variation in haplotype diversity occurred within the 3 regions (> 99%, $P < 0.53$) (Table 5.3).

Table 5-2: Pairwise F_{ST} values of genetic differentiation between Beaufort, Gulf and Keys populations in Clade 2 reported below the diagonal. The low values indicate that the populations in this clade are genetically indistinct. Negative values treated as zeros.

	Bf	Gf	Keys
Beaufort	0		
Gulf	0.000	0	
Keys	-0.006	-0.002	0

Table 5-3: Hierarchical AMOVA comparing genetic variation of Beaufort, Gulf and Keys regions in Clade 2. All F statistics are non significant.

Source of variation	df	variance components	% variation	F statistic	*P* value
Among groups (Bf-Gf vs Keys)	1	0.0169 Va	0.85	0.001	0.32
Among pops in groups (Bf vs Gf)	1	-0.0144 Vb	-0.73	-0.007	0.53
Within populations (Bf vs Gf vs Keys)	73	1.980 Vc	99.88	0.009	0.53

Phylogenetic analysis of the mtDNA COI gene revealed two well supported clades for *L. variegatus* corresponding to *L. variegatus variegatus* (Clade 1) and *L. variegatus carolinus* (Clade 2) (Fig. 5.1). Similar topologies were obtained from the Maximum Likelihood (ML) analysis in GARLI and Maximum Parsimony (MP), Maximum Likelihood (ML) and the Neighbor Joining (NJ) analyses in MEGA. Well supported (> 90) MP and ML bootstrap values were obtained between outgroups and the genus *Lytechinus*, between the Pacific and Atlantic clades and between Clades 1 & 2. The sympatric congener *L. williamsi* nested within the *L. v. variegatus* clade in all analyses. These results are consistent with the results of Zigler and Lessios [2004].

Clade 1 includes 13 haplotypes representing 26 individuals from the Keys whereas Clade 2 includes 42 haplotypes representing 76 individuals from Beaufort (38), Gulf (25) and the Keys (13). Haplotypes from all three regions are intermingled throughout the clade, showing no association between haplotype relatedness and region of origin. Seven of the 13 haplotypes from Keys urchins are shared with Beaufort and/or

Gulf urchins and six are unique. In both clades, the number of unique haplotypes is greater than the number of shared haplotypes.

