The Long Arm of the Larva:
Evolutionary Responses to Resource Availability

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ABSTRACT

JUSTIN S. MCALISTER: The Long Arm of the Larva: Evolutionary Responses to Resource Availability

(Under the direction of Joel G. Kingsolver, Ph.D.)

How do organisms adapt to environmental heterogeneity? We know that organisms can respond to environmental heterogeneity by expressing phenotypes that are either phenotypically plastic or constant. However, which strategy of phenotypic expression (plasticity versus constancy) will evolve depends on many factors including: the fitness costs of a given strategy, the degree of environmental heterogeneity, and the degree of association among other life history traits that are also evolving to maximize organismal fitness. Food resource availability is an environmental parameter that is frequently heterogeneous. Echinoid echinoderm larvae are one group of organisms, among many, that have been demonstrated to modify the expression of food collection structures depending on food availability. In my dissertation I examined how the aforementioned factors are associated with the evolution of plastic or constant expression of food collection structures using larval echinoids as a model system.

I investigated whether plastic genotypes pay a fitness cost for expressing phenotypic plasticity of food collection structures. I reared multiple genotypes (families) of larvae of the sea urchin, *Lytechinus variegatus*, and examined whether the degree of plasticity of food collection structures is negatively correlated with two fitness measures: total energetic content and larval stomach length (a site of energy storage). I demonstrated genetic variation

for plasticity among families but did not demonstrate a cost of plasticity, suggesting either that plasticity is inexpensive or that costs of plasticity are difficult to detect.

I investigated whether historical changes in the availability of food resources are associated with evolved differences in the constant and/or plastic expression of food collection structures. I examined larval development of seven "geminate species pairs" of sea urchins located in coastal waters on both sides of the Isthmus of Panama. These species have been evolving in the different environments, with respect to planktonic food for larvae, of the eastern Pacific Ocean and western Caribbean Sea for the past approximately 3 million years. I demonstrated that Caribbean species have evolved to grow longer arms relative to body length than Pacific species regardless of food treatment level (a constant response), and also that none of the species have evolved phenotypic plasticity of food collection structures.

I investigated whether the evolution of constancy or plasticity of different life history traits are correlated. Specifically, I examined whether evolved and experimentally induced differences in egg size, which represents an endogenous energetic resource for larvae, are associated with the expression of differences in the length and plasticity of length of larval feeding structures. Using two species from the sea urchin genus *Strongylocentrotus* that differ in egg size, I demonstrated that evolved and experimentally induced differences in egg size are associated with the expression of larval arm length and that evolved differences in egg size are associated with the degree of plasticity of larval arm length.

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I leave you now to ponder a quote that has helped me to keep life in perspective:

"We sat on a crate of oranges and thought what good men most biologists are, the tenors of the scientific world – temperamental, moody, lecherous, loud-laughing, and healthy. Once in a while one comes on the other kind – what used in the universities to be called a 'dry-ball' – but such men are not really biologists. They are the embalmers of the field, the picklers who see only the preserved form of life without any of its principle. Out of their own crusted minds they create a world wrinkled with formaldehyde. The true biologist deals with life, with teeming boisterous life, and learns something from it, learns that the first rule of life is living. The dry-balls cannot possibly learn a thing every starfish knows in the core of his soul and in the vesicles between his rays. He must, so know the starfish and the student biologist, who sits at the feet of living things, proliferate in all directions. Having certain tendencies, he must move along their lines to the limit of their potentialities. And I have known biologists who did proliferate in all directions: one or two have had a little trouble about it. Your true biologist will sing you a song as loud and off-key as will a blacksmith, for he knows that morals are too often diagnostic of prostatitis and stomach ulcers. Sometimes he may proliferate a little too much in all directions, but he is as easy to kill as any other organism, and meanwhile he is very good company, and at least he does not confuse a low hormone productivity with moral ethics."

- John Steinbeck: *The Log from the Sea of Cortez* -

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ABBREVIATIONS

AL	**anterolateral arm length**
ANOVA	**analysis of variance**
ASW	**artificial seawater**
BL	**body length at midline**
C	**Celsius**
CaMg-FSW	**calcium and magnesium free seawater**
d	**day**
FE	**fertilization envelope**
Fig	**Figure**
FSW	**filtered seawater**
h	**hours**
l	**liters**
min	**minutes**
ml	**milliliters**
mm	**millimeters**
PO	**postoral arm length**
SL	**stomach length**
µl	**microliters**
µm	**micrometers (microns)**
ppt	**parts per thousand**

CHAPTER ONE

INTRODUCTION

Organisms do not live in a vacuum; they have an intricate relationship with the environment(s) in which they reside. Organisms produce phenotypes that facilitate interaction with their environments. The expression of a given phenotype hinges in part on the genetic programming for that particular phenotype, but also on the organism's ability to incorporate specific information about the quality of the environment. To ensure the production of an appropriate phenotype, the availability of a specific resource can be used as an assessment of environmental quality. Some organisms have the ability to modulate the expression of a given phenotype depending on environmental conditions, i.e. they express phenotypes that are plastic across environments, whereas other organisms express constant phenotypes that do not vary across environments. Evolution of a particular strategy, i.e. phenotypic plasticity or phenotypic constancy, for the expression of phenotypes can occur, given significant genetic variation in a population for different strategies; selection will favor the production of phenotypes, and the strategies for producing phenotypes, that maximize fitness. Which strategy is most successful and will evolve in a system depends on the fitness costs and/or benefits of a given strategy, the degree of environmental heterogeneity, and the degree of association among other life history traits that are also evolving. The goal of this dissertation is to examine how these factors have contributed to the evolution of the

expression of plastic or constant feeding structures, using larval echinoid echinoderms as a model system.

Background

Environmental heterogeneity

Environments routinely change and organisms often experience these changes in spatial and/or temporal patches. Although the magnitude of patch size is usually unknown, the degree of patch heterogeneity, i.e. the amount of environmental change, experienced by an organism can often be predicted. This predictability in patch heterogeneity is referred to as the grain of the environment (Levins, 1968). Environmental grain is organism specific; the same environment may be differently grained to different organisms. Environmental grain is also variable specific: a single organism may experience different environmental variables at different grains. An organism that experiences no heterogeneity exists in an environment that is coarse-grained. Alternatively, organisms that experience differing degrees of environmental heterogeneity do so at fine grain. The grain at which an organism experiences environmental heterogeneity influences the ecological strategy that organism assumes to cope with change.

Organisms have developed different strategies with which to cope with environmental heterogeneity. Traditionally, four different strategies have been recognized by evolutionary ecologists (DeWitt & Langerhans, 2004): (1) specialization, (2) generalization, (3) bet-hedging, and (4) phenotypic plasticity. Organisms specialize by producing a single phenotype when environmental heterogeneity is low. Generalization occurs when an organism produces a phenotype that is moderately successful in multiple environments, but

not optimal in any one. Bet-hedgers produce either multiple phenotypes or single phenotypes probabilistically, whereas phenotypically plastic strategists produce alternative phenotypes depending on the environment. The benefits of adopting one strategy over another are variable and there has been considerable theoretical work on these strategies (Levins, 1968; Lively, 1986; van Tienderen, 1991, 1997; DeWitt & Langerhans, 2004; see reviews by Wilson & Yoshimura, 1994, and Kassen, 2002). *Sensu* DeWitt & Langerhans (2002), bet-hedging is not necessarily a unique strategy because it can be thought of simply as adding variance to any of the other three strategies. For this reason, only specialization, generalization, and phenotypic plasticity are distinct ecological strategies for coping with environmental heterogeneity.

Of these three strategies, phenotypic plasticity may seem unbeatable in heterogeneous environments. An organism possessing the ability to consistently develop environment-specific phenotypes should be favored by natural selection (Schmalhausen, 1949; Bradshaw, 1965). However, this statement fails to incorporate the fact that with differing environmental grain, different strategies may have varying effects on organismal fitness (Pigliucci, 2001). For example, if an organism experiences primarily coarse-grained environmental heterogeneity during its lifetime, then adopting a specialist strategy may provide highest fitness. Organisms that experience fine-grain environmental heterogeneity may adopt a generalist strategy by producing an intermediate phenotype, thereby providing marginal within-environment but higher across-environment fitness. Alternatively, organisms in fine-grain environments can develop a phenotypically plastic strategy, which may provide higher fitness both within and across environments (assuming costs of plasticity are minimal: see next section). Environmental grain notwithstanding, natural selection cannot produce

different ecological strategies if there is no across-patch genetic variance in, or genotype-by-environment interaction variance for fitness among ecologically similar individuals (Kassen, 2002).

Assuming these variances exist, researchers have developed models for the evolution of ecological strategies using the reaction norm concept (Via and Lande, 1985; van Tienderen, 1991; Gomulkiewicz & Kirkpatrick, 1992; Kisdi et al., 1998; Pigliucci & Murren, 2003). Simple, two environment reaction norms are graphical representations that depict the range of phenotypic values produced by genotypes across environments. Reaction norms can have either no slope or some degree of positive or negative slope. When the optimal phenotypic values in the two environments differ, a specialist genotype is one that produces an optimal phenotype in one environment, and the same, albeit sub-optimal phenotype in the second environment. In this scenario a specialist will exhibit a reaction norm with no slope. However, a genotype that produces near-optimum phenotypic values in both environments is a generalist and will exhibit a sloped reaction norm (Figure 1.1A). Alternatively, when the optimal phenotypic values in the two environments are equal, a generalist genotype is one that produces near-optimum phenotypes across environments. In this second scenario, a generalist will exhibit a reaction norm with no slope. However, a genotype with a sloped reaction norm is a specialist because it produces an optimum phenotype in only one of the environments (Figure 1.1B).

In the two scenarios (dissimilar versus equal phenotypic optima) either generalists or specialists can be phenotypically plastic. In the dissimilar phenotypic optima scenario the generalist is phenotypically plastic (Figure 1.1A). Counter intuitively, in the scenario with equal phenotypic optima, the specialist is phenotypically plastic (Figure 1.1B). Phenotypic

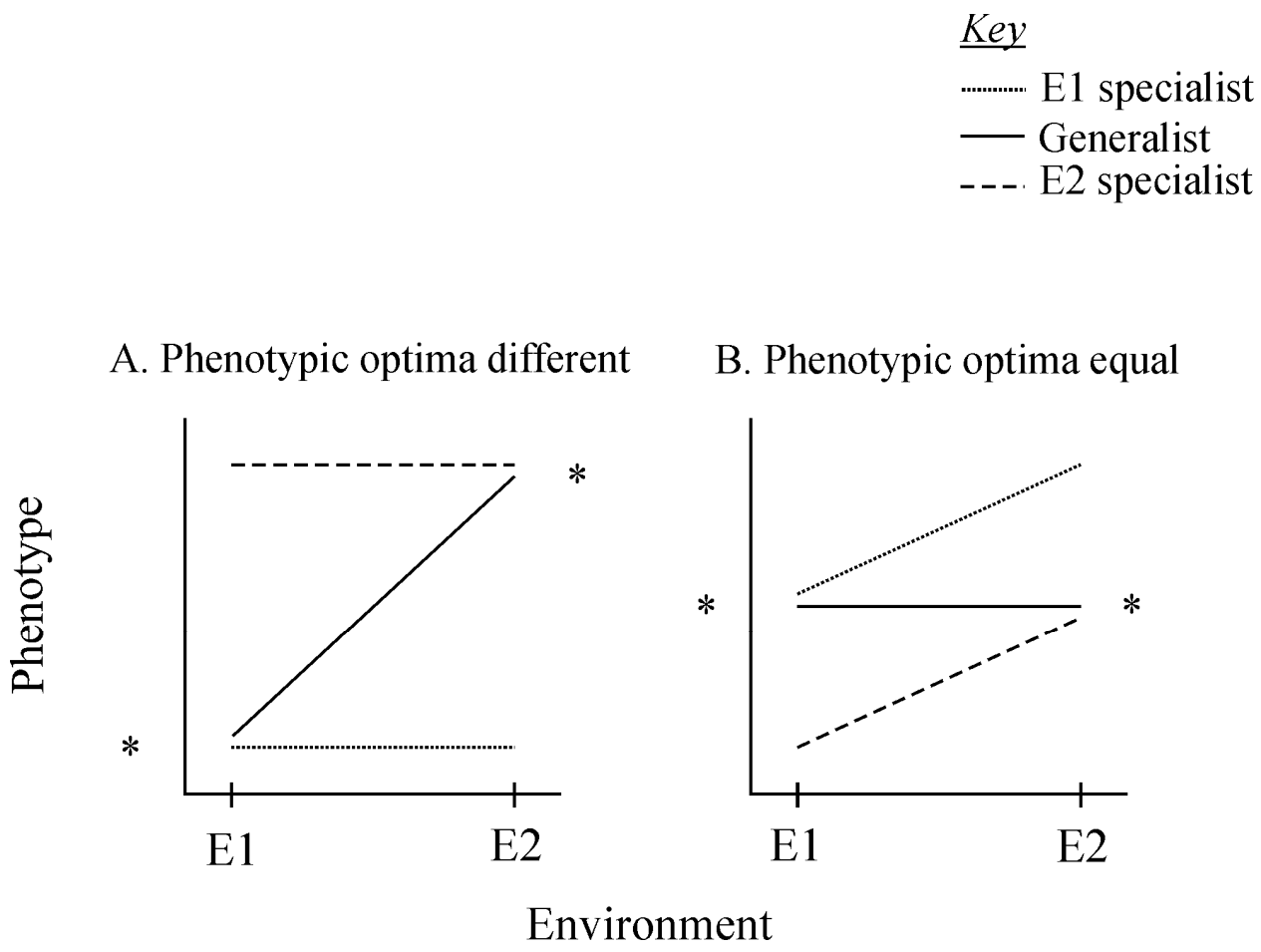


Figure 1.1: Distributions of reaction norms in relation to phenotypic optima (denoted by an *) in each environment. In A, the phenotypic optima are different between environments, whereas in B, the phenotypic optima are the same. Different genotypes are indicated by the solid and dashed lines in each scenario (see key). Fixed and phenotypically plastic genotypes assume different ecological strategies in relation to the phenotypic optima. Figure adapted from van Tienderen (1991).

plasticity has often been associated with generalization, because some of the initial models of the evolution of phenotypic plasticity were based on a two environment scenario with different optima (Via and Lande, 1985; Moran, 1992), whereas the production of constant phenotypes has often been ascribed to specialization. In both situations however, there exist genotypes that produce the same phenotype across environments, and genotypes that produce different phenotypes across environments. I consider therefore, that organisms can evolve one of two unique strategies for the expression of a phenotype: plasticity and constancy.

Costs of phenotypic plasticity

In most biological systems, individuals rarely produce phenotypes that optimally match the degree of environmental heterogeneity (Levins, 1968). One reason for incomplete phenotype-environment matching is that possessing the ability to change one's phenotype, i.e. being phenotypically plastic, may be inherently costly (Bradshaw, 1965; van Tienderen, 1991; DeWitt, 1998; DeWitt et al., 1998; Scheiner & Berrigan, 1998; Tucić et al., 1998; Dorn et al., 2000; van Kleunen et al., 2000; Tucić & Stojković, 2001; Agrawal et al., 2002; Poulton & Winn, 2002; Relyea, 2002; Steinger et al., 2003). By definition, a cost of phenotypic plasticity is incurred when more-plastic genotypes are comparably less fit than less-plastic or constant genotypes when producing the same phenotype in a given environment (DeWitt et al., 1998). Costs of plasticity may be incurred in the (1) maintenance or (2) production of a phenotype, in (3) acquiring information about the environment, via (4) imprecision during development of a phenotype, and/or (5) when the genes responsible for plasticity are associated with other genes conferring low fitness (DeWitt et al., 1998).

Costs of phenotypic plasticity have been incorporated into theoretical models of the evolution of phenotypic plasticity (Via and Lande, 1985; Lively, 1986; Via, 1987; van Tienderen, 1991, 1997; Gomulkiewicz & Kirkpatrick, 1992; Moran, 1992; Leon, 1993; Padilla & Adolph, 1996). Generally these models predict that adaptive phenotypic plasticity will evolve under four conditions (as outlined by Relyea, 2002): (1) the fitness of alternative phenotypes is affected by the degree of environmental heterogeneity a population experiences, i.e. the environmental grain as discuss above; (2) the cues that an organism uses to detect environmental quality or state are reliable; (3) there are no optimal phenotypes that confer superior fitness in all environments; and (4) the costs of phenotypic plasticity are relatively low. Despite the prominent position of costs of phenotypic plasticity in these models and careful research to test for costs using a variety of biological systems, the detection of overwhelming evidence for ubiquitous costs of phenotypic plasticity has been elusive. In general, empirical results have demonstrated that plasticity costs are either absent (Scheiner & Berrigan, 1998; Tucić & Stojković, 2001; Relyea, 2002), infrequent (Dorn et al., 2000), or are negligible in scale and/or limited to specific traits in particular environments (DeWitt, 1998; Tucić et al., 1998; Dorn et al., 2000; van Kleunen et al., 2000; Agrawal et al., 2002; Poulton & Winn, 2002; Relyea, 2002; Steinger et al., 2003). None of these studies however, have focused on measures of phenotypic plasticity that result from changes in food resource levels.

Food resource availability and the expression of phenotypic plasticity

The level of food resources available to an organism is an environmental characteristic that is frequently heterogeneous. Heterogeneity of food resources can induce

phenotypic plasticity of trophic structures in some organisms. Within a population however, other genotypes may express less-plastic or constant phenotypes. The availability of food resources can be defined in terms of the magnitude (the mean) and the variability (the variance) of the resource. The expression of phenotypic plasticity has been demonstrated across a wide range of taxa in response to across-environment differences in both of these parameters.