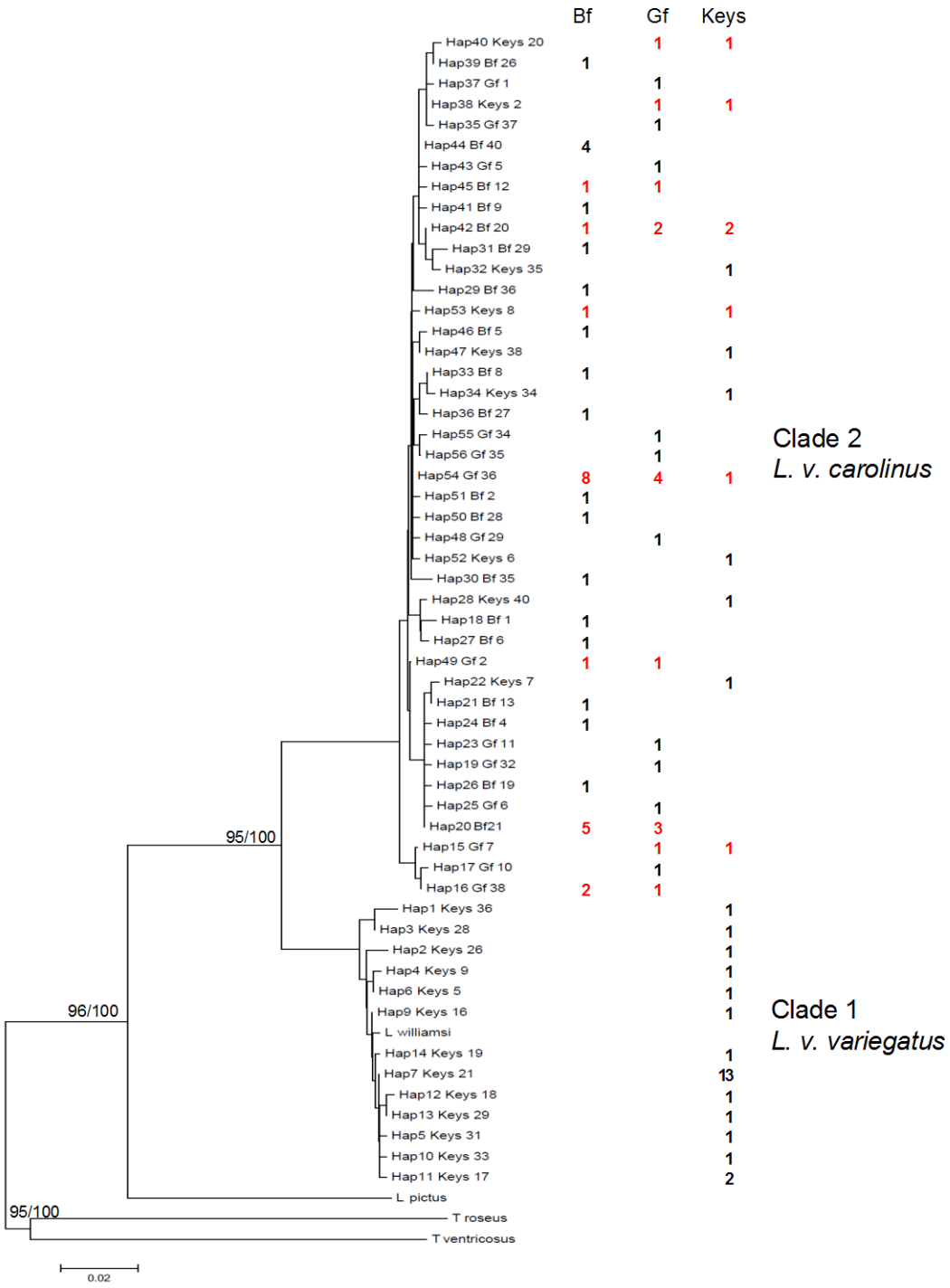

Figure 5-1: Neighbor-joining tree of *L. variegatus* based on 536 bp of the COI mitochondrial gene. Bootstrap values for Maximum Likelihood/Maximum Parsimony between outgroups and the genus *Lytechinus*, between the Pacific and Atlantic clades and between Clades 1 & 2 (100 ML and 500 MP replicates). Haplotypes shared between regions (Bf, Gf and Keys) are indicated in red.

The parsimony network of haplotypes built in TCS is congruent with the phylogenetic tree revealing two distinct unconnected clades: Clade 1 *L. v. variegatus* and Clade 2 *L. v. carolinus* (Fig. 5.2). Clade 1 is strictly comprised of urchins from the Keys, corresponding to the subspecies *L. v. variegatus*. In contrast, Clade 2 is comprised of urchins from Beaufort and Gulf, corresponding to the subspecies *L. v. carolinus* with the addition of 13 Keys urchins phenotypically resembling the subspecies *L. v. variegatus*.

The relationship between the haplotypes and the regions of origin reveals no evident structure in Clade 2 with haplotypes from Beaufort, Gulf and Keys urchins intermingled throughout the clade. Figure 5.2 highlights the regional composition of shared haplotypes.

The relationship between genetic diversity and color phenotype is illustrated in the identical haplotype network in Figure 5.3. Color also shows a lack of structure with respect to haplotypes. Color phenotypes are intermingled throughout both clades. The same color can be found on urchins from different regions. All shared haplotypes are a mix of more than one color phenotype.

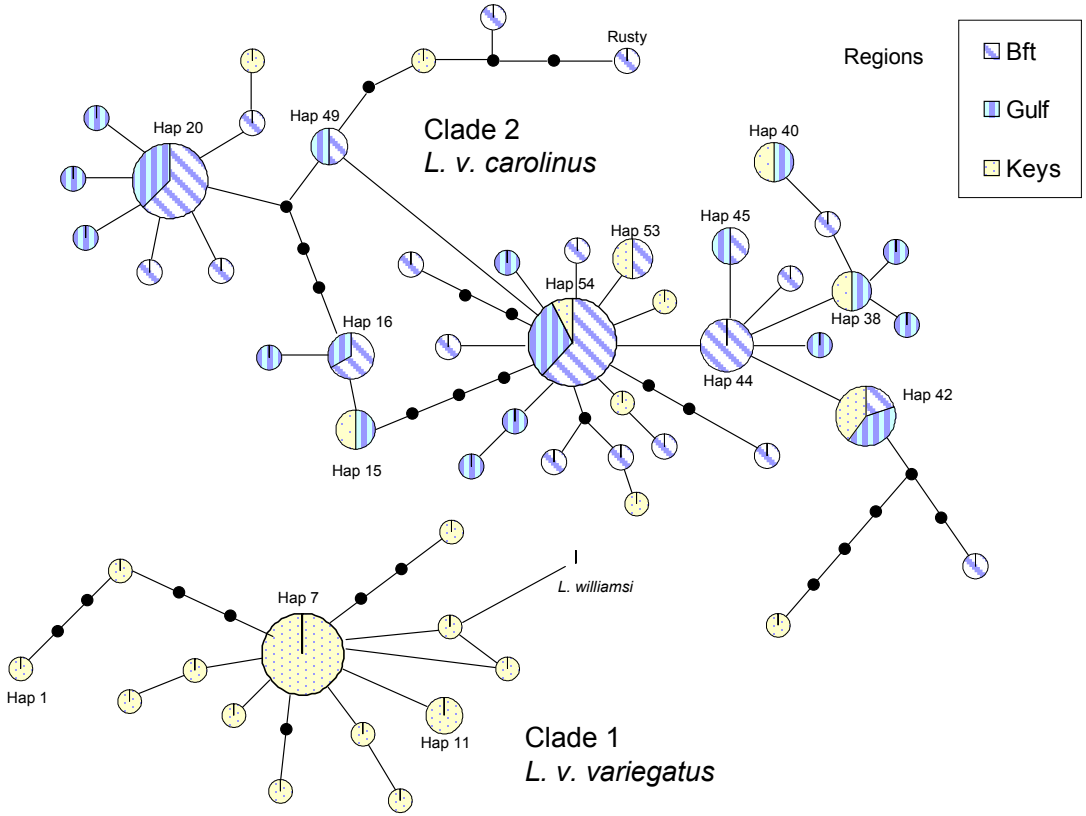

Figure 5-2: Parsimony haplotype networks of Clade 1 and Clade 2, constructed with the TCS program (Clement et al. 2000). The small black circles indicate hypothetical haplotypes not observed in the data. Each small circle represents a unique haplotype (1 individual). Larger circles are drawn in proportion to the number of individual having that haplotype and the colors represent the region of origin. Lines indicate single sequence differences (mutations) joining haplotypes or hypothetical haplotypes.