An association between resource level magnitudes with the expression of phenotypic plasticity has been demonstrated for life history traits. These traits include the age and size at metamorphosis of larval anurans (Wilbur and Collins, 1973; Smith-Gill and Berven, 1979; Alford and Harris, 1988; Hensley, 1993; Leips and Travis, 1994; Beck, 1997; Newman, 1998; Nicieza, 2000; Doughty, 2002), pitcher plant mosquitoes (Bradshaw and Johnson, 1995), copepods (Twombly, 1996), gastropod molluscs (Pechenik et al., 1996), barnacles (Hentschel and Emlet, 2000), polychaete annelids (McEdward and Qian, 2001), mud crabs (Suprayudi et al., 2002), and damselflies (Johansson et al., 2001), and similarly for the age and size to maturity of Daphnid crustaceans (Reinikainen and Repka, 2003).

Differences in resource type are also associated with the expression of morphological phenotypic plasticity. Resource polymorphisms have been demonstrated in vertebrates (in particular fish, amphibians, and birds) and have been reviewed by Robinson and Wilson (1994), Skúlason and Smith (1995), and Smith and Skúlason (1996). As an example of the type of plasticity found in many of these studies, sunfish fed diets which differed in the proportion or type of food exhibited differences in pharyngeal and/or jaw morphology (Mittlebach et al., 1999; Hegrenes, 2001). The phenotypic plasticity demonstrated in many

of these studies may have resulted from differences in the magnitude of some characteristic (e.g. protein content) of the food resource.

In invertebrate systems, empirical research has demonstrated that diet induces changes in the shape of snail radular teeth (Padilla, 2001), in insect jaw morphology (Bernays, 1986; Greene, 1989; Thompson, 1992), and in the shape of crustacean chelae (Smith and Palmer, 1994). Research in filter-feeding bivalves has shown that the ratio of gill-to-labial palp mass changes with the degree of sediment coarseness (Drent et al., 2004). Drent et al.'s (2004) study represents an area of research that has demonstrated morphological (Pfennig, 1990, 1992; Piersma and Lindstrom, 1997; Stark, 1999; Dekinga et al., 2001; McWilliams and Karasov, 2001; Piersma and Drent, 2003; Relyea and Auld, 2004) and enzymatic (Bock and Mayer, 1999) changes in the alimentary canal of both vertebrates and invertebrates in response to changes in food conditions.

The association of variability of resource levels with the expression of phenotypic plasticity has not been examined as broadly. However, theoretical results suggest that longer-term predictable variations in resource level (e.g. seasonal fluctuations) can have effects on life history (Cohen, 1967; Levins, 1968; Fretwell, 1972; Colwell, 1974). Empirical work indicates that resource level variability can influence behavior in zebrafish (Grant and Kramer, 1992) and convict cichlids (Grand and Grant, 1994), gut length plasticity and the differential allocation of resources to growth or reproduction in fathead minnows (Siems and Sikes, 1998), age and size at metamorphosis in spadefoot toad tadpoles (Newman, 1998), and the expression of arm length plasticity in echinoid echinoderm larvae (Miner and Vonesh, 2004).

Food resource availability and the evolution of phenotypic plasticity

The association between food resource level and phenotypic plasticity has been studied at several levels of evolutionary inquiry. Researchers have demonstrated or documented phenotypic plasticity in response to resource levels (Boidron-Metairon, 1988; Fenaux et al., 1988; Hart and Scheibling, 1988; see reviews by Robinson and Wilson, 1994; Skúlason and Smith, 1995; and Smith and Skúlason, 1996). In addition, studies have demonstrated variation in the degree of phenotypic plasticity among populations (or species associations) in response to variation in resource level (DeBenedictis, 1974; Kaitala, 1991; Blouin, 1992; Leips and Travis, 1994; Buchholz and Hayes, 2000, 2002; Leips et al., 2000; Langerhans et al., 2003; Reinikainen and Repka, 2003; Morey and Reznick, 2004; Stauffer and Van Snik Gray, 2004). However, demonstrations of whether differences in plasticity have evolved in response to historical changes in the availability of food resources are scant (but see Morey and Reznick, 2004). Morey and Reznick (2004) evaluated the effect of food supply on the plastic response of age and size at metamorphosis in three spadefoot toads (Pelobatidae: *Spea intermontana*, *Sp. hammondii*, and *Scaphiopus couchii*) whose larvae inhabit bodies of water with different degrees of permanence. The results from their comparative approach indicate that each species exhibited a different degree of plasticity of age and size at metamorphosis that was associated with the degree of habitat permanence. What remains unknown in this system (and many others) is the relationship and times of divergence among the different species, and how they have adapted to unique larval habitats since separation. These unknown variables make this level of evolutionary inquiry of greatest interest because no research has demonstrated an association between historical environmental changes in resource levels and the repeated evolution of phenotypic plasticity.

Feeding structures in planktonic larvae

Morphological phenotypic plasticity in response to food resource level has been demonstrated in planktotrophic pluteus larvae from several species in the echinoderms (echinoids: Boidron-Metairon, 1988; Fenaux et al., 1988; Hart and Scheibling, 1988; Strathmann et al., 1992; Hart and Strathmann, 1994; Eckert, 1995; Bertram and Strathmann, 1998; Heyland and Hodin, 2004; Miner and Vonesh, 2004; Reitzel and Heyland, 2007; asteroids: George, 1994, 1999; and ophiuroids: Podolsky and McAlister, 2005), molluscs (bivalves: Strathmann et al., 1993; and gastropods: Estrella Klinzing and Pechenik, 2000), and freshwater Daphnid crustaceans (Lampert, 1994; Reinikanen and Repka, 2003). These larvae depend on exogenous phytoplankton food, and in response to low food availability, can increase the length of the ciliated band used for collecting food. Echinoid and ophiuroid echinoderms accomplish this by growing longer larval arms; plasticity of ciliated band length is correlated with lengthening of skeletal arm rods in echino- and ophio-plutei. Increased ciliated band length enhances larval ability to capture phytoplankton, and increases in ciliated band length under low food conditions have been demonstrated to be adaptive because larvae with longer ciliated bands have greater maximum clearance rates (Hart and Strathmann, 1994). In addition, by increasing ciliated band length, the larval surface-to-volume ratio increases, which could increase intake of dissolved organic matter (Manahan et al., 1983). Plasticity in arm length has also been used as a measure of larval feeding history in the field (Strathmann et al., 1992).

However, increases in energetic investment to lengthen feeding structures in low food conditions result in a decreased energetic investment in the development of juvenile structures required for metamorphosis. Consequently, food-limited larvae exhibit delayed

time to metamorphosis, a potentially dangerous prospect for planktonic feeding organisms (Thorson, 1950; Rumrill, 1990; Morgan, 1995). Selection will be strong for traits that ameliorate the effects of adverse feeding conditions (Doughty, 2002) by decreasing the duration of time larvae spend in the plankton. These traits are those associated with the utilization of energetic resources available to the larva from: 1) the exogenous food resources acquired from the larval feeding environment or 2) the endogenous energetic reserves obtained from the parent.

Previous work indicates that arm length plasticity is expressed during early larval development (Boidron-Metairon, 1988; Hart & Scheibling, 1988; Eckert, 1995; Sewell et al., 2004), suggesting that larvae may utilize endogenous resources for the initial production of food collecting structures, then move to exogenous resources for the development of other, later-appearing structures. Endogenous resources are provided to individual offspring within the egg and egg size is positively correlated with the level of investment (Jaeckle, 1995). Herrera et al. (1996) have demonstrated variation in feeding period with egg size; development time to metamorphosis is inversely related to egg size among various echinoid species. The capacity for plasticity of arm length early in development may therefore depend on the amount of maternally provisioned energetic reserves, and thus on egg size (Herrera et al., 1996).

The planktotrophic pluteus larvae of many echinoids provide an ideal system in which to examine the evolution of the expression of different phenotypic strategies for food resource acquisition. Gametes are easy to obtain, can be fertilized externally to produce multiple genetic families, and large numbers of larvae can be reared easily in a small laboratory space. In addition, echinoids are easy to collect, species exist with known

relationships and times of divergence, and species differ in egg size. Because of these benefits, I have used this system in my dissertation research to examine how organisms adapt to environmental variation. More specifically, my empirical research investigate the fitness costs for one resource acquisition strategy, phenotypic plasticity; examines whether historical changes in resource levels are associated with the repeated evolution of plastic or constant phenotypes; and investigates whether the evolution of a trait is correlated with the evolution of other life history traits, i.e. is the evolution of feeding structure (larval arm length) plasticity correlated with evolved differences in the amount of endogenous energetic materials available to a developing larva. I summarize these efforts briefly below.

Summary of Experiments

The dissertation research is divided into three sections, contained in Chapters II, III, and IV. Chapter II is an investigation of the fitness costs of phenotypic plasticity. Twenty-nine full-sib half-sib families of larvae of the echinoid *Lytechinus variegatus* were reared under replicated high food or low food conditions for two weeks. Morphological measurements of arm length and body length were collected on days 2, 4, 6, and 8 post-fertilization. Two measures of fitness, stomach length on day 8 and total energetic content on day 14, were also collected. The degree of phenotypic plasticity of arm length relative to body length was calculated for larvae from each family for each day. Utilizing a statistical methodology outlined by DeWitt et al. (1998) I used these data to examine whether more-plastic genotypes had lower fitness measures than less-plastic genotypes, which would indicate a cost of plasticity.

Chapter III addresses whether historical environmental changes in resource levels are associated with the repeated evolution of plastic or constant phenotypes. Larval development of three echinoid "geminate species pairs," formed when previously continuous species were separated before or during the raising of the Isthmus of Panama 2.8-3.1 million years ago, were examined in this study. Three of the echinoid species, *Diadema mexicanum*, *Echinometra vanbrunti*, and *Eucidaris thouarsi*, were collected from the heterogeneous (with respect to phytoplankton food for larvae) waters of the eastern Pacific Ocean. Their geminate counterparts, *Diadema antillarum*, *Echinometra lucunter*, *Echinometra viridis*, and *Eucidaris tribuloides*, were collected from the constantly low, oligotrophic waters of the western Caribbean Sea. Multiple full-sib families of larvae from all seven species were reared under replicated high food or low food conditions for approximately 10 days. Morphological measurements of arm length and body length were collected on days 2, 3, 4, 5, 6, 8, and 10 post-fertilization. The degree of phenotypic plasticity of arm length relative to body length was calculated for larvae from each family for each day.

Chapter IV examines whether the degree of expression of feeding structure (larval arm length) plasticity is correlated with differences in the size of the egg. Larvae from the congeneric sea urchins *Strongylocentrotus franciscanus* and *Strongylocentrotus purpuratus*, which differ in egg volume by 5-fold, were used in this study. In addition, the egg size of *S. franciscanus* (the larger-egged species) was experimentally manipulated by separating blastomeres, a simple embryological protocol, at the 2-cell stage to produce half-sized larvae. Normal-sized and half-sized larvae were reared under replicated high food or low food conditions for 20 days post-fertilization. Morphological measurements of arm length and body length were collected on days 3, 5, 7, 10, 13, 16, and 20 post-fertilization. The degree

of phenotypic plasticity of arm length relative to body length was calculated for larvae from each species and treatment for each day.

Significance

My dissertation research has produced significant additions to our understanding of the evolution of the strategies used to express feeding structures in larval echinoderms. First, using the echinoid *Lytechinus variegatus* I demonstrate marginally significant genetic variation of larval arm length plasticity in response to food limitation for this species. Genetic variation of plasticity is an underlying requirement for adaptation, and facilitates the evolution of varied responses to food limitation among close relatives that occupy habitats which differ in food availability. Indeed, the number of known, genetically distinct families reared in this experiment represents an approximately 10-fold increase in the number of known sib-ships used in any previous study of larval development in marine invertebrates. Arm length and plasticity of arm length did not correlate with either of the two fitness measures (total energy content and stomach length) I collected however; thus, I did not detect a cost of plasticity. The results of this study contribute to a growing body of evidence that costs of plasticity are absent or are difficult to detect.

Second, the results from the studies of Central American echinoids indicate that none of the geminate species expressed larval arm plasticity. While these results are unexpected, because plasticity has been demonstrated in many other echinoid species, they indicate that plasticity is not guaranteed to evolve within all species. The results need explanation and open up new questions regarding the evolution of plasticity in this system: Are tropical species severely food limited? Is there latitudinal variation in the degree of plastic

expression? Is there strong selection on other life history characters, to ameliorate the effects of an adverse feeding environment, e.g. egg size, that may supersede selection on feeding structures? Caribbean species in my studies did grow longer larval arms relative to body size than Pacific species, regardless of food level treatment, however. These results are the first to demonstrate that differences in the evolution of constant phenotypes can occur repeatedly across taxa.

Third, the results of the egg size manipulation and comparative larval growth experiments with species of *Strongylocentrotus* indicate that egg size affects larval arm length plasticity; larger eggs produce more-plastic larvae both in an experimental and a comparative context. However, evolved differences in the pattern of plasticity expressed by each species over time can not be accounted for by changes in egg size alone. These results provide insight into the related question of whether changes solely in egg size are sufficient to induce an evolutionary transition from feeding (planktotrophic) to non-feeding (lecithotrophic) larval development.

CHAPTER TWO

COSTS OF PHENOTYPIC PLASTICITY DURING LARVAL DEVELOPMENT OF THE SEA URCHIN *LYTECHINUS VARIEGATUS* (LAMARCK)

Summary

In response to food limitation, larvae of some echinoid species grow longer arms and thereby elongate a food collecting ciliated band, which can increase feeding rate. One potential cost of arm length plasticity could be detected if more-plastic genotypes had lower fitness than less-plastic genotypes of the same phenotype. To test for this cost, I reared multiple families of larvae of *Lytechinus variegatus* (Lamarck) in a full-sib, half-sib breeding design under different food conditions. Low-fed larvae grew longer arms than high-fed larvae through day 6 with a maximum difference on day 6; I detected marginally significant variation across families for this response, i.e. marginally significant genetic variation for arm length plasticity. Arm length and plasticity of arm length were not correlated with two fitness measures (total energy content and stomach length); thus, I did not detect a cost, or a clear benefit, of plasticity. Plasticity in arm length may be more closely associated with other, unmeasured, fitness components, such as development time to metamorphosis.

Introduction

In order to increase fitness, many organisms change the expression of a phenotype in response to environmental cues, a process known as adaptive phenotypic plasticity. However, organisms rarely express phenotypes that are optimal for all environments (Levins, 1968). One potential reason for the expression of sub-optimal phenotypes is that adaptive phenotypic plasticity may be inherently costly (Bradshaw, 1965; van Tienderen, 1991; DeWitt et al., 1998). Costs of plasticity can be associated with the maintenance and production of a phenotype, with acquiring information about the environment, with developmental instability in producing a phenotype, or with genetic correlations between plasticity and genes conferring low fitness (DeWitt et al., 1998). Such costs of phenotypic plasticity would be demonstrated if more-plastic genotypes were less fit than less-plastic genotypes when they produced the same phenotype in a given environment (DeWitt et al., 1998).

Empirical studies have searched for plasticity costs in several plant species in which plasticity can be induced by competition or light availability (Tucić et al., 1998; Dorn et al., 2000; van Kleunen et al., 2000; Tucić & Stojković, 2001; Agrawal et al., 2002; Poulton & Winn, 2002; Steinger et al., 2003), and in a few animals where plasticity can be induced by predators (DeWitt, 1998; Scheiner & Berrigan, 1998; Relyea, 2002; Merila et al., 2004). Few of these studies, however, have detected significant costs of adaptive phenotypic plasticity. However, because such costs are predicted by theory, empirical tests in diverse biological systems and environmental conditions will help to better understand whether costs play a significant role in the evolution of phenotypic plasticity. Additionally, no previous

empirical studies have tested for costs of phenotypic plasticity in marine invertebrates or for morphological changes of feeding structures in response to changes in food availability.

Morphological phenotypic plasticity in response to food availability has been demonstrated in planktonic pluteus larvae from several species in the echinoderm classes Echinoidea (Boidron-Metairon, 1988; Fenaux et al., 1988; Hart & Scheibling, 1988; Strathmann et al., 1992; Hart & Strathmann, 1994; Eckert, 1995; Sewell et al., 2004) and Ophiuroidea (Podolsky & McAlister, 2005). These larvae consume phytoplankton, and in response to low food availability they grow longer arms, thereby increasing the length of the ciliated band used for food collection. An increase in larval arm and ciliated band length enhances the ability of larvae to capture phytoplankton, a response that is adaptive under limiting food conditions because larvae with longer arms and ciliated bands clear food from suspension at greater maximum rates (Hart & Strathmann, 1994). In addition, an increase in surface area generated by longer arms could increase the uptake of dissolved organic matter (Manahan et al., 1983). For these reasons, arm length has been used as an indicator of larval nutritional history in the field, and plasticity of feeding structures may be important in the recurrent evolution of non-feeding larvae (Strathmann et al., 1992).

Plasticity of larval feeding structures hinges on a trade-off in investment between larval and juvenile structures: increased investment in arms can result in decreased investment in other structures, such as the juvenile rudiment. As noted, this response can enhance feeding under low food conditions (Hart, 1994) and reduce time to metamorphosis (Boidron-Metairon, 1988). Reducing development time can be an important survival strategy for low fed larvae, because delayed time to metamorphosis increases the duration of larval exposure to planktonic predation (Rumrill, 1990; Morgan, 1995). However, costs to

larvae of retaining the ability to be phenotypically plastic, for example the additional costs of producing longer arms beyond what a non-plastic genotype would have to invest (i.e. excess production costs *sensu* DeWitt et al., 1998), could counter the theoretical benefits and thereby constrain the evolution of plasticity.

The planktotrophic pluteus larvae of many echinoids provide an ideal system for testing for costs of plasticity. Gametes can be fertilized externally to produce multiple genetic families, and large numbers of larvae can be reared easily in a small laboratory space. Previous work indicates that larval arm length plasticity is expressed in some species during early larval development (Boidron-Metairon, 1988; Hart & Scheibling, 1988; Hart & Strathmann, 1994; Eckert, 1995; Sewell et al., 2004; Podolsky & McAlister, 2005). Thus data on larval arm length and plasticity can be collected in a short period of time (1-2 weeks post-fertilization for most species), allowing for the rearing of a large number of genetic families using successive experimental blocks.