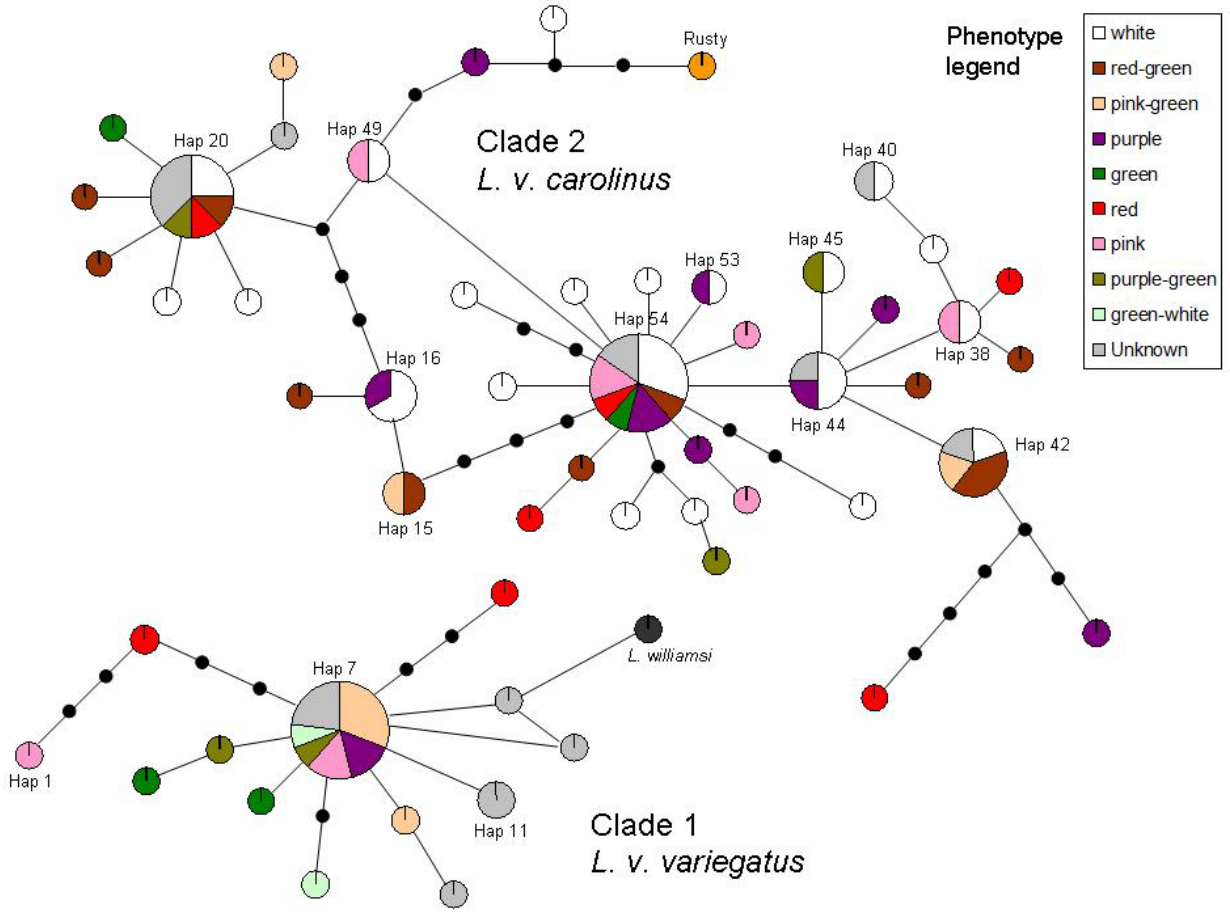

Figure 5-3: The same parsimony haplotype network as in Figure 5.1 but with the color phenotype represented. Grey indicates urchins of unknown phenotype. The size of the colored wedges in the shared haplotypes is proportional to the number of urchins of that phenotype. "Rusty" indicates the unusual rusty-colored urchin found offshore Beaufort.

5.4 Discussion

The data presented in this chapter illustrates the level of genetic diversity within *L. variegatus* from the 3 regions sampled. Population genetic indices and phylogenetic data demonstrate significant divergence between the subspecies *L. v. variegatus* and *L. v. carolinus*, upholding the separation proposed by Mortensen [1943] and Serafy [1973]. However, the inclusion of Keys urchins phenotypically resembling *L. v. variegatus* into the *L. v. carolinus* clade suggests that the Keys region is either a hybrid zone or the clades represent cryptic species. Genetic differentiation does not concord with phenotypic and morphological differentiation.

5.4.1 Genetic diversity and population structure

Population genetic diversity indices demonstrate the amount of genetic diversity within and between regions and confirmed the historical separation of *L. v. variegatus* from *L. v. carolinus* urchins (F_{ST} 0.89, $P < 0.001$). However, the presence of a sizable number of Keys urchins within the *L. v. carolinus* clade (Clade 2) complicates the matter of subspecies identification based on phenotype. Pairwise genetic distance F_{ST} values of 0 in Clade 2 between the three regions indicate no genetic structure. Moreover, the level of haplotypic diversity (Hd) within each of the 3 regions—Beaufort, Gulf and Keys—is high indicating that the proportion of genetic variability is greater within region than between regions (Table 5.3 A). The lack of genetic differentiation between regions within

Clade 2 (Tables 5.1 and 5.2) strongly suggests a single population of *L. v. carolinus* that is continuously distributed from Beaufort to the Gulf.

Tajima's D values were negative in both Clade 1 and Clade 2 but were significant only for Clade 1 and the Beaufort population in Clade 2. Negative values signify a departure from the hypothesis of equilibrium and selective neutrality implying that the frequency of polymorphic variants is unequal and may result from recent population bottlenecks or selection against deleterious alleles [Hartl, et al., 2007]. The significant negative Tajima's D for Beaufort and Clade 1 populations may be due to the recurrent cycles of population expansion and contraction that are characteristic of many echinoderm populations in general [Lessios, et al., 1984; Scheibling, et al., 1984; Uthicke, et al., 2009] and *L. variegatus* populations in particular [Beddingfield, et al., 1994; Camp, et al., 1973; Macia, et al., 1999; Moore, et al., 1963; Rose, et al., 1999]. The recorded disappearance of urchins in Beaufort in 2007 and 2010 (pers. obs.), in Tampa in 2007 (Rittschof, pers. comm.) and in St. Joseph Bay in 1994 [Beddingfield, et al., 1994] combined with population explosions in Pepperfish Key on the Gulf coast of Florida [Camp, et al., 1973] and in Outer Florida Bay [Macia, et al., 1999; Rose, et al., 1999] demonstrate the wide population fluctuations that may alter gene frequency leading to an excess of rare alleles and a negative D value [Hartl, et al., 2007]. The same process of population extinctions followed by rapid expansions could explain the difference in color morphs across the regions if it alters the frequency of the color genes such that some alleles are lost and others become overrepresented.

5.4.2 Phylogenetic structure

The separation of Clade 1 and Clade 2 in both the phylogenetic tree and the parsimony haplotype network mirrors results obtained by Zigler and Lessios [2004]. The high F_{ST} value (0.89) between Clade 1 and Clade 2 suggests differentiation levels on the order of separate species [e.g., Johnson, et al., 2006], thus extending the level of differentiation reported since Mortensen [1943]. Values greater than 0.25 generally indicate very great levels of genetic differentiation [Hartl, et al., 2007]. High F_{ST} values have recently been found in a number of other echinoderms, suggesting a greater level of genetic differentiation among geographically widespread species. For example, previously assumed panmictic species *Astrotoma agassizii*, a brittle star found in the Southern Ocean and Antarctica, and the Antarctic crinoid *Promachocrinus kerguelensis* were shown to be genetically discontinuous and composed of several separate lineages [Hunter, et al., 2008; Wilson, et al., 2007]. F_{ST} for among clade comparisons for *A. agassizii* was 0.84 and for *P. kerguelensis* ranged from 0.188–0.42. Likewise, the crown-of-thorns starfish *Acanthaster planci*, previously considered a single taxonomic unit spanning the Indo-Pacific, is now considered a species complex with 4 distinct lineages [Vogler, et al., 2008].

The phylogenetic split between Beaufort/Gulf populations into the *L. v. carolinus* clade and Keys populations into the *L. v. variegatus* clade corresponds to a phylogeographic break midway along both the Florida Atlantic (Cape Canaveral) and

Gulf coasts (Naples) [Avise, 1992; 2000], splitting Florida into two separate biogeographic zones. These separation points coincide with a biogeographical/ecological transition zone with warm temperate sea water above and subtropical/tropical temperatures below the zone [Avise, 2000]. Genetic discontinuities have been reported for a number of taxa whose ranges extend from the Atlantic to the Gulf of Mexico [Avise, 2000]. In many cases, the break at approximately the latitude of Cape Canaveral constitutes the point of greatest genetic divergence separating the taxa into Atlantic and Gulf species.