In this study, I investigated whether costs are associated with the expression of phenotypic plasticity of feeding structures in larvae of the sea urchin *Lytechinus variegatus*. I predicted that families with a greater capacity for expression of plasticity (across environments) would have lower fitness for a given phenotype expressed in a given environment. Using a quantitative genetic breeding design and a statistical methodology outlined by DeWitt et al. (1998), I tested for a relationship between the degree of plasticity in larval arm length and two fitness-related measures: total energy content and larval stomach length.

Materials and Methods

Adults of the sea urchin *Lytechinus variegatus* were collected in May 2004 by dredging from a sub-tidal population located off the coast of Morehead City, NC. The urchins were placed in disposable plastic containers (3-4 urchins per container) filled with a small amount of seawater. The containers holding urchins were stacked in a cooler and transported to Chapel Hill, NC where the urchins were maintained in recirculating aquaria filled with artificial sea water (ASW: Instant Ocean, Aquarium Systems). Adult urchins were fed carrots *ad libitum* for the duration of the experiment, which was approximately 6 weeks.

Larval Culture

Gametes were obtained from adult urchins by peristomial injection into the body cavity of approximately 1 ml of 0.5 M KCl. Eggs were collected and washed once in ASW, and sperm were collected by mouth pipette and kept on ice until use. Gametes were used for controlled fertilizations in a 6 male by 2 female full-sib, half-sib breeding design that was replicated in 3 temporally separated blocks (similar to Newman, 1988) using each time different males and females to produce a total of 36 full-sib, half-sib families. This breeding design maximized the number of males, and thus the amount of additive genetic variation among families, given the space and time constraints associated with rearing multiple larval cultures. Due to larval mortality in one block, I obtained data from approximately 2½ of the blocks for a total of 29 full-sib, half-sib families. For each family, fertilized embryos and larvae were reared in one of two food environments (1 or 5 algal cells μl^{-1}), with two replicate cultures per treatment. These food treatment levels are representative of low, food-

limiting and high, satiating conditions for echinoid larvae (Boidron-Metairon, 1988; Fenaux et al., 1988). Each larval culture was fed the unicellular alga *Dunaliella tertiolecta* (UTEX Algal Supply, Austin, TX) daily, starting at 24 h (all ages reported are post–fertilization). All cultures were reared in ASW in 1l plastic tri-pour beakers at densities of 1 larva ml^{-1} and water was changed every other day. The cultures were maintained in an environmental chamber at 25°C and were continually stirred with acrylic paddles at a rate of approximately 10 strokes min^{-1} to keep larvae and food in suspension (Strathmann, 1987). The alga was cultured at room temperature in autoclaved ASW enriched with a modified Guillard's f/2 medium (Florida Aqua Farms, Inc.), and was re-suspended in fresh ASW before use.

Measures of Phenotype

Echinoid pluteus larvae are bilaterally symmetrical and possess a calcitic endoskeleton, which includes two short body rods and four pairs of arm rods. As larvae mature, they initiate in succession and lengthen simultaneously pairs of arm rods. Depending on conditions, larvae can mature at different rates to reach different developmental stages (based on the number of arm pairs initiated) at a given age, creating the potential for confounding size and stage. However, all comparisons to detect a cost of plasticity were made among genotypes within a single environment, not between environments.

Every other day through day 8, approximately 10 larvae were removed from each culture, immobilized on a glass slide with a dilute (<10%) solution of buffered formalin in ASW, and covered with a glass cover slip raised on clay feet. Three-dimensional Cartesian coordinates of several morphological landmarks were recorded for 5 larvae from each culture (Fig. 2.1). These landmarks included the tip and base of each anterolateral and postoral arm

rod, the posterior tip of the larva, the tip of the oral hood (i.e. the mid-point of the soft-tissue that stretches between the pair of anterolateral arms), and points at the anterior and posterior ends of the stomach. To collect data from each larva, I used a digitizing tablet (Hyperpen 12000U, Aiptek Inc.) to capture *x* and *y* coordinates of morphological landmarks, while simultaneously obtaining *z* coordinates from a rotary encoder (U.S. Digital) coupled to the fine focus knob of a compound microscope (McEdward, 1985). Using these 3-D Cartesian coordinates, I reconstructed individual arm, body, and stomach lengths for each larva. Because the postorals and anterolaterals were the most prominent arms at the stages when I collected measurements, our analysis focuses on plasticity in the summed length of these arms ("total arm length").

To assess the effects of arm length plasticity on fitness, I measured a proxy of fitness, energy content, on day 14. At this time high-fed larvae had initiated formation of the juvenile rudiment, indicating that they had begun to shift energetic investment from larval structures to juvenile structures that persist beyond metamorphosis. Low-fed larvae had not initiated juvenile rudiment formation on day 14. I collected a sub-sample of larvae from each culture to measure total energy content (a measure of growth related to fitness) per larva from each family reared in each food environment (Gosselin & Qian, 1999). For each culture, 2 larvae were placed in each of 3 glass culture tubes, washed approximately 3 times with an isosmotic 3.5% solution of ammonium formate to remove residual chloride (Gosselin & Qian, 1999), freeze dried and stored at -20°C. I used a modification (Allen et al., 2006) of Gosselin & Qian's (1999) wet oxidation assay to obtain measures of total energy content.

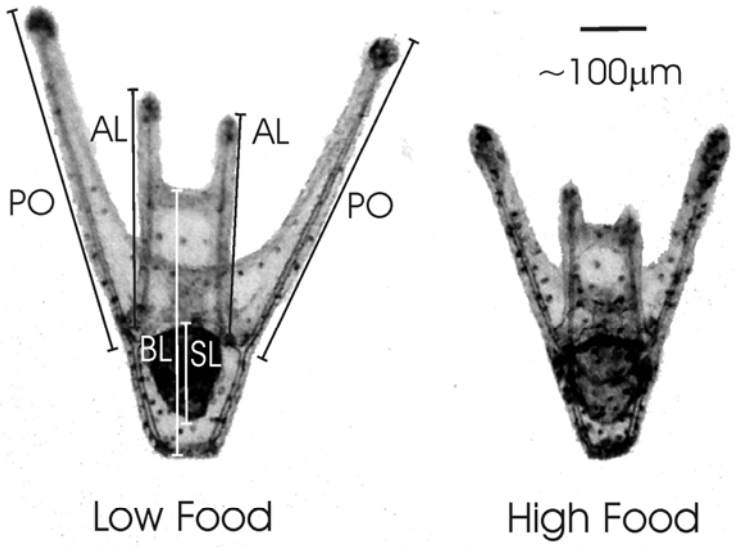


Figure 2.1: Low- and high-fed *Lytechinus variegatus* larvae from the same family on day 4 showing morphological characters that I measured on days 2, 4, 6, and 8: AL = Anterolateral arm, PO = Postoral arm, BL = Body length at midline, SL = Stomach length. Both larvae are displayed at the same magnification; scale bar represents approximately 100 microns.

Measures of Fitness

I examined a second performance measure involving a trade-off in energy allocation between larval arms and the size of the larval stomach. Miner (2005) detected this trade-off in 5-day old larvae of *Strongylocentrotus franciscanus* and *S. purpuratus*: high-fed larvae had shorter arms relative to their stomach length, a result of growth rather than distension by food. Miner (2005) suggested that larger stomachs with larger surface areas could increase assimilation rates in high-fed larvae, whereas longer arms could increase particle capture rates in low-fed larvae. In addition, the wall of the larval stomach can store lipids (Burke, 1981), which are used by juveniles during and after metamorphosis. For these reasons, I used measures of stomach length on day 8 as a second measure of investment related to fitness.

Statistical Analyses

I tested the effect of variation among genotypes (family), food level (food), culture replicate (culture), and day of development (day) on total arm length (sum of the postoral and anterolateral arms) using analysis of variance (PROC MIXED: SAS Institute, Cary, NC). The statistical model also included terms to account for variation due to the interactions of family with food, day with food, family with day, and the three-way interaction of family by food by day. Day was coded as a repeated measure with culture as the subject; the covariance structure of the R matrix was specified as Compound Symmetry (CS). Degrees of freedom were calculated using the DDFM=BW (Between-Within) option in PROC MIXED. Body length was included in the model as a quantitative covariate. I considered food, day, day with food, and body length as fixed effects. Family, family with food, family with day, family by food by day, and culture were specified as random effects. The factor

culture was nested within family and food. I defined "plasticity" as a difference between food levels (low minus high) in total arm length (postoral plus anterolateral), and "adaptive plasticity" as a positive difference in this measure between low and high food levels, on any given day. Arm and body length values for individual larvae were natural log transformed prior to analysis to meet the assumptions of normality.

Previous studies have demonstrated that larval arm length plasticity is most apparent early in development (Boidron-Metairon, 1988; Hart & Scheibling, 1988; Hart & Strathmann, 1994; Eckert, 1995; Sewell et al., 2004; Podolsky & McAlister, 2005). In a prior study on a different population of *Lytechinus variegatus*, adaptive plasticity was significant on day 4, but not when next measured on day 7 (Boidron-Metairon, 1988). To determine on what days of development low-fed larvae had longer arms than high-fed larvae (i.e., when adaptive plasticity was apparent), I calculated the mean across all 29 families of both the absolute difference in total arm length and the percentage difference (relative to their mean) between food treatments on each measurement day. Using these mean values, I determined the range of plasticity expressed across all families on days 2, 4, 6, and 8 and calculated an overall mean percent difference in arm length for each day.

To test for a cost of plasticity, I used the method of Dewitt et al. (1998) to determine whether more-plastic genotypes had lower fitness measures than less-plastic genotypes, controlling for phenotype. Analyzing the two food levels separately, I first regressed family mean fitness (total energy content on day 14 or stomach length on day 8) on family mean phenotype (total larval arm length). I then plotted the residuals from this regression against each family's degree of plasticity (mean arm length in low food minus mean arm length in high food). A significantly negative association between fitness residuals and the degree of

plasticity would support the hypothesis of a cost of plasticity. Alternatively, a positive relationship, i.e. lower fitness for less-plastic genotypes, would be consistent with a cost of canalization or homeostasis for those traits (Dorn et al., 2000; Poulton & Winn, 2002). For each food level environment, I tested the regression coefficient from this analysis using a two-tailed significance test (SPSS, Inc. Chicago).

In addition to using fitness residuals, I also tested the hypothesis that fitness (uncorrected for arm length) is correlated with arm length plasticity. I regressed family mean fitness (total energy content on day 14 or stomach length on day 8) against each family's degree of plasticity, as calculated above. For each food level environment, I tested the regression coefficient from this analysis using a two-tailed test. Finally, in order to control for body size, I repeated each of the analyses described above using the arm to body length ratio, i.e. relative arm length (to correct arm length for body size), instead of absolute arm length.

One assumption of this analysis is that genotypes are distributed homogeneously across phenotypic space. I tested this assumption by regressing mean trait value against the degree of plasticity for each family. Mean trait value (arm length on day 6) was calculated by averaging the natural logs of arm length values of all individuals within each family reared in either food environment. Degree of plasticity was calculated for each family as described above. I tested the regression coefficient from this analysis using a two-tailed significance test (SPSS, Inc. Chicago).

Results

ANOVA detected significant fixed effects of food, day, body length, and the interaction of food with day. ANOVA detected significant covariance parameter estimates for the random effects of family with day interaction and culture (nested in family with food interaction). ANOVA detected marginally significant covariance parameter estimates due to the random effects of family and family with food interaction. ANOVA did not detect a significant covariance parameter estimate due to the random three-way interaction effect of family by food by day (Table 2.1). Larvae from both low and high food treatments had developed by day 2 to the four arm stage (postoral and anterolateral arm pairs present) and by day 6 to the eight arm stage (postoral, anterolateral, posterolateral, and posterodorsal arm pairs present). Low-fed larvae had longer arms than high-fed larvae on days 2, 4, and 6 (Fig. 2.2). Similarly, calculation of the mean percent difference between low and high food in absolute arm length averaged across all families indicates that low fed larvae had longer arms than high fed larvae through day 6: day 2 mean 4.89% (range -6.63% to 16.46%); day 4 mean 11.08% (range -7.59% to 35.30%); day 6 mean 11.77% (range -5.20% to 26.59%); day 8 mean -2.45% (range -15.17% to 21.62%). High-fed larvae had developed longer arms than low-fed larvae (negative value) by day 8, likely because food is both the cue that induces plasticity in arm length and one resource used for arm growth. After 14 days, high-fed larvae had initiated formation of the juvenile rudiment, representative of a shift in investment from larval to juvenile structures.

Table 2.1: Analysis of Variance (ANOVA) results among 29 full-sib half-sib families. Dependent variable is total arm length with body length as a quantitative covariate.

Type 3 Tests of Fixed Effects

Effect	df Num	df Den	F value	Pr > F
Food	1	115	91.22	<.0001
Day	3	339	190.94	<.0001
Food*Day	3	339	33.89	<.0001
Body Length	1	1265	1822.66	<.0001

Covariance Parameter Estimates of Random Effects

Covariance Parameter	Estimate	Std. Err.	Z value	Pr Z
Family	.000595	.000402	1.48	0.0694
Family*Food	.000285	.000210	1.36	0.0870
Family*Day	.001854	.000331	5.60	<.0001
Family*Food*Day	.000038	.000094	0.41	0.3422
Culture (Family*Food)	.000551	.000155	3.54	0.0002

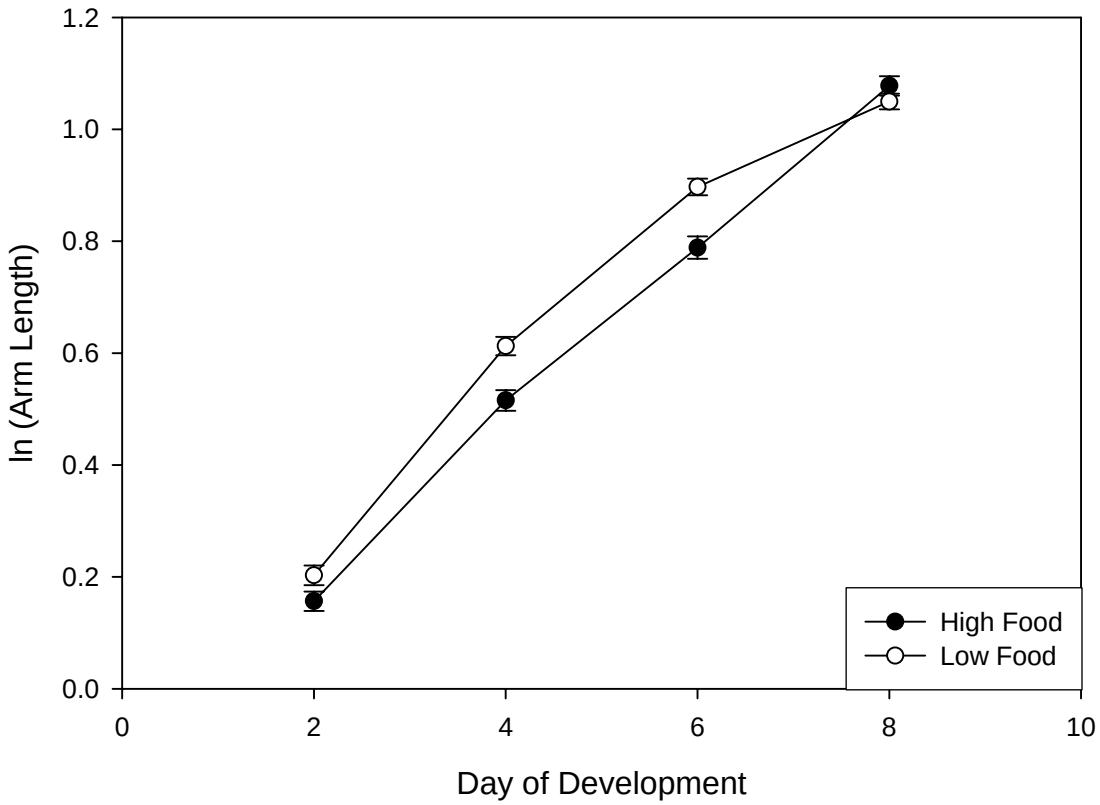


Figure 2.2: Mean summed length of Postoral and Anterolateral arms (± 2SE) for all High-fed (*filled* symbols) and Low-fed (*open* symbols) larvae (averaged across all 29 families) over time. Arm length values for individual larvae were natural log transformed before means were calculated.

Averaged across families, the degree of plasticity of larval arm length was greatest on day 6. Figure 2.3 depicts differences among families over time between low-fed and high-fed larval arm length. Positive values indicate adaptive phenotypic plasticity, i.e. low-fed larvae have longer arms than high-fed larvae at a given age; the largest positive deviation from zero occurs on day 6.

Costs of Plasticity

Energy content and arm length were marginally negatively associated in the low food environment ($F_{1, 27} = 3.158$, $p = 0.087$, $R^2 = 0.1047$) and unassociated in the high food environment, ($F_{1, 28} = 0.015$, $p = 0.995$, $R^2 = 0.0005$) (Fig. 2.4A). Similarly, stomach length was significantly negatively associated with arm length in the low food environment ($F_{1, 27} = 4.836$, $p = 0.037$, $R^2 = 0.1519$) and unassociated in the high food environment ($F_{1, 28} = 2.227$, $p = 0.147$, $R^2 = 0.0737$) (Fig. 2.4B).

Using the residuals obtained from the regressions of energy content and stomach length on arm length (Figs. 2.4A and 2.4B), I tested for a cost of plasticity in larval arm length (Figs. 2.5A and 2.5B). The energy content residuals were not significantly associated with the degree of larval arm length plasticity in either the low food ($F_{1, 27} = 0.000$, $p = 0.995$, $R^2 = 1E\text{-}06$) or high food ($F_{1, 27} = 0.171$, $p = 0.683$, $R^2 = 0.0063$) environments (Fig. 2.5A). The stomach length fitness residuals were significantly positively associated with the degree of larval arm length plasticity in the low food environment ($F_{1, 27} = 8.393$, $p = 0.007$, $R^2 = 0.2371$) and not significantly associated in the high food environment ($F_{1, 27} = 0.209$, $p = 0.651$, $R^2 = 0.0077$) (Fig. 2.5B).