The presence of Keys urchins in Clade 2 and the complete lack genetic differentiation within the clade is unclear but may have several explanations. One possibility is that hybridization between *L. v. variegatus* and *L. v. carolinus* occurs but cross fertilization occurs preferentially in one direction over the other. Gametic incompatibility between sympatric and allopatric urchin species has been noted before, especially in the genus *Echinometra* [Lessios, et al., 1990; McCartney, et al., 2002]. *Echinometra* has two sympatric Caribbean species, *E. lucunter* and *E. viridis* and an eastern Pacific species *E. vanbrunti*. The eggs of *E. lucunter* have a much stronger block against cross-species fertilization than the other two species and consequently cross fertilization occurs in only one direction with respect to *E. lucunter*. The other two species have equal rates of cross fertilization of each others eggs and *E. lucunter* sperm can fertilize the eggs of the other two species. However, this is not sustained in the case of *L. v. variegatus* and *L. v. carolinus* since experiments demonstrate no significant

difference in sperm preference in competitive fertilization experiments [Zigler, et al., 2004], and crosses between Beaufort and Keys urchins, in both directions (Ch 4), demonstrate that F_1 hybrids are produced and are viable and fertile. However, it is not known whether Keys urchins used in the crosses cluster in Clade 1 or Clade 2. If the urchins cluster in Clade 2 then the production of F_1 offspring is easily explained. If they cluster in Clade 1, then the ease of fertilization between the clades signals that a block against cross-clade fertilization is not present. The absence of a block does not indicate a lack of genetic differentiation as no such block exists between *L. variegatus* and the Pacific species *L. pictus* [Minor, et al., 1991].

The presence of Keys urchins in Clade 2 may be the result of introgression of mtDNA genes from the two clades with the Florida Keys as the zone of contact. Gene flow between the Atlantic and Gulf populations of *L. v. carolinus* around the Florida peninsula may allow larvae to recruit into the habitat of *L. v. variegatus* and become part of the population. Lack of genetic incompatibility in fertilization, as mentioned previously, may allow for hybrids. The hybrids could carry the mitochondrial DNA signature of *L. v. carolinus* or *L. v. variegatus* depending on the maternal parent. Thus there could be urchins of *L. v. variegatus* phenotype with *L. v. carolinus* mitochondrial genotype (Keys urchins in Clade 2) or potentially *L. v. carolinus* phenotype with *L. v. variegatus* genotype (not sampled). The other two possibilities would not differ from their respective subspecies. Given that the range of Gulf *carolinus* urchins extends to the Gulf side of the Keys in Florida Bay the possibility for hybridization is high. The absence

of Beaufort and Gulf haplotypes in Clade 1 may be due to insufficient sampling. Increasing the number of sampling sites in the Keys may uncover the *carolinus* phenotype/*variegatus* genotype hybrids. Alternatively, introgression may not be happening since it is unlikely to occur in only one direction. This would imply that the clades are separate species and the presence of Clade 2 in the Keys suggests a range expansion. Clade 1 and Clade 2 likely diverged 2-3 million years ago in allopatry [Palumbi, et al., 2005], with the subsequent range expansion of Clade 2 into southern Florida.

The difficulty of distinguishing Clade 1 and Clade 2 Keys urchins phenotypically suggests the urchins are in fact cryptic species. Phenotypic indistinguishability is a hallmark of cryptic species [Knowlton, 1993]. This point was explored in great detail for marine organisms by Knowlton [1993, 2000] to demonstrate that "excessive lumping rather than excessive splitting, characterizes the current systematic situation in many groups." Knowlton [2000] asserts that sympatric cryptic species can be identified by characteristic differences "in ecology or life history, and can, in retrospect, be identified by subtle differences in morphology or color pattern."