Separate analyses of the association between fitness and degree of plasticity revealed that energy content (not residuals) was not associated with the degree of larval arm length plasticity in either low food ($F_{1, 27} = 0.277$, $p = 0.603$, $R^2 = 0.0102$) or high food ($F_{1, 27} = 0.111$, $p = 0.742$, $R^2 = 0.0041$) environments. Similarly, stomach length was not associated with the degree of larval arm length plasticity in either low food ($F_{1, 27} = 3.261$, $p = 0.082$, $R^2 = 0.1078$) or high food ($F_{1, 27} = 1.975$, $p = 0.171$, $R^2 = 0.0682$) environments. Furthermore, I obtained results that were qualitatively the same as those described above when I conducted each of the cost of plasticity analyses using energy content or stomach length as measures of fitness with arm length corrected for body size (relative arm length) as the phenotype. Finally, our test of the assumption that genotypes are distributed homogeneously across phenotypic space revealed no association between mean trait value (arm length on day 6) across environments and degree of plasticity ($F_{1, 27} = 2.489$, $p = 0.126$, $R^2 = 0.0844$).

Discussion

Plasticity of Arm Length

My results demonstrate phenotypic plasticity of larval arm length in response to food level, as found in previous studies of echinoid (Boidron-Metairon, 1988; Fenaux et al., 1988; Hart & Scheibling, 1988; Strathmann et al., 1992; Hart & Strathmann, 1994; Sewell et al., 2004) and ophiuroid (Podolsky & McAlister, 2005) pluteus larvae. As in other species, the expression of this plastic response appears to be restricted to early development in *Lytechinus variegatus*: low-fed larvae had longer arms through day 6 but not on day 8. Although morphological measurements were not collected after day 8, high-fed larvae appeared to have longer arms than low-fed larvae through day 14 when the experiment was terminated.

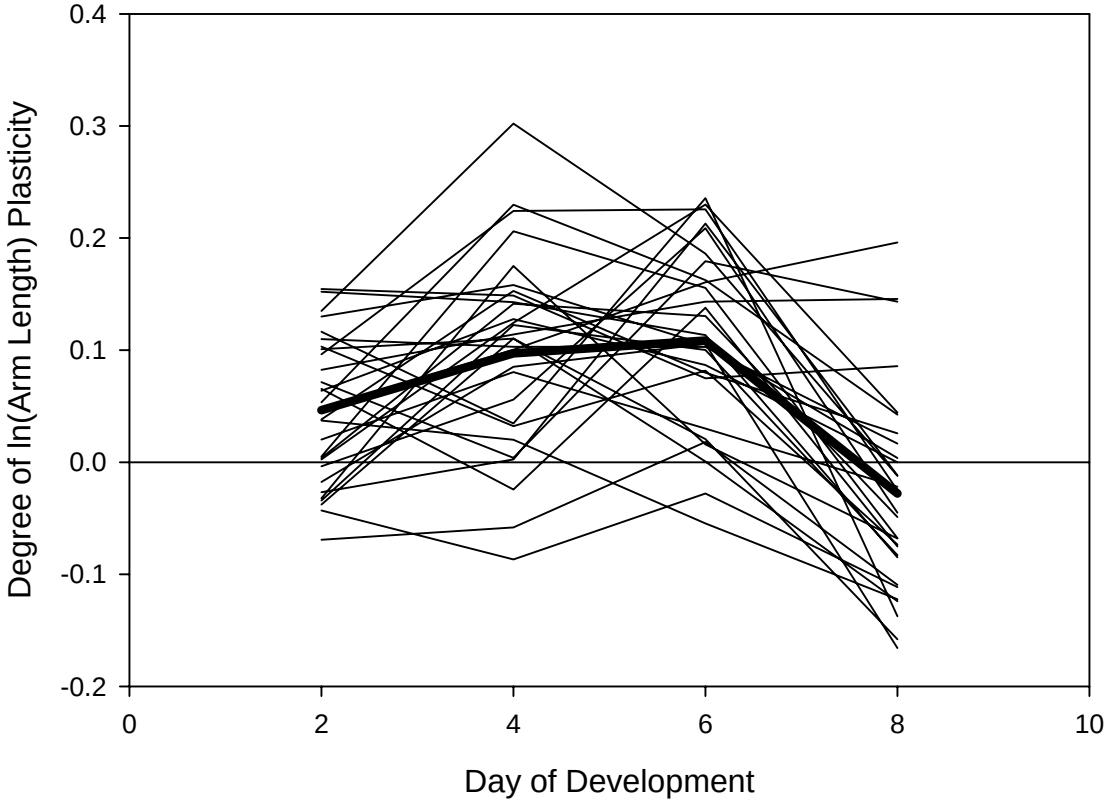


Figure 2.3: Degree of plasticity for 29 larval families over time. Degree of plasticity was calculated by subtracting the mean natural log-transformed arm lengths expressed by larvae reared in the high food environment from the mean natural log-transformed arm lengths expressed by larvae reared in the low food environment for each family. Each thin line represents one family. The mean degree of plasticity averaged across all 29 families is indicated by the bold line. Positive deviations from zero, with a maximum mean deviation on day 6, indicate low-fed larvae have longer arms than high-fed larvae.

Figure 2.4: A: Total energy content on day 14 as a function of arm length (Sum of the Postoral and Anterolateral arms) on day 6. **B:** Fitness (stomach length on day 8) versus arm length (Sum of the Postoral and Anterolateral arms on day 6). Values of energetic content, stomach length, and arm length for individual larvae were natural log-transformed before means were calculated. Each symbol represents the mean for one of 29 families used in this analysis. Filled symbols indicate larval families reared in high food. Open symbols indicate larval families reared in low food. Filled and open symbols of the same shape indicate families sharing the same mother (dam). Five separate dams were used (one half-block, representing one dam, was lost). The linear regression equations are for A: High food $y = 0.0861x + 0.1988$ and Low food $y = -2.1862x + 1.7268$; and for B: High food $y = -0.2277x - 1.3071$ and Low food $y = -0.3834x - 1.3047$.

Figure 2.4A

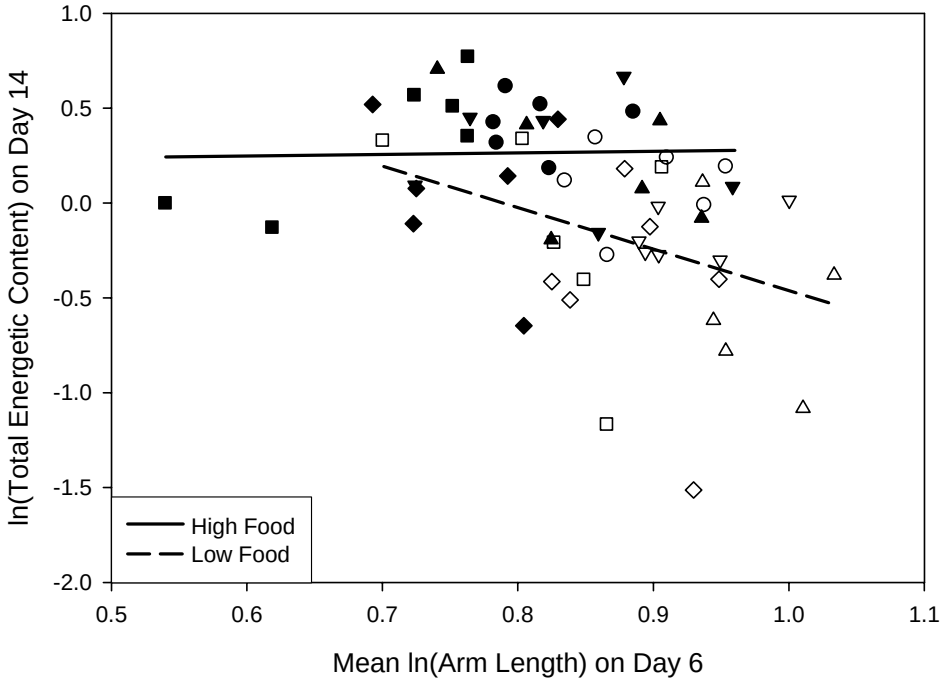


Figure 2.4B

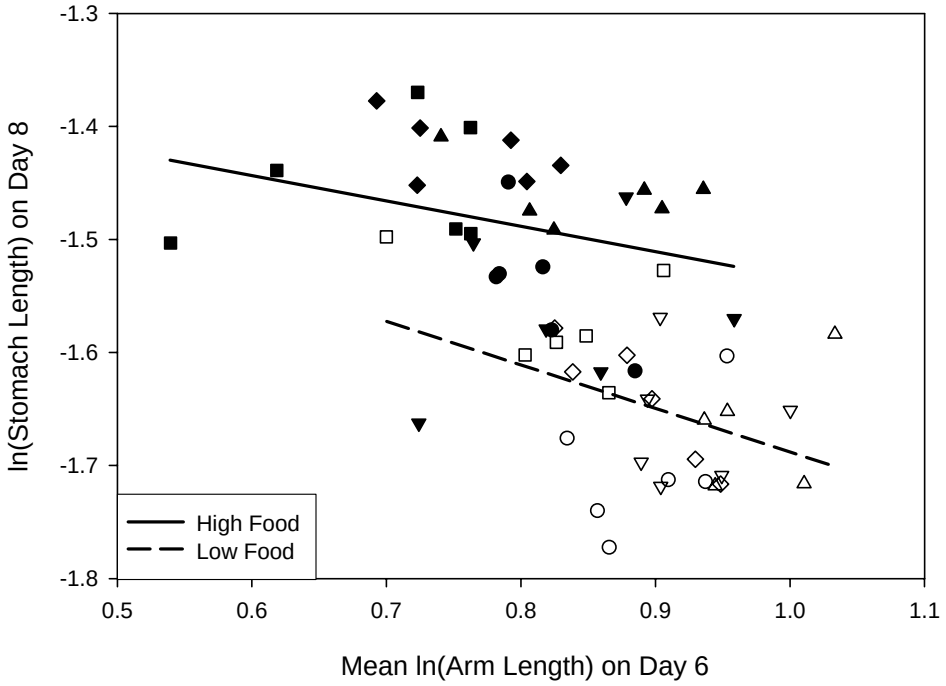


Figure 2.5: A: Fitness residual (from regression in Fig. 4A) versus degree of plasticity. **B:** Fitness residual (from regression in Fig. 4B) versus degree of plasticity. Each point represents one of 29 families used in this analysis. Values of arm length for individual larvae were natural log-transformed before degree of plasticity was calculated. Each symbol represents the mean for one of 29 families used in this analysis. Filled symbols indicate larval families reared in high food. Open symbols indicate larval families reared in low food. Filled and open symbols of the same shape indicate families sharing the same mother (dam). Five separate dams were used in this study. The linear regression equations are for A: High food $y = 0.3294x – 0.0297$ and Low food $y = -0.0062x + 0.0007$; and for B: High food $y = 0.079x – 0.0106$ and Low food $y = 0.3805x – 0.0423$.

Figure 2.5A

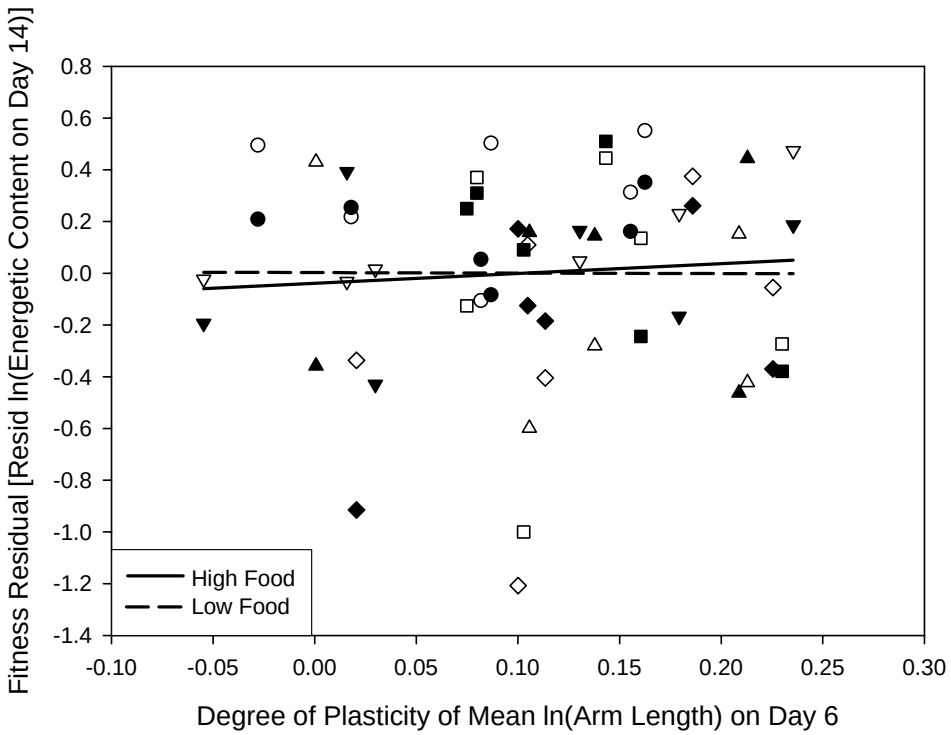
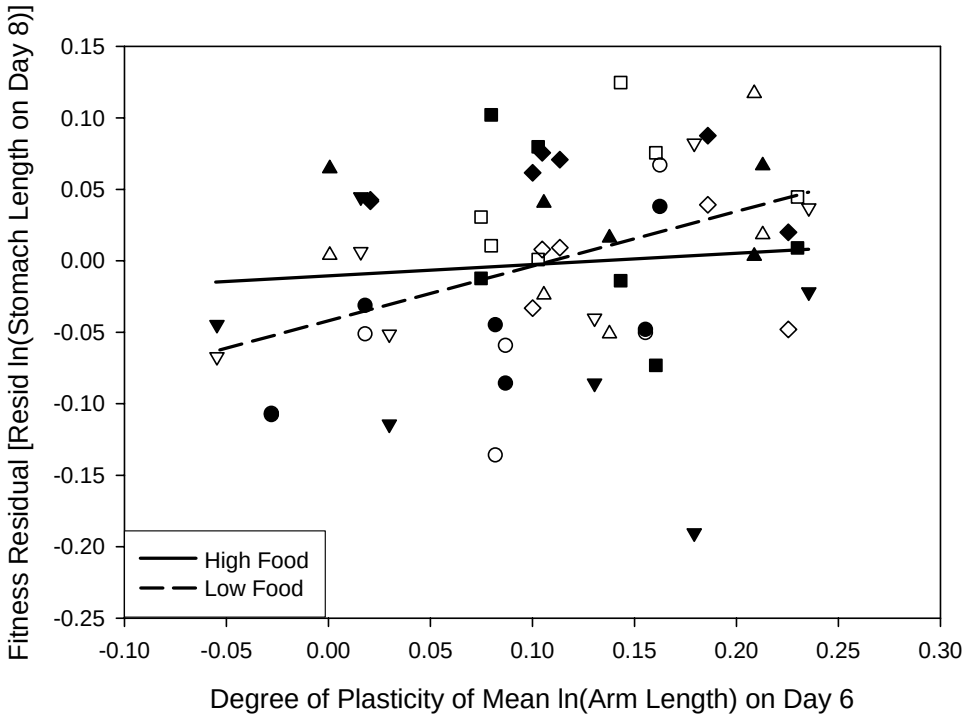


Figure 2.5B



These results are similar to those of Boidron-Metairon (1988), who found plasticity through day 4 but not on day 7, when next measured, for the same species in a population from Puerto Rico. In addition, the mean values of percent difference in absolute arm length on days 2, 4, and 6, which show a percent increase in arm length for low fed larvae, are comparable to values reported or calculated for pluteus larvae from other echinoid and ophiuroid species (see Podolsky & McAlister, 2005).

Previous studies documenting the expression of larval arm length plasticity have used single (Sewell et al., 2005) or few (Boidron-Metairon, 1988; Hart & Scheibling, 1988; Strathmann et al., 1992; Hart, 1994; Eckert, 1995; Podolsky & McAlister, 2005) male-female crosses (families) and have not focused on genetic variation among male-female pairs (see Podolsky & McAlister, 2005 as an exception). In our study, I obtained measures of larval arm length over time from 29 families from a single population. The results from the ANOVA (PROC GLM) found a marginally significant genotype-by-environment (family by food) interaction for arm length, i.e. genetic variation for plasticity of arm length. Although this result was not significant at an alpha=0.05 level, the result suggests that genetic variation for phenotypic plasticity of feeding structures exists for these organisms.

Arm Length & Fitness

I assessed the relationship between arm length (measured on day 6) on two different fitness measures: total energy content, a measure of growth (on day 14), and stomach length, a measure of relative allocation to post-larval structures (on day 8). I chose to use arm length measures on day 6 because plasticity was maximized on this day when averaged across all families (Fig. 2.3). I chose to measure energy content on day 14 for two reasons. First, I

expected that a fitness benefit of possessing longer arms early in development would manifest itself in a greater total energy content later in development. Second, because I planned to rear a large number of larvae in successive blocks, I decided to halt data collection from each block on a day that roughly corresponded to an easily identifiable developmental landmark. Day 14 provided a good end-point for each block because most high-fed larvae had initiated juvenile rudiment formation at this time. Furthermore, stopping the experiment on this day ensured that larvae in both treatments had not exceeded this developmental landmark. Despite the fact that high and low-fed larvae had reached different developmental stages by day 14, the important comparison in this study was among genotypes within a single environment.

The lack of a significant relationship between arm length and energy content (Fig. 2.4A) suggests that energy content on Day 14 may not be a relevant measure of fitness for this analysis in this system. Total energy content at this time might not differ as a function of arm length within each food environment if there is a trade-off in allocation to different structures. Miner (2005) demonstrated such a tradeoff between arm length and stomach length across environments on day 5 in larvae of the sea urchins *Strongylocentrotus franciscanus* and *S. purpuratus*. Such a trade-off could mask differences among families because larvae may grow by allocating energy towards the development of arms or other morphological structures, thereby resulting in similar levels of total energy among larvae at this point in development. A more appropriate measure may be total energy content later in larval development or at metamorphosis. Despite the fact that both of my fitness measures were not significantly positively associated with arm length, my results do suggest a trade-off

between larval arm length and stomach length, as high-fed larvae had both larger stomachs and shorter arms than low-fed larvae, consistent with Miner's observation (Fig. 2.4B).

Larval arm length may therefore be more closely related to fitness measures other than total energy content. I addressed this possibility by using stomach length on day 8 as a measure of fitness in our analyses. The larval stomach can serve as a site of lipid storage, which is carried through metamorphosis to the juvenile stage (Burke, 1981). I used stomach length measurements from day 8 because this was the last day from which I obtained morphological data and because stomach length was greatest at this time. Investment in stomach length early in development, although negatively correlated with and occurring at the expense of investment in arm length (Miner, 2005), may translate into higher fitness for an individual later in development or upon metamorphosis.

The trade-off between arm length and stomach length described by Miner (2005) is a between-environment pattern; larvae invest preferentially in one structure or another depending on the environment. Interestingly, I detected a significantly negative within-environment association between stomach length and arm length in low-fed larvae (Fig. 2.4B). This result for low-fed larvae, coupled with the negative, albeit non-significant, association between these two parameters in high-fed larvae, suggests that there may be a negative genetic correlation between these traits.

Analysis of Costs of Plasticity

My analyses detected no significant association between total energy content and the degree of arm length plasticity in high or low food environments (Fig. 2.5A), nor did they detect a significant association between stomach length and the degree of arm length

plasticity in the high food environment (Fig. 2.5B). However, in the low food environment I detected a significantly positive association between stomach length and arm length plasticity (Fig. 2.5B). Although my results show that longer-armed larvae reared in a low food environment have smaller stomachs (Fig. 2.4B), more-plastic families reared in a low food environment have stomachs that are relatively large for their arm lengths (Fig. 2.5B). This result may indicate a possible benefit of plasticity, or a cost of developmental canalization or homeostasis (Dorn et al., 2000; Poulton & Winn, 2002). For example, larvae from more-plastic families may be better able to modulate the distribution of energy reserves towards the production of food gathering (arms) or food processing (stomach) structures in order to more optimally match and benefit from environmental conditions than can less-plastic families.

The lack of a detectable cost of plasticity suggests that plasticity of larval arm length could be more closely associated with a temporal fitness measure, such as development time to metamorphosis or development time to juvenile rudiment formation, than with total energy content or stomach length. Alternatively, plasticity of larval arm length could entail fitness costs or "latent effects" (Pechenik, 2006) that are not realized until after metamorphosis, such as effects on juvenile size, quality, or growth rate. Although previous researchers have demonstrated that food limitation is associated with developmental time to metamorphosis (Strathmann et al., 1992; Fenaux et al., 1994), and that plasticity of arm length is functionally adaptive (Hart & Strathmann, 1994) what remains unknown is whether arm length plasticity affects development time, and is thus evolutionarily adaptive because it has fitness consequences. Furthermore, no study has addressed whether more-plastic genotypes take longer to reach developmental end-points than less-plastic genotypes.

A test of the adaptive nature of the plastic response of arm length to food level would require an uncoupling of the plastic response from the effects of food (Hart & Strathmann, 1994). This could be accomplished by generating larvae that express short arms when reared in low food and the reverse. Results of a study by Heyland & Hodin (2004) investigating the role of thyroid hormones in larval development and metamorphosis suggest that larvae reared with low levels of both food and thyroid hormone develop to metamorphosis via a short-armed phenotype. Their protocol may prove useful for testing the fitness consequences, and for better understanding the costs, of larval arm length plasticity.

Other recent studies that tested for costs of plasticity similarly did not detect costs or have detected costs that do not fully support theoretical predictions regarding their importance (DeWitt, 1998; Scheiner & Berrigan, 1998; Tucić et al., 1998; Dorn et al., 2000; van Kleunen et al., 2000; Tucić & Stojković, 2001; Agrawal et al., 2002; Poulton & Winn, 2002; Relyea, 2002; Steinger et al., 2003; Merila et al., 2004). My study contributes to this growing body of evidence that costs of plasticity are absent or are difficult to detect. An important limitation of most empirical studies is that they only consider the range of plasticity currently expressed in populations; experimental manipulations of plasticity using hormones or genetic engineering to increase the range of plasticity expression may prove more fruitful in revealing costs of plasticity (Reznick & Ghalambor, 2001; Heyland & Hodin, 2004; van Kleunen & Fischer, 2005).

CHAPTER THREE

EVOLUTIONARY RESPONSES TO ENVIRONMENTAL HETEROGENEITY IN CENTRAL AMERICAN ECHINOID LARVAE: PLASTIC VERSUS CONSTANT PHENOTYPES.

Summary

Do changes in food resources lead to evolutionary changes in phenotypic plasticity or in different constant phenotypes? I addressed this question by studying plasticity of larval feeding arms for "geminate species pairs" in three echinoid genera. These closely related species were geographically isolated when the Panamanian Isthmus raised 2.8-3.1 million years ago, creating two different food level environments: high but variable food levels in the eastern Pacific versus chronically low food levels in the western Caribbean. I reared larvae of geminate species in different replicated food environments for 10 days post-fertilization, collected morphological measurements of individual arm and body lengths, and calculated degrees of plasticity of relative arm length for each species. In contrast to previous studies with temperate echinoids, there was no significant plasticity of arm length in either the Pacific or Caribbean species considered here. Caribbean species, however, had significantly longer relative arm lengths than Pacific species, regardless of food levels. These results suggest that historical changes in food levels have led to the evolution of constant rather than

plastic differences between Pacific and Caribbean echinoids. The evolution of plasticity may be limited by the timing of reproduction or by egg size in this system.

Introduction

The expression of a phenotype is intricately associated with the environment in which an organism resides. In some cases, the phenotype expressed by a given genotype can be influenced by environmental conditions, a phenomenon known as phenotypic plasticity (Bradshaw 1965; Stearns 1989). Alternatively, a genotype can produce the same phenotype across environments, indicating that the expression of the phenotype is constant. In heterogeneous environments, phenotypic plasticity may allow an organism to maximize fitness (Gotthard and Nylin 1995); given appropriate genetic variability for plasticity and predictable environmental cues in a population, adaptive phenotypic plasticity is expected to evolve (Via et al. 1995). Conversely, expression of a constant phenotype is expected to confer high fitness and to evolve in environments with low heterogeneity and constant environmental characteristics.

The association between environmental changes and the expression of plasticity has been studied at several levels of evolutionary inquiry. Researchers have documented plasticity in response to different environments in many taxa (Boidron-Metairon 1988; Fenaux et al. 1988; Hart and Scheibling 1988; see reviews by Robinson and Wilson 1994; Skúlason and Smith 1995; and Smith and Skúlason 1996, West-Eberhard 2003). In addition, studies have demonstrated variation in the degree of plasticity among populations (or species associations) in response to the degree of variation in the environment (DeBenedictis 1974; Kaitala 1991; Blouin 1992; Leips and Travis 1994; Buchholz and Hayes 2000, 2002; Leips et

al. 2000; Langerhans et al. 2003; Reinikainen and Repka 2003; Morey and Reznick 2004; Stauffer and Van Snik Gray 2004). However, few studies explore whether historical changes in environments are associated with the evolution of phenotypic plasticity or of different constant phenotypes (see Morey and Reznick 2004 for one example). What remains unknown in many systems are the relationships and times of divergence among different species, and how they have adapted to unique habitats since separation. These unknown variables make this level of evolutionary inquiry of greatest interest because no research has demonstrated an association between historical environmental changes and the repeated evolution of plastic or constant phenotypes. A comparison of phenotypic expression between close relatives that occupy habitats with different patterns of resource availability would therefore provide a crucial empirical test of the environmental factors underlying the evolution of alternative mechanisms for the expression of a phenotype.

A comparison of this type is provided by "geminate species pairs," formed when previously continuous species were separated before or during the raising of the Panamanian Isthmus 2.8-3.1 million years ago (Duque-Caro 1990; Keigwin 1982). Geminate species pairs occur in multiple phyla (Jordan 1908), and although their time of divergence is variable (Knowlton and Weigt 1998; Marko and Jackson 2001), they have been evolving in isolation for at least 3 million years since the final rise of the Isthmus (Coates and Obando 1996). The rise of the Isthmus also separated the tropical western Atlantic (the western Caribbean Sea) and tropical eastern Pacific oceans, producing two environments that are markedly different with regard to productivity, which equates to food for plankton-feeding organisms. The eastern Pacific is characterized by strong, seasonal upwelling that produces variable yet predictably high phytoplankton food levels, whereas the western Caribbean experiences little

upwelling, has low primary production, and is thus constantly nutrient poor and low in phytoplankton food (Glynn 1982; Keigwin 1982). Transisthmian geminate species offer a unique, replicated natural research system (Moran 2004) that can be used to address the evolution of adaptive phenotypic plasticity in response to the heterogeneity of food resource levels.

Morphological phenotypic plasticity in response to food level has been demonstrated in planktotrophic pluteus larvae from several species in the echinoderm class Echinoidea (Boidron-Metairon 1988; Strathmann et al. 1992; Hart and Strathmann 1994). These larvae depend on exogenous phytoplankton food, and, in response to low food availability, larvae increase the length of the ciliated band used for collecting food by growing longer larval arms; plasticity of ciliated band length is correlated with lengthening of skeletal arm rods in echinoplutei. Increased ciliated band length enhances larval ability to capture phytoplankton, and increases in ciliated band length under low food conditions have been demonstrated to be adaptive because larvae with longer ciliated bands have greater maximum clearance rates (Hart and Strathmann 1994). In addition, by increasing ciliated band length, the larval surface-to-volume ratio increases, which could increase intake of dissolved organic matter (Manahan et al. 1983). For this reason, plasticity in arm length has been used as a measure of larval feeding history in the field (Strathmann et al. 1992). A recent study demonstrates genetic variation of larval arm length plasticity in response to food limitation in the echinoid *Lytechinus variegatus* (McAlister unpub. data).

Here I examine the evolution of phenotypic plasticity of larval feeding structures in response to differences in environmental heterogeneity for planktotrophic larvae of the echinoid geminate species pairs found off the coasts of Panama. For each geminate pair, one

species lives in the highly productive but variable eastern Pacific, while the other inhabits the minimally productive and constant western Caribbean. Three sets of hypotheses can be made regarding the effect of food level heterogeneity on plasticity of larval arm length. First, the "Plasticity" Hypothesis posits that all species will exhibit some degree of phenotypic plasticity of larval arm length. This expectation can be justified by the fact that plasticity of larval arm length has been demonstrated in a large number of echinoid species in which it has been examined (Boidron-Metairon 1988; Hart and Scheibling, 1988; Strathmann et al. 1992; Hart and Strathmann 1994; Sewell et al. 2004; Reitzel and Heyland 2007).

Second, the "Differential Plasticity" Hypothesis posits that larvae evolving in the western Caribbean, which has constant low phytoplankton food levels, will exhibit low to no degrees of phenotypic plasticity of arm length. Conversely, larvae evolving in the eastern Pacific, characterized by variable phytoplankton food levels, will exhibit greater degrees of phenotypic plasticity of arm length. In support of this hypothesis, larval echinoid species from tropical or subtropical waters with low food levels show minimal plasticity (Boidron-Metairon 1988; Eckert 1995; Reitzel and Heyland 2007), whereas species from cold temperate waters with more variable food levels show greater degrees of plasticity (Boidron-Metairon 1988; Hart and Scheibling 1988). None of these studies are comparative or examined many taxa however.

Third, the "Constant Differences" Hypothesis posits that larvae evolving under constantly low food levels, characteristic of the western Caribbean, will grow longer arms relative to body length than larvae evolving in the variable food levels of the eastern Pacific. If phenotypic plasticity confers a benefit only in heterogeneous environments, then there may be no benefit of plasticity for larvae evolving in the homogeneous environment of the

Caribbean. A better evolutionary strategy for resource acquisition may be to evolve longer arms under all conditions, especially if there is a cost of phenotypic plasticity (DeWitt et al. 1998). The number of examples in the literature is too small to thoroughly test the patterns described by these hypotheses, nor have these ideas been tested in a rigorous phylogenetic context. My results indicate that historical changes in food availability can lead to the repeated evolution of differences in the expression of constant phenotypes between species, and suggest that the evolution of phenotypic plasticity may hinge in part on selection for other life history characteristics associated with resource acquisition, e.g. egg size.

Materials and Methods

I investigated whether heterogeneity of food level is correlated with the expression of plastic and/or constant larval arm length by studying three geminate pairs of marine sea urchins in the genera *Diadema*, *Echinometra*, and *Eucidaris*. These species are found in coral reef habitats off the Caribbean and Pacific coasts of the Republic of Panama (Lessios 1979; Lessios 1981; Bermingham and Lessios 1993; McCartney et al. 2000). I performed two sets of experiments over the course of two summer field seasons in Panama. The first set of experiments examined larval morphological plasticity under two different food levels in two true geminate pairs, *Diadema antillarum* in the Caribbean with *D. mexicanum* in the Pacific and *Eucidaris tribuloides* in the Caribbean with *Eu. thouarsi* in the Pacific; genetic divergence among these species pairs is pegged to the final closure of the Central American Seaway approximately 2.8-3.1 million years ago (Lessios et al. 1999; 2001). In addition, I included in this experiment the *Echinometra* complex: *Ec. lucunter* and sister taxa *Ec. viridis* in the Caribbean with *Ec. vanbrunti* in the Pacific. The most recent common ancestor of *Ec.*

lucunter and *Ec. viridis* is thought to be the geminate partner of *Ec. vanbrunti*, diverging approximately 3.1 million years ago; *Ec. lucunter* and *Ec. viridis* diverged approximately 1.27-1.62 million years ago (McCartney et al. 2000). Although these three pairings are not the only echinoid geminates, they represent the genera with planktotrophic larvae that are most easily collected and spawned, and were therefore most amenable to this analysis. A second set of experiments examined the effects of food limitation on growth of *Ec. vanbrunti* and *Ec. viridis* larvae reared in one of five different food levels, including satiating and starvation conditions.

Adults of the sea urchins *D. mexicanum*, *Ec. vanbrunti*, and *Eu. thouarsi* were collected from the Pacific Ocean in June and July 2005 by SCUBA from populations located in waters off Isla Taboguilla near Panama City, Panama (see Figure 3.1). Pacific species were placed in coolers filled with seawater and transported by boat to the Smithsonian Tropical Research Institute's (STRI) Naos Island Laboratories (Naos) near Panama City. Adults of their geminate species counterparts, *D. antillarum*, *Ec. lucunter*, *Ec. viridis*, and *Eu. tribuloides* were collected from the Caribbean Sea by snorkel in the vicinity of STRI's Galeta Marine Laboratory near Colon, Panama (see Figure 3.1). Caribbean species were placed in disposable plastic containers (3-4 urchins per container) filled with a small amount of seawater. The containers holding Caribbean urchins were stacked in a cooler and transported by vehicle to Naos. All species were maintained in flow-through seawater aquaria at Naos.

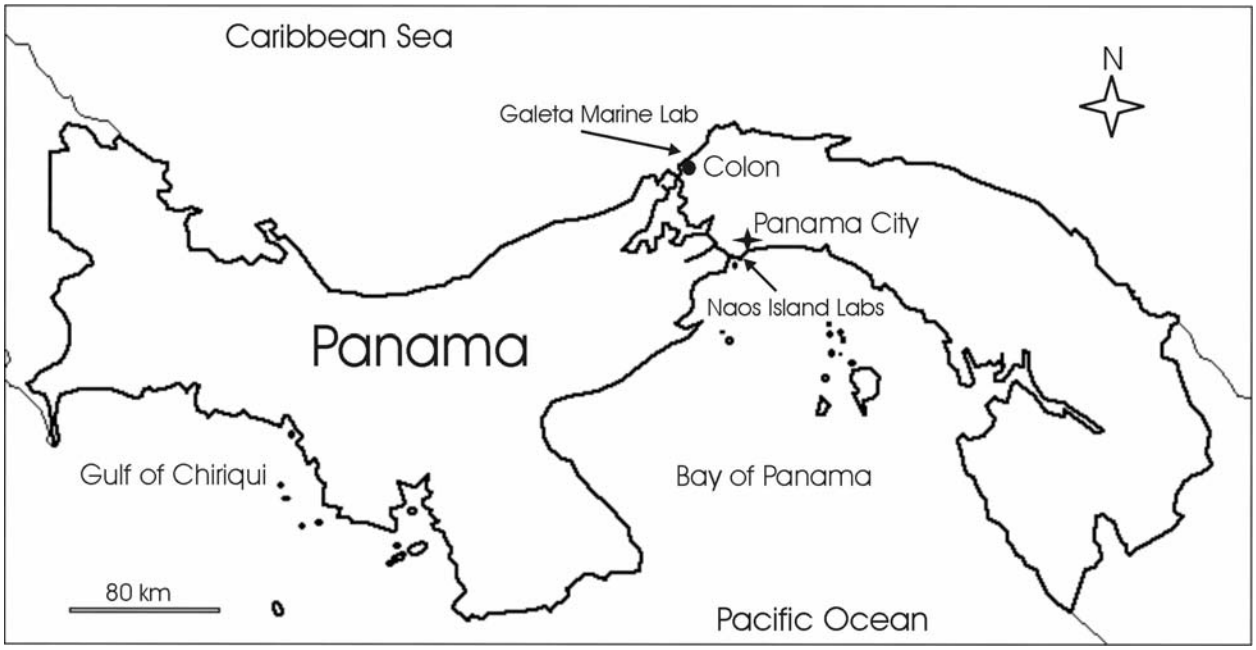


Figure 3.1: Map of the Republic of Panama indicating the locations of the Smithsonian Tropical Research Institute's Galeta Marine Laboratory on the Caribbean coast and the Naos Island Laboratories on the Pacific coast. Adult urchins used to obtain gametes and produce larvae for this study were collected in waters in the immediate vicinity of Galeta Marine Laboratory and at Isla Taboguilla, located approximately 10 km offshore from Naos Island Laboratories.