5.4.3 Relationship between genetic and phenotypic structure

Analysis of the mitochondrial COI gene in this chapter does not concord with the phenotypic and morphological data outlined in chapters 2 and 3. Although the absence of patterning and the reduction in the overall spectrum of color phenotypes in the

northern populations (Ch 2) at first glance seems to be consistent with the split in mitochondrial COI haplotypes, placing them within the *L. v. carolinus* subspecies. However, as I've shown color patterns vary across all regions, those within the subspecies *carolinus* (Beaufort and Gulf) and those within the subspecies *variegatus* (Keys, Panama and Brazil). This suggests that color patterns are influenced on a more proximate scale, by environmental/habitat factors. Moreover, in the Florida Keys the zone of contact between the two clades, pockets of phenotypically *L. v. carolinus* are found in areas predominated by the other subspecies *L. v. variegatus* (Brian Keller, Florida Keys National Marine Sanctuary and Ken Nedimyer, Sea Life Florida, personal comm.). This suggests that the subspecies are easily identifiable and separate entities. Genetic data complicate this picture since urchins from Beaufort, Gulf and Keys regions in Clade 2 are phenotypically very different but are genetically indistinguishable. This means that Keys urchins in Clade 2 and Keys urchins in Clade 1 would not be recognized as genetically divergent based on their phenotype and would both be classified as the *variegatus* subspecies.

The haplotype network in Fig. 5.3 shows no structure with regards to color phenotype. Many of the color morphs are found in both clades and therefore not limited to one region (Ch 2) and shared haplotypes are a mix of more than one color phenotype. This is especially true for phenotypes pink, purple, pink-green, green and red. White is rare outside of Beaufort and its low frequency in the Keys (12.5%) could account for its absence in Clade 1. Despite the low frequency of white and green phenotypes in many

regions, they may reflect the ancestral phenotype of the genus *Lytechinus*. White is common to *L. pictus (L. anamesus)* in the Pacific and *L. williamsi* in the Caribbean, whereas, green is common to *L. semituberculatus (L. panamensis)* also in the Pacific as well as *L. williamsi*. The reds and purples may be derived phenotypes in *L. variegatus* after the genus split into Pacific and Atlantic clades. The greater variability in color phenotypes in *L. variegatus* in comparison to other *Lytechinus* species may be due to a higher mutation rate in pigment genes that is sustained by positive selection [Schluter, 2000].

The association of color and genetic differentiation has been examined in disparate taxa with various levels of concordance. In echinoid species such as the cosmopolitan ophiuroid *Amphipholis squamata* color and genetic differentiation are not concordant [Sponer, et al., 2001]. The same color varieties are found in different clades and in different locations. For the Indo-Pacific asteroid *Linkia laevigata* there is a consistent genetic and phenotypic pattern: the phenotypic divergence of populations in the Pacific Ocean/Western Australia from those of the Indian Ocean is broadly consistent with the genetic split [Williams, et al., 1998]. In the colonial ascidian *Pseudodistoma crucigaster*, high levels of genetic divergence of sympatric color morphs suggests genetic isolation leading to speciation [Tarjuelo, et al., 2004].

For other organisms, the adaptive significance of color polymorphism helps resolve the discrepancy between genetic and geographic variation. In the pocket mouse *Chaetodipus intermedius* the Mc1r allele that codes for coat color is strongly correlated with habitat while showing no correlation with mtDNA markers [Hoekstra, et al., 2004].

Habitat-dependent selection on coat color matches melanic and light colored mice to their respective dark and light colored rock substrates. A similar strong habitat effect (shallow, high light versus deeper, darker areas) was associated with genetic differentiation and UV absorbance of pink and green morphs of the sea anemone *Condylactis gigantea* [Stoletzki, et al., 2005].

The discrepancy between genetic (often mitochondrial) and phenotypic data underscores the autosomal origin of color phenotype. This indicates that at this scale of resolution, phenotype and genotype are often not congruent. Mitochondrial genes are a good marker to infer population separation at phylogenetic timescales, whereas, a better measure of phenotypic differentiation at ecological timescales would be informative alleles or other variable nuclear genes that can detect the difference between the clades [Hellberg, 2009]. Since color is a bi-parentally inherited trait in many organisms, identifying the color genes would give more accurate estimates of allele frequency, gene flow and natural selection leading to genetic divergence. Understanding the adaptive significance of color variability in organisms, *L. variegatus* included, would greatly aid us in understanding the patterns seen in the field and in the genes.