Larval culture

Gametes were obtained from adult urchins by injecting approximately 1ml of 0.5M KCl through the peristomium into the body cavity. Eggs were collected and washed once in 0.45µm filtered seawater and sperm were collected by mouth pipette and kept on ice until use. Full-sib larval families of all species were established by separately fertilizing eggs from 1 female with sperm from 1 male. Four separate full-sib families were established for *Ec. lucunter*. Three separate full-sib families were established for *D. mexicanum*, *Ec. vanbrunti*, *Ec. viridis*, and *Eu. tribuloides*. Due to the difficulty in finding reproductively mature adult females, one full-sib family was established for both *D. antillarum* and *Eu. thouarsi*. Initial mean ($\pm$ 1 S.E.) egg diameters (means of 25 eggs each) and egg volumes (assuming a sphere) for females from each species are given in Table 3.1.

Fertilized embryos and larvae of each species were reared in one of two replicated food environments (5 and 1 algal cells/µl). Each food level was then replicated among three cultures. Each larval culture was fed the unicellular alga *Dunaliella tertiolecta* (UTEX Algal Supply, Austin, TX) daily, starting at 48h (all ages reported are post-fertilization). All cultures were reared in 0.45µm filtered seawater in 1-l plastic tri-pour beakers at densities of 1 larva ml^{-1} and water was changed every day. The cultures were maintained in a recirculating water bath held at 28°C and were continually stirred at approximately 10 strokes min^{-1} with acrylic paddles to homogenize food and to keep larvae in suspension (Strathmann, 1987). *D. tertiolecta* was cultured at room temperature in microwaved 0.45µm filtered seawater enriched with a modified Guillard's f/2 medium (Florida Aqua Farms, Inc.). Algae were separated from the growth medium by centrifugation and then re-suspended in fresh 0.45µm filtered seawater before use.

Table 3.1: Initial mean (±1SE) egg diameters (**bold** text; units = micrometers) and volumes (normal text; units = nanoliters) for females of each species used to produce different larval families. Values were calculated using 25 eggs from each female.

Species	Ocean	Female used for each Full-Sib Family				
		1	2	3	4	Average
D. antillarum	C	**74.56 (.45)** 0.22 (.00)	N/A	N/A	N/A	**74.56 (.45)** 0.22 (.00)
D. mexicanum	P	**64.96 (.34)** 0.14 (.00)	**64.8 (.45)** 0.14 (.00)	**68.16 (.19)** 0.17 (.00)	N/A	**65.97 (.27)** 0.15 (.00)
E. lucunter	C	**83.2 (.47)** 0.30 (.00)	**82.08 (.40)** 0.29 (.00)	**79.84 (.66)** 0.27 (.00)	**87.28 (.56)** 0.35 (.00)	**83.1 (.38)** 0.30 (.00)
E. viridis	C	**90.8 (.41)** 0.39 (.00)	**90.08 (.38)** 0.38 (.00)	**89.44 (.45)** 0.38 (.00)	N/A	**90.11 (.25)** 0.38 (.00)
E. vanbrunti	P	**67.84 (.19)** 0.16 (.00)	**67.76 (.29)** 0.16 (.00)	**69.76 (.43)** 0.18 (.00)	N/A	**68.46 (.21)** 0.17 (.00)
E. tribuloides	C	**92.64 (.58)** 0.42 (.00)	**92.16 (.70)** 0.41 (.00)	**93.44 (.40)** 0.43 (.00)	N/A	**92.74 (.34)** 0.42 (.00)
E. thouarsi	P	**86.08 (.40)** 0.33 (.00)	N/A	N/A	N/A	**86.08 (.40)** 0.33 (.00)

Measures of Phenotype

On days 2, 3, 4, 5, 6, 8, and 10 approximately 10 larvae were removed from each culture. Larvae were placed on a glass slide, immobilized with a dilute (<10%) solution of buffered formalin in seawater, and covered with a glass cover slip raised on clay feet. Three-dimensional Cartesian coordinates were recorded of multiple morphological features for 5 larvae from each culture (Figure 3.2). These landmarks included the tip and base of each anterolateral, postoral, posterolateral, and posterodorsal arm rod, the posterior tip of the larva, and the tip of the oral hood (i.e. the mid-point of the soft-tissue that stretches between the pair of anterolateral arms). To collect data from each larva, I used a camera lucida (drawing tube) and a digitizing tablet (Hyperpen 12000U, Aiptek Inc.) to capture *x* and *y* coordinates of morphological landmarks. Simultaneously, I obtained *z* coordinates from a rotary encoder (U.S. Digital) coupled to the fine focus knob of a Wild M-20 compound microscope (McEdward 1985). Using these 3-D Cartesian coordinates, I geometrically reconstructed individual arm and body lengths (measured in millimeters) for each larva. Because the postoral arms were the first arm pair to develop in all species used in this study, and were the most prominent arms at all developmental stages when I collected measurements, my analysis focuses on plasticity in their summed length ("sum of postoral arms").

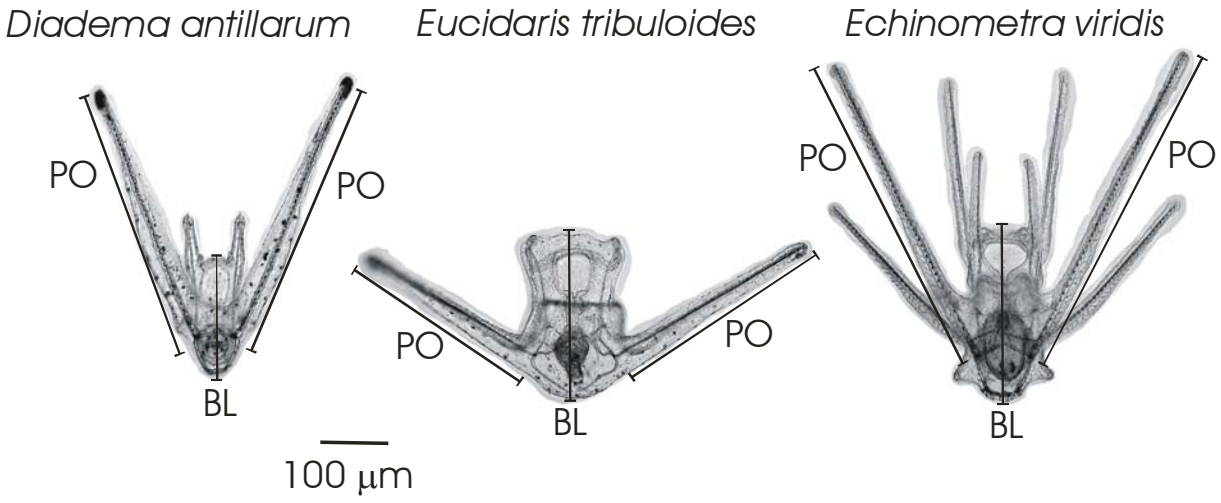


Figure 3.2: Low-fed *Diadema antillarum*, *Echinometra viridis*, and *Eucidaris thouarsi* larvae at 10 days of development post-fertilization. Morphological characters that I measured on days 2, 3, 4, 5, 6, 8, and 10: PO = Postoral arm, BL = Body length at midline. All larvae are displayed at the same magnification; scale bar represents 100 microns.

Statistical Analysis

Analysis of variance (PROC MIXED: SAS Institute, Cary, NC) tests were conducted 1) across all species and geminate pairs ("ocean analysis") and 2) for each geminate species pairing ("paired species analyses"), using the natural log corrected sum of the postoral arm lengths (arm length) as the response variable in all statistical models. For the ocean analysis, I tested for the effect of variation among ocean, genus, family, day of development (day), food level (food), and culture replicate (culture) on arm length. The statistical model included the following interaction terms: ocean with food, ocean with day, day with food, ocean with genus, genus with food, and the three-way interactions of ocean by day by food and ocean by genus by food. Ocean, genus, day, food, and the interaction terms were coded as fixed effects and family and culture as random effects. The factor culture was nested within ocean, family, and food.

For the paired species analyses, I tested for the effect of variation among species, family, day, food, and culture on arm length. The model included terms to account for variation due to the interaction of species with food, day with food, species with day, and the three-way interaction of species by day by food. Species, day, food, and the interaction terms were coded as fixed effects and family and culture as random effects. The factor culture was nested within species, family, and food. The following paired species analyses were conducted: *D. antillarum – D. mexicanum*; *Ec. vanbrunti – Ec. lucunter*; *Ec. vanbrunti – Ec. viridis*; and *Eu. thouarsi – Eu. tribuloides*.

In both the ocean analysis and the paired species analyses, day was coded as a repeated measure with culture as the subject; the type of covariance structure of the R matrix was specified as Compound Symmetry (CS). Degrees of freedom were calculated using the

DDFM=BW (Between-Within) option in PROC MIXED. Natural log corrected midline body length (body length) was included in all models as a quantitative covariate. I compared models both with and without the body length interaction terms and used the models (no interaction terms) that provided the better fit to the data using Akaike's information criteria (AIC) (Littell et al., 1996).

Test of food limitation

I conducted a second experiment to test the effects on larval development of food levels lower than 1 algal cell/µl. Adult *Ec. vanbrunti* and *Ec. viridis* sea urchins were collected in August 2006 from the same respective Pacific and Caribbean field sites as described for the 2005 study (see above). Transportation of adult urchins to Naos and their maintenance in flow-through seawater aquaria were similar for this experiment. Gametes were obtained from adult urchins by peristomial injection of 0.5M KCl. Fertilizations were conducted by combining eggs from 7 female with sperm from 4 male *Ec. viridis*, and in a separate container, eggs from 2 female with sperm from 4 male *Ec. vanbrunti*. Initial mean (± 1 S.E.) egg diameters (means of 10 eggs each) for *Ec. vanbrunti* females were 70.17 (± 0.45) and for *Ec. viridis* females were 86.53 (± 0.35). Assuming a sphere, mean egg volumes (± 1 S.E.) were 0.18 (± 0.00) and 0.34 (± 0.00) nl, respectively.

Fertilized embryos and larvae of each species were reared in one of five replicated food environments (High - 5, Low - 1, Half - 0.5, Limit - 0.1, and Zero - 0 algal cells/µl). Each food level was then replicated among three cultures. Larval cultures were fed the unicellular alga *D. tertiolecta* (UTEX Algal Supply, Austin, TX) daily, starting at 48h. All cultures were reared in 0.45µm filtered seawater in 1-l plastic tri-pour beakers at densities of

1 larva ml^{-1} and water was changed every day. Larval cultures were maintained in the same manner (i.e. placed in a recirculating water bath held at 28°C, etc.), and *D. tertiolecta* cultures were reared and dispensed to larvae as described for the 2005 experiment.

For this second experiment, measures of phenotype were collected on days 2, 3, 4, 6, and 8. Analyses of variance (PROC MIXED: SAS Institute, Cary, NC) test were conducted between the two species and five food levels using the natural log corrected sum of the postoral arm lengths (arm length) and/or midline body length (body length) as the response variables in the statistical models. I tested for the effect of variation among species, day, food, and culture on arm length in one ANOVA and on body length in a separate ANOVA. The statistical models included terms to account for variation due to the interaction of species with food, day with food, species with day and the three-way interaction of species by day by food. Species, day, food, and the interaction terms were coded as fixed effects and culture as a random effect. The factor culture was nested within species and food. An analysis of variance (PROC MIXED) was also conducted between the two species and only the high and zero food levels using arm length as the response variable. Body length was included in the models testing for differences in arm length as a known quantitative covariate. In all statistical models for the food limitation experiment, day was coded as a repeated measure with culture as the subject and the covariance structure of the R matrix was specified as Compound Symmetry (CS). Degrees of freedom were calculated using the DDFM=BW (Between-Within) option in PROC MIXED.

Results

ANOVA among larvae from all Pacific and all Caribbean species (the ocean analysis) fed High (5 algal cells / µl) or Low (1 algal cell / µl) food levels in the 2005 experiment using arm length as the response variable detected significant effects due to genus, ocean, day, body length, and the interactions of ocean with day and ocean with genus (Table 3.2). There was no effect due to food, the interactions of ocean with food, day with food, genus with food, or to the three-way interactions of ocean by day by food and ocean by genus by food. The least square mean ($\pm$ 1 S.E.: units = ln mm) estimate of arm length corrected for body length was -0.5542 $\pm$ 0.062 (t_{38}=-8.96; $p < 0.0001$) for the Caribbean and -0.6314 $\pm$ 0.063 (t_{38}=-10.03; $p < 0.0001$) for the Pacific.

Longer absolute arm lengths were expressed by Caribbean species of the genera *Echinometra* (Figure 3.3A) and *Diadema* (Figure 3.4A) over all developmental days post-fertilization as compared to their Pacific geminate counterparts. Conversely, longer absolute arm lengths were expressed by the Pacific *Eucidaris thouarsi* through Day 6 (Figure 3.5A); between Day 6 and Day 10, the Caribbean *Eu. tribuloides* exhibited an increase in absolute arm length (Figure 3.5A). Longer relative arm to body lengths were expressed by both Caribbean *Echinometra* species (Figure 3.3B) compared to the Pacific species over all days. A similar pattern was exhibited by the Caribbean *Diadema antillarum* as compared to the Pacific *D. mexicanum* after approximately 4-5 days of development (Figure 3.4B). Trajectories of arm to body length for both *Eucidaris* species indicate that larvae of the Caribbean *Eu. tribuloides* have larger bodies than the Pacific *Eu. thouarsi* throughout the period of measurement (Figure 3.5B). The distinct arm length relative to body length growth

Table 3.2: Analysis of Variance (ANOVA) results for all Caribbean versus all Pacific species larvae. Dependent variable is the natural log of the sum of postoral arm lengths. Natural log corrected midline body length was included in the model as a known quantitative covariate.

Effect	**df: N, D**	**F Value**	**Pr > F**
Genus	2, 40	1247.93	<.0001
Ocean	1, 38	9.92	0.0032
Day	6, 228	467.05	<.0001
Food	1, 38	0.33	0.5686
Ocean*Food	1, 38	0.57	0.4556
Ocean*Day	6, 228	10.90	<.0001
Day*Food	6, 228	1.18	0.3156
Ocean*Genus	2, 40	397.93	<.0001
Genus*Food	2, 40	3.13	0.0547
Ocean*Day*Food	6, 228	0.51	0.8025
Ocean*Genus*Food	2, 40	3.01	0.0608
ln (Body Length)	1, 3347	732.62	<.0001

Figure 3.3: A. Mean (± 1SE) natural log corrected summed length of Postoral arms for High-food (*filled* symbols) and Low-food (*open* symbols) larvae from the genus *Echinometra* over time. **B.** Mean (± 1SE) natural log corrected summed length of Postoral arms versus mean (± 1SE) natural log corrected Body Length at midline for High-food (filled symbols) and Low-food (open symbols) larvae from the genus *Echinometra*. In both A and B, circle symbols indicate values for *Echinometra vanbrunti*, triangle symbols indicate values for *Echinometra viridis*, and square symbols indicate values for *Echinometra lucunter*. Units are ln millimeters.

Figure 3.3 A

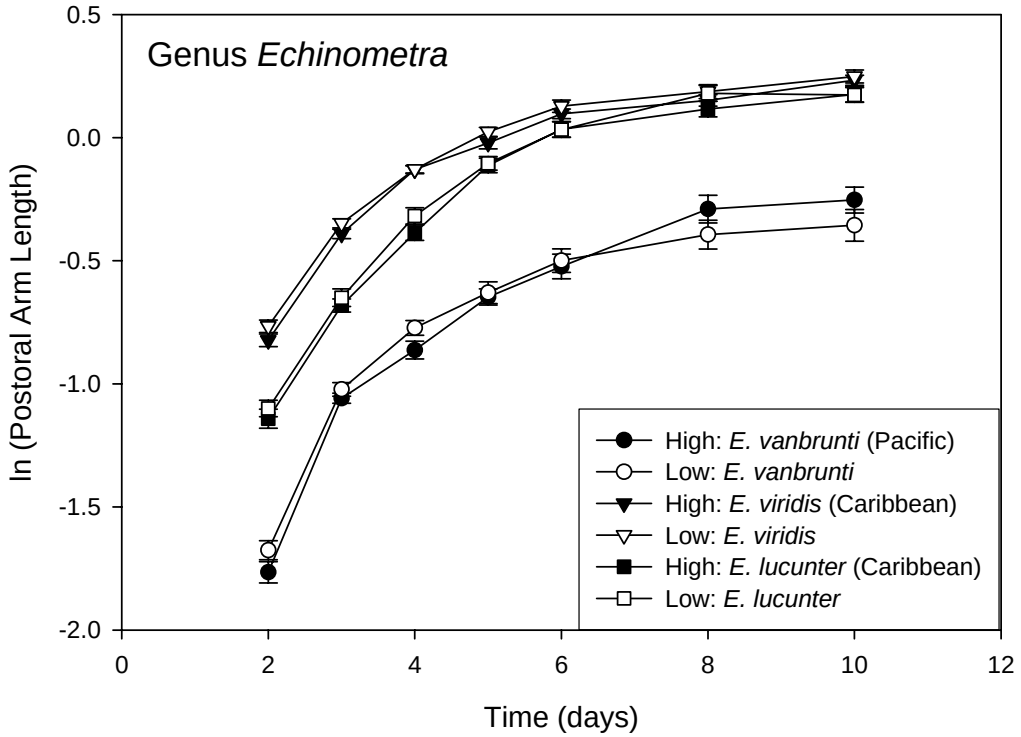


Figure 3.3 B

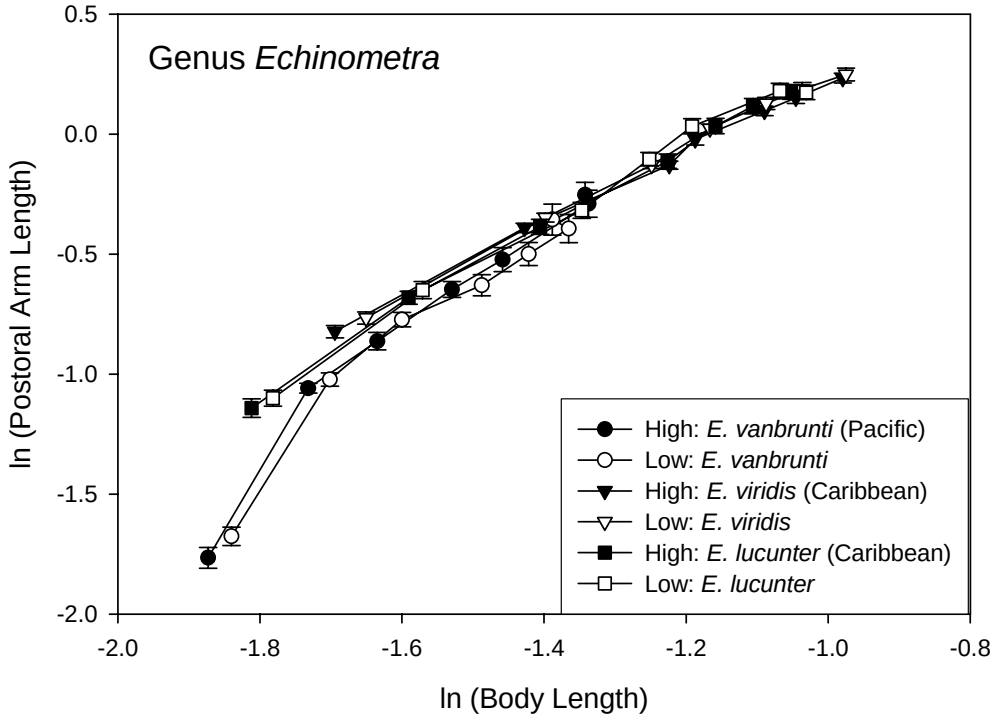


Figure 3.4: **A.** Mean (± 1SE) natural log corrected summed length of Postoral arms for High-food (*filled* symbols) and Low-food (*open* symbols) larvae from the genus *Diadema* over time. **B.** Mean (± 1SE) natural log corrected summed length of Postoral arms versus mean (± 1SE) natural log corrected Body Length at midline for High-food (filled symbols) and Low-food (open symbols) larvae from the genus *Diadema*. In both A and B, circle symbols indicate values for *Diadema mexicanum* and triangle symbols indicate values for *Diadema antillarum*. Units are ln millimeters.

Figure 3.4 A

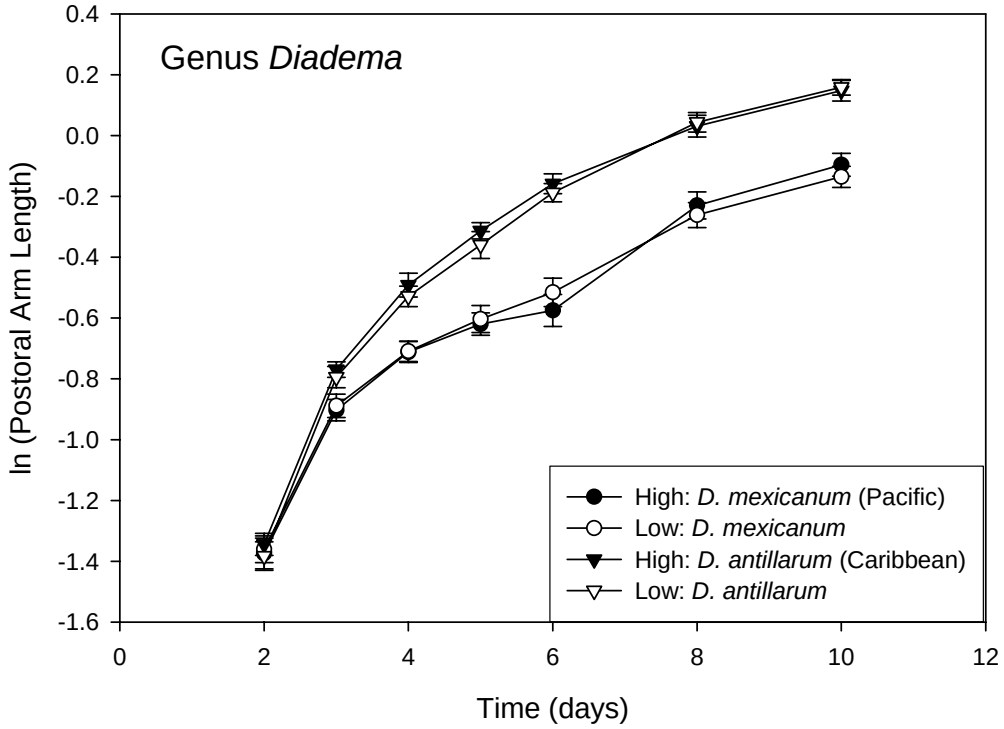


Figure 3.4 B

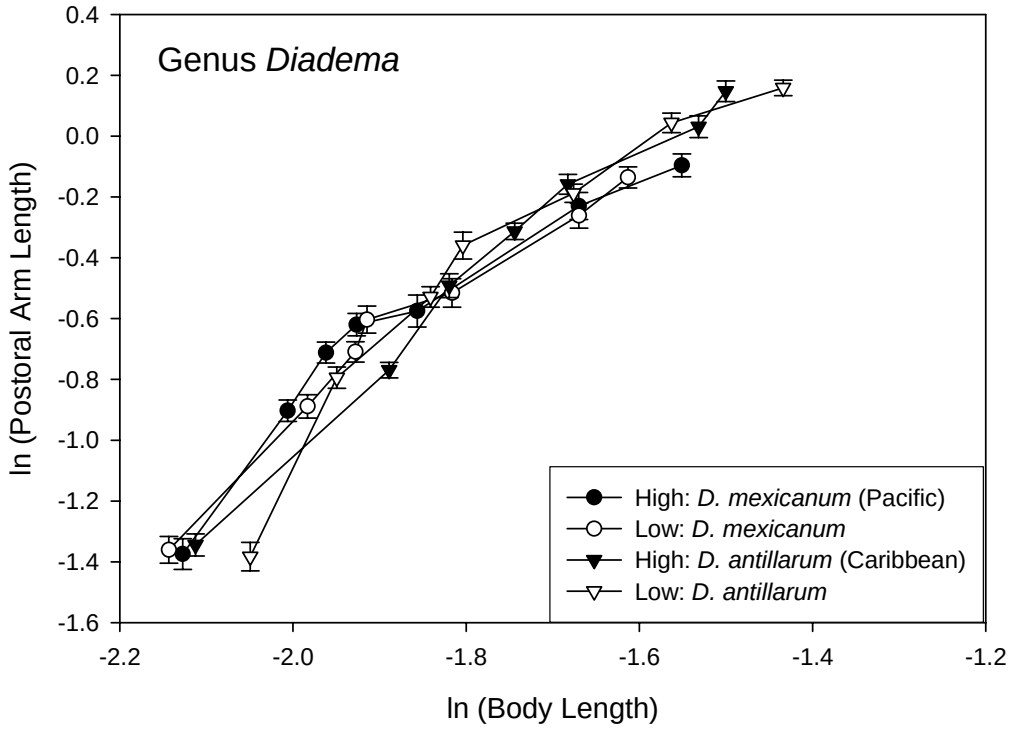


Figure 3.5: A. Mean (± 1SE) natural log corrected summed length of Postoral arms for High-food (*filled* symbols) and Low-food (*open* symbols) larvae from the genus *Eucidaris* over time. **B.** Mean (± 1SE) natural log corrected summed length of Postoral arms versus mean (± 1SE) natural log corrected Body Length at midline for High-food (filled symbols) and Low-food (open symbols) larvae from the genus *Eucidaris*. In both A and B, circle symbols indicate values for *Eucidaris thouarsi* and triangle symbols indicate values for *Eucidaris tribuloides*. Units are ln millimeters.

Figure 3.5 A

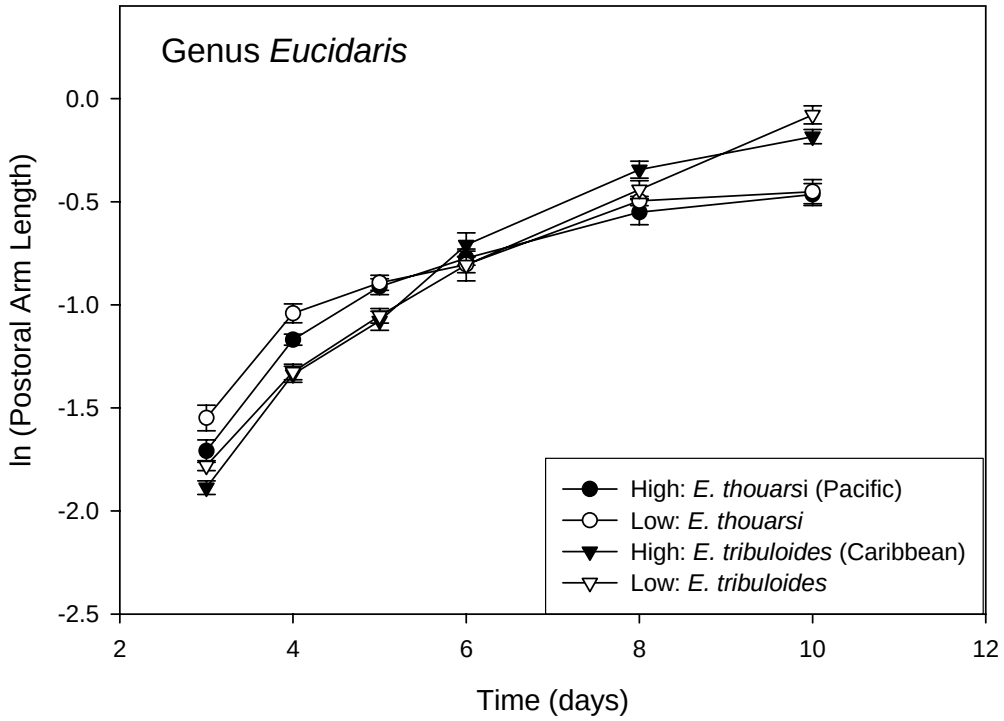


Figure 3.5 B

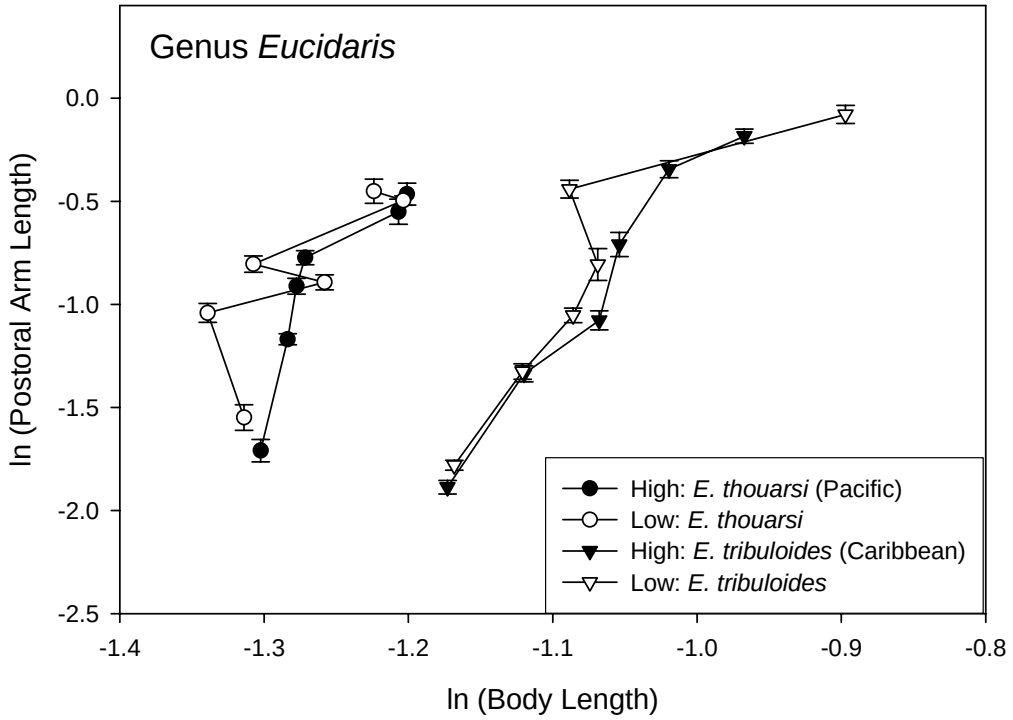


Table 3.3: Analysis of Variance (ANOVA) results for larvae from geminate species pairs i.e. separate tests between a given Caribbean species versus its Pacific species geminate. Dependent variable in each model is the natural log of the sum of postoral arm lengths. Natural log corrected midline body length was included in each model as a known quantitative covariate. Effect abbreviations: S=Species, D=Day, F=Food, lnBL=natural log of body length. Test abbreviations: df=degrees of freedom, n=numerator, d=denominator.

	Species pairs tested: Analysis of Variance											
	E. viridis vs. *E. vanbrunti*			*E. lucunter* vs. *E. vanbrunti*			*D. antillarum* vs. *D. mexicanum*			*E. tribuloides* vs. *E. thouarsi*		
Effect	**df: n, d**	**F value**	**Pr > F**	**df: n, d**	**F value**	**Pr > F**	**df: n, d**	**F value**	**Pr > F**	**df: n, d**	**F value**	**Pr > F**
S	1, 32	153.41	<.0001	1, 38	105.35	<.0001	1, 20	215.43	<.0001	1, 20	17.13	0.0005
D	6, 185	153.97	<.0001	6, 221	197.03	<.0001	6, 109	312.59	<.0001	5, 77	466.66	<.0001
F	1, 32	0.12	0.7353	1, 38	0.08	0.7723	1, 20	0.24	0.6319	1, 20	1.01	0.3267
S*F	1, 32	0.22	0.6395	1, 38	0.19	0.6660	1, 20	0.67	0.4218	1, 20	0.60	0.4470
S*D	6, 185	58.32	<.0001	6, 221	15.40	<.0001	6, 109	10.24	<.0001	5, 77	21.50	<.0001
D*F	6, 185	1.65	0.1365	6, 221	1.90	0.0813	6, 109	0.16	0.9864	5, 77	1.95	0.0960
S*D*F	6, 185	1.71	0.1201	6, 221	1.59	0.1512	6, 109	0.59	0.7412	5, 77	0.76	0.5819
lnBL	1, 1151	1234.13	<.0001	1, 1351	1104.55	<.0001	1, 722	199.09	<.0001	1, 558	43.94	<.0001

patterns expressed by both *Eucidaris* species, as compared to the other species used in this study (Figures 3.3B, 3.4B, and 3.5B), may reflect the fact that over the time-frame of this study neither of these species projected distinct anterolateral arms with rigid structural elements from the oral hood (the soft-tissue area between the anterolateral arms; see Figure 3.2). The anterolateral arms help to lengthen and support the larval bodies in most species, providing for more accurate linear body measurements. The bodies of *Eucidaris* larvae tended to curl inwards as they grew larger, even while alive and before slide preparation procedures were conducted. This slight curling of the body affected measurements of *Eucidaris* sp. body lengths, making them artificially shorter.

Paired-species ANOVAs using arm to body length ratio as the response variable between larvae fed High (5 algal cells / µl) or Low (1 algal cell / µl) food levels in the 2005 experiment support the visual interpretations of Figures 3.3-3.5 and detected the following patterns (see Table 3.3 for values). The ANOVAs between larvae of *Echinometra vanbrunti* and *Ec. viridis*, *Ec. vanbrunti* and *Ec. lucunter*, *D. mexicanum* and *D. antillarum*, and *Eu. thouarsi* and *Eu. tribuloides* detected significant effects of species, day, body length and species with day within each analysis. In each analysis, there was no effect due to food, species with food, day with food, or to the three-way interaction of species by day by food. Visual inspection of the arm to body length trajectories for each species support this result (Figures 3.3B, 3.4B, and 3.5B). The least square mean ($\pm$ 1 S.E.) estimates of arm length to body length ratio for species from each analysis are given in Table 3.4.

Table 3.4: Least square mean estimates (units = ln mm) from each of the analyses of variance (ANOVAs) between oceans (Table 3.2) and geminate species pairs (see Table 3.3).

Analysis	**Effect**	**Ocean/Species**	**Estimate (±1S.E.)**	**df**	**t Value**	**Pr > |t|**
Ocean Analysis	Ocean	Caribbean	-0.5542 ± 0.062	38	-8.96	<.0001
		Pacific	-0.6314 ± 0.063	38	-10.03	<.0001
Paired Species Analysis	Species	*E. viridis*	-0.2575 (±0.056)	32	-4.59	<.0001
		E. vanbrunti	-0.5902 (±0.056)	32	-10.51	<.0001
	Species	*E. lucunter*	-0.3552 (±0.066)	38	-5.35	<.0001
		E. vanbrunti	-0.5846 (±0.067)	38	-8.68	<.0001
	Species	*D. antillarum*	-0.3468 (±0.1219)	20	-2.85	0.0010
		D. mexicanum	-0.6222 (±0.1209)	20	-5.15	<.0001
	Species	*E. tribuloides*	-0.9604 (±0.046)	20	-20.70	<.0001
		E. thouarsi	-0.7705 (±0.058)	20	-13.27	<.0001

Food limitation analysis

Caribbean *Ec. viridis* larvae did not respond significantly to limiting food conditions; arm to body length trajectories are comparable across all five food treatments (Figure 3.6A). The growth of larval arms relative to body in Pacific *Ec. vanbrunti* larvae was affected by food concentrations lower than 1.0 algal cell / µl; trajectories for the lower food treatments do not extend as far as for the higher food treatments (Figure 3.6B). This result suggests that food is limiting for Pacific species but not for Caribbean species. In support of this finding, the ANOVA between *Ec. vanbrunti* and *Ec. viridis* larvae fed High (5 algal cells / µl), Low (1 algal cell / µl), Half (0.5 algal cell / µl), Limit (0.1 algal cell / µl), or Zero (0 algal cell / µl) food levels in the 2006 experiment using body length as the response variable detected significant effects of species ($F_{1, 20} = 165.19$, $p < 0.0001$), day ($F_{4, 74} = 572.49$, $p < 0.0001$), food ($F_{1, 20} = 6.50$, $p = 0.0016$), species with day ($F_{4, 74} = 13.70$, $p < 0.0001$), day with food ($F_{16, 74} = 4.76$, $p < 0.0001$), and the three-way interaction of species by day by food ($F_{16, 74} = 2.68$, $p = 0.0022$). There was no effect due to species with food ($F_{4.20} = 2.30$, $p = 0.0938$).