6. Summary

Phenotypic variability in urchins from the 5 regions (Beaufort, Gulf, Keys, Panama and Brazil) was assessed by *in situ* sampling. Overall, I assigned 14 color morphs to 3954 urchins across the geographic region from Beaufort in the north to Brazil in the south. The expected frequency distribution of color phenotypes across all regions assuming no association of color and location would have all color morphs present in all locations. However, contingency analysis of the color morphs reveals a dramatic difference in distribution of the color morphs that is statistically highly significant and region dependent ($\chi^2 = 8105$, df = 36, $P < 0.0000$). Keys is the only region in which all 14 color morphs are present. Beaufort has the fewest with 5. In all regions except Keys the disparity in the frequency of local color morphs is great, with 1 or 2 morphs taking up the largest share of the total.

Color variability measured on the spines and test also reveals regional differences. Of 139 different colors observed on the spines and test of 297 urchins from 4 of the 5 regions, absolute numbers of colors were roughly equal but the identity and frequency of individual colors differed.

Color variability for 508 tests from Beaufort and Tavernier Key differed despite comparable frequencies of color categories. Of the 93 colors observed, the distribution differed by test-area, with the 3rd color categories differing by region. Color variability within the Beaufort population was low for the inshore sites but significantly different ($\chi^2 = 178$, df = 9, $p < 0.0001$) with the offshore site.

Morphology of spine length and width, test shape, thickness and lantern size differed for all regions. A total of 16 characters associated with physical measures of the tests, spines and lanterns were examined on 417 urchins from the 5 regions. The mean test diameter was largest for Brazil urchins, test wall thickness was greatest for Beaufort urchins and the height-diameter ratio was largest on Gulf urchins. Aboral spines were longest on Keys, Panama urchins, whereas, ambital spines were longest on Beaufort urchins. Spines were thickest on Beaufort urchins.

The physical measure of 10 characters on 498 tests from Beaufort and Tavernier Key revealed similar differences. Beaufort tests had thicker walls but were flatter than Tavernier tests. Differences within the Beaufort population revealed local scale variability, with offshore urchins being larger overall and having thicker test walls and the largest height-diameter ratio. Urchins from the Turning Basin had the largest peristome ratio.

Crosses were created from Beaufort and Tavernier Key urchins to assess heritability of color phenotype. 30 crosses produced 745 F_1 offspring, 10 F_1 crosses produced 350 F_2 offspring. Cross color phenotypes resemble native site phenotypes into the F_2 generation. Hybrid crosses produced both Beaufort and Tavernier phenotypes. Color variability as measured on the spines and tests also parallels native site variability with hybrid crosses showing colors common to both Beaufort and Tavernier Key.

Spine color, test color and test patterning were inherited independently and trait ratios conformed to a simple Mendelian model. The white spine phenotype was dominant to both purple and green. The latter phenotypes, recessive to white, were co-

expressed creating the dual purple-green phenotype. Dark tests were dominant to light tests and test patterning was dominant over non-patterning.

Morphological differentiation between crosses of 16 characters associated with spine, test and lantern measures mirror field populations. Significant differences were limited to H/D ratio, lantern L/W ratio and spine length. Keys crosses have taller tests and longer spines but Beaufort crosses have larger lanterns. Hybrid crosses have intermediate values.

Phylogenetic analysis revealed 2 distinct clades for urchins from the 3 regions examined (Beaufort, Gulf and Keys). Clade 1 consists exclusively of urchins from the Keys, whereas, Clade 2 consists of urchins from all 3 regions. Genetic distance between the two clades was very high ($F_{ST} = 0.89$). In contrast, genetic distance between the regions in Clade 2 was zero, indicating that urchins in these regions are genetically identical. The genetic separation of phenotypically indistinguishable Keys urchins from Clades 1 and 2 suggests they are cryptic species.

Genetic differentiation does not concord with color and morphological differentiation seen in Chapters 2 and 3. Many color morphs are found in both clades. Shared haplotypes are a mix of more than one color morph.

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Published Abstracts

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