Low food levels did not induce statistically significant phenotypically plastic responses in larvae from any food treatment lower than or equal to 1.0 algal cell / µl; there was no effect due to food in the ANOVA using arm length as the response variable. This ANOVA was structured the same as the ANOVA for body length described above, albeit including body length as a quantitative covariate, and detected significant effects of species, day, body length, and the interactions of species with day, species with food, day with food, and the three-way interaction of species by day by food (see Table 3.5). A smaller ANOVA between *Ec. vanbrunti* and *Ec. viridis* larvae fed High (5 algal cells / µl) or Zero (0 algal cell / µl) food levels using arm length as the response variable and body length as a covariate

Figure 3.6: Mean (± 1SE) natural log corrected summed length of Postoral arms for High-food (5 algal cells / µl : *filled circle* symbols), Low-food (1 algal cell / µl : *open circle* symbols), Half-food (0.5 algal cell / µl : *filled triangle* symbols), Limit-food (0.1 algal cell / µl : *open triangle* symbols), and Zero-food (0.0 algal cell / µl : *filled square* symbols) larvae versus mean (± 1SE) natural log corrected Body Length at midline. In **A.**, values for Caribbean *Echinometra viridis* larvae are indicated. In **B.**, values for Pacific *Echinometra vanbrunti* larvae are indicated. Larvae from both species were reared in these food treatments during the subsequent food-limitation experiment conducted in 2006. Units are ln millimeters.

Figure 3.6 A

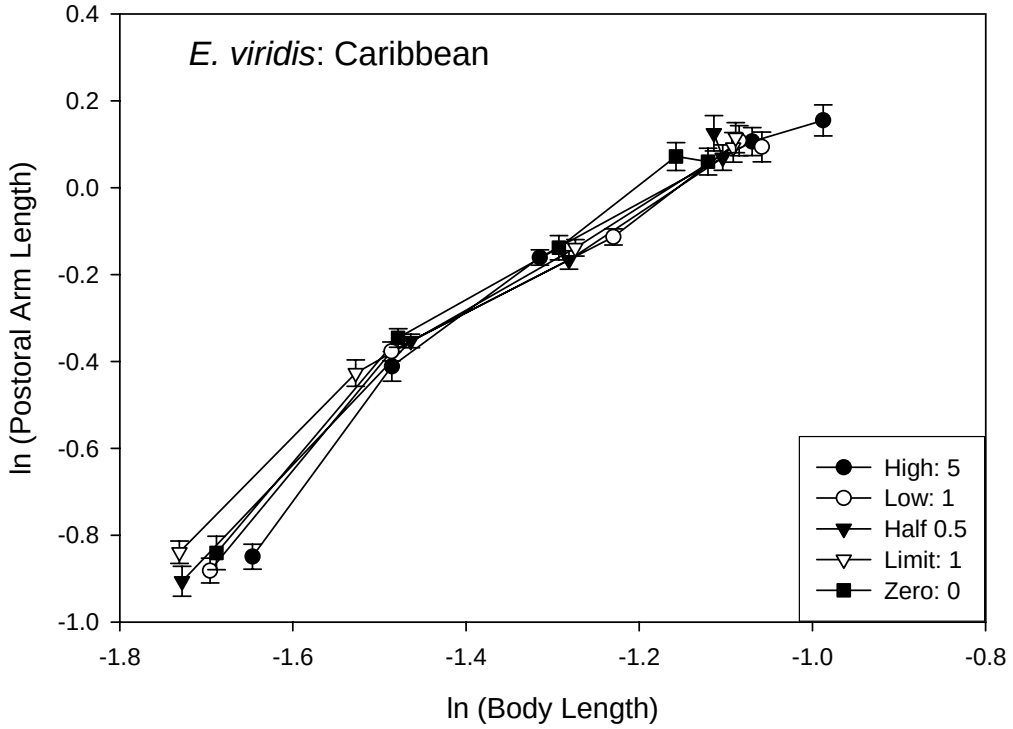


Figure 3.6 B

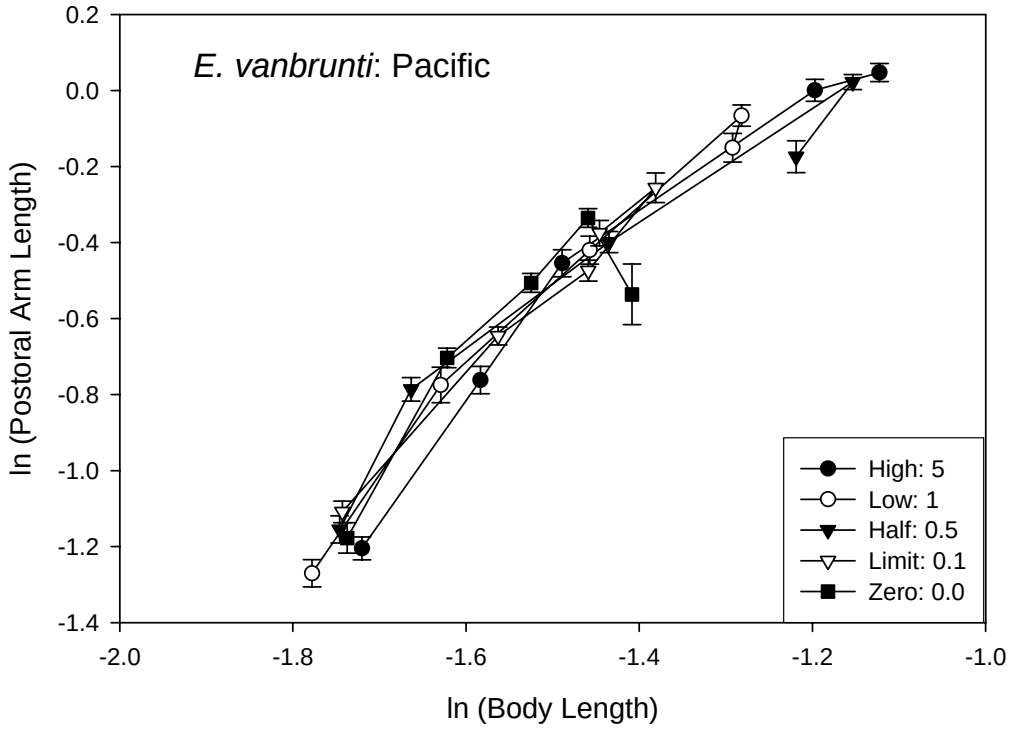


Table 3.5: Analysis of Variance (ANOVA) results for Caribbean *Echinometra viridis* versus Pacific *Echinometra vanbrunti* larvae fed either High (5.0 algal cells / µl), Low (1.0 algal cell / µl), Solow (0.5 algal cell / µl), Limit (0.1 algal cell / µl), or Zero (0.0 algal cell / µl) food levels. Dependent variable is the natural log of the sum of postoral arm lengths. Natural log corrected midline body length was included in the model as a known quantitative covariate.

Effect	**df: N, D**	**F value**	**Pr > F**
Species	1, 20	306.12	<.0001
Day	4, 74	320.78	<.0001
Food	4, 20	1.84	0.1605
Species*Food	4, 20	3.69	0.0209
Species*Day	4, 74	21.34	<.0001
Day*Food	16, 74	6.24	<.0001
Species*Day*Food	16, 74	4.60	<.0001
ln (Body Length)	1, 649	308.07	<.0001

detected significant effects of species ($F_{1, 8} = 78.97$, $p < 0.0001$), day ($F_{4, 29} = 147.66$, $p < 0.0001$), body length ($F_{1, 256} = 79.42$, $p < 0.0001$), the interactions of species with day ($F_{4, 29} = 5.69$, $p = 0.0017$), species with food ($F_{1, 8} = 6.50$, $p = 0.0342$), day with food ($F_{4, 29} = 16.77$, $p = < 0.0001$), and the three-way interaction of species by day by food ($F_{4, 29} = 9.84$, $p = < 0.0001$). There was no effect due to food ($F_{1, 8} = 3.89$, $p = 0.0842$).

Discussion

In the Introduction, I posed three hypotheses regarding how differences in the heterogeneity of phytoplankton food levels may influence the evolution and expression of larval arm length. The results presented in this study do not support the two plasticity hypotheses; however they do support the constant differences hypothesis.

Constant Differences

The results from the 'ocean' analysis (Tables 3.2 and 3.4) indicated that larvae from the Caribbean had longer arms relative to body length than larvae from the Pacific. Similarly, the results from the 'paired species' analyses of variance indicated that larvae from Caribbean species in the genera *Diadema* and *Echinometra* had longer arms relative to body length than their Pacific geminate counterparts. There were significant effects due to species on postoral arm length corrected for body length in each of these 'paired species' analyses of variance (Table 3.3) and the least square mean estimates were greater for each Caribbean species than for each Pacific species, within each respective comparison (Table 3.4). However, the result from the 'paired species' analysis of variance between the Caribbean and Pacific *Eucidaris* species indicated the opposite; *Eucidaris thouarsi* from the Pacific had

longer arms relative to body length than *Eucidaris tribuloides* from the Caribbean (Tables 3.3 and 3.4). The result for *Eucidaris* may reflect the fact that only one *Eu. thouarsi* family was used in the analysis, as opposed to three *Eu. tribuloides* families. I had considerable difficulty obtaining mature gametes from *E. thouarsi* during the time I conducted the experiments (mid-June through early September in 2005 and August of 2006); I injected over 120 individuals with 0.5M KCl to induce spawning and obtained mature gametes from only 1 male and 1 female.

The significant difference in relative larval arm length detected across ocean basins when incorporating all species (the large 'ocean' analysis of variance) must be interpreted carefully (Table 3.2). Analysis of variance incorporates the magnitude as well as the direction of differences between categories; therefore it is possible that a large, directional difference in one (or more) geminate species pairings could have lead to the overall significant difference detected across ocean basins. I conducted the paired species analyses to account for this possibility and to aid in the interpretation of this result. Note that 3 of the 4 species pairs I examined exhibited the same pattern: longer relative arm lengths for the Caribbean species in each separate pairing of *Echinometra* sp. and with the *Diadema* (Table 3.3). *Eucidaris* was the only genus that showed the opposite pattern, perhaps influenced in part due to the body length measurement issue mentioned in Results (above). A signed-rank test would aid in the interpretation of the overall pattern, however there are not enough easily collectable echinoid geminate species pairs with feeding larvae (i.e. at least six independent pairs) in this system to perform this type of test.

The results from the 'ocean' analysis and the 'paired species' analyses for *Diadema* and *Echinometra* support the Constant Differences Hypothesis; larvae evolving in the

constantly low phytoplankton food levels of the western Caribbean grew longer arms relative to body length than larvae evolving in the variable food levels of the eastern Pacific. In an environment with little to no heterogeneity in food resources, as characterized by the Caribbean, an appropriate evolutionary strategy for resource acquisition may be to express constantly long arms relative to body length. Expressing a constant long arm phenotype may produce a better return (in terms of exogenous energy acquisition) on the investment in long arms (in terms of materials to produce and metabolism to maintain) than expressing a plastic arm length phenotype. If phenotypic plasticity confers a benefit in heterogeneous environments, as characterized by the Pacific, then there may be no benefit from plasticity of arm length for larvae evolving in the homogeneous environment of the Caribbean; a cost of plasticity may also constrain the expression of arm length plasticity in a homogeneous environment.

Phenotypic Plasticity

Contrary to the published findings of several researchers using various, diverse echinoid species (Boidron-Metairon 1988; Hart and Scheibling 1988; Strathmann et al. 1992; Hart and Strathmann 1994; Sewell et al. 2004; Reitzel and Heyland 2007), none of the species I reared in this study exhibited phenotypic plasticity of larval arm length. There was no significant effect due to food on postoral arm length corrected for body length detected in either the 'ocean' analysis of variance (Table 3.2) or any of the 'paired species' analyses of variance (Table 3.3). This surprising finding begs the question as to why there was no, or minimal, i.e. no statistically significant, phenotypic plasticity of larval arm length exhibited by any of the seven species reared in this study?

The simplest explanation for this result may be that the low food level I used (1.0 algal cell / µl) was not low enough to induce a phenotypically plastic response. In other words, this food level may not have been representative of a food limiting condition for these larvae; however, this food level falls within the range of "low" food levels used in other studies demonstrating arm length plasticity in larval echinoids (2 algal cells / µl: Miner 2005; Reitzel and Heyland 2007; ~1.3 algal cells / µl: Boidron-Metairon 1988; 0.6 algal cells / µl: Sewell et al. 2004; 0.5 algal cells / µl: McAlister Ch. 4; 0.3 cells / µl: Hart and Strathmann 1994). I chose 1.0 algal cell / µl as a low food treatment because lower food levels have been demonstrated to result in stalled larval development in some invertebrate species (Pechenik et al. 1984; Eckert 1995; Herrera et al. 1996). The results from the food limitation experiment I conducted in 2006 (the second experiment described above) using *Ec. viridis* and *Ec. vanbrunti* indicated that these species did not express plasticity of larval arm length at food treatments lower than 1.0 algal cell / µl; there was no significant effect due to food on postoral arm length corrected for body length detected by the analysis of variance among the five different food treatments (Table 3.5). There was no effect on larval body length with decreasing food ration on Caribbean *Ec. viridis* (see Figure 3.6A). However, the two lowest food rations (0.1 algal cell / µl and 0.0 algal cell / µl) did limit development of larval body length in Pacific *Ec. vanbrunti* (see Figure 3.6B). The results from the food limitation experiment suggest that the lack of measurable levels of phenotypic plasticity of arm length within this system (i.e. all of the species used in the 2005 experiment) is a true finding. As mentioned, these results run counter to the published findings of plasticity from multiple other echinoderm species (Boidron-Metairon 1988; Hart and Scheibling, 1988; Strathmann et al. 1992; Hart and Strathmann 1994; Sewell et al. 2004; Reitzel and Heyland 2007). These

results do not preclude the fact that there may be additional species that are similarly non-plastic; other negative findings may have no record of publication.

None of the species examined in this study demonstrated phenotypic plasticity of larval arm length; the results indicated that there are no differences in degree of plasticity across all species and do not support either the plasticity or differential plasticity hypotheses. The results do suggest that despite the well documented and historical differences in productivity between the eastern Caribbean and western Pacific (Glynn 1982; Keigwin 1982), there may be less difference in the variability of food resources between these environments, on a scale that is relevant to larvae. Additionally, when differences in egg size across the geminate pairs are considered, the lack of plasticity in these species suggests that selection may have acted on other life history characteristics to account for differences in the levels of exogenous phytoplankton food. While the negative finding of a lack of plasticity is the main strength of this study, interpreting this result, and the interesting trends in relative arm length, as evolutionary responses must be tempered, however, by the fact that we do not know, and cannot determine in this system, what the ancestral conditions were with regard to plasticity, relative arm length, or egg size. I discuss these possibilities, concerns, and the collective results of my experiments below.

Environmental variation in resource levels: Local and latitudinal considerations

Tropical coastal marine ecosystems are commonly oligotrophic with patchy food resources (Koblentz-Mishke et al. 1970; Mackas et al. 1985) for planktonic larvae. Alternatively, levels of primary productivity in temperate coastal ecosystems can cycle between low levels in winter and large peaks during spring and summer algal blooms (Lalli

and Parsons 1994). Values of chlorophyll *a* concentration, a measure of phytoplankton concentration, for coastal waters suggest that larvae, regardless of ecosystem, are usually food-limited to some degree (Paulay et al. 1985), although comparison among ecosystems is crude because of different assemblages of algal species and lack of information about natural dietary preferences. Published chlorophyll *a* concentrations are 1 to 2 orders of magnitude lower in tropical (0.01 to 0.35 μg/l for Moorea, Society Islands; Ricard, 1981; 0.19 to 0.52 μg/l in waters of the Great Barrier Reef; Lucas, 1982; 5.9^{-4} μg/l during the rainy season and 1.48^{-3} during the dry season of upwelling in the Bay of Panama, and 4.1^{-4} during the rainy season and 3.6^{-4} during the dry season at San Blas Point in the Caribbean; calculated from values reported in mg m^{-3} by D'Croz and Robertson 1997) than in temperate ecosystems (<1 ug/l in winter to >15 μg/l in spring blooms off the Washington and Oregon coasts; Richards 1950; Anderson 1964; Harrison et al. 1983; and 1.3 to 3.8 μg/l in August in Long Island Sound; Whitledge and Wirick 1983). These values suggest that larvae of tropical species may be severely food limited.

Faced with constant low food levels, the tropical planktotrophic larvae from the Caribbean species examined in this study may have evolved to express a constant long larval arm length phenotype instead of plasticity of arm length. In tropical habitats with widespread resource patchiness, expressing a constant long arm length phenotype likely increases the food gathering capability of a given larva. Conversely, plasticity of arm length may be an evolutionary strategy that results in greater food gathering capability for larvae in temperate habitats. Matched against the patterns of ecosystem productivity, plasticity of arm length in pluteus larvae has been demonstrated primarily in temperate species. Some of the highest magnitudes of larval arm length plasticity are recorded for species from cold

temperate waters (Boidron-Metairon 1988; Hart and Scheibling 1988; Sewell et al. 2004) whereas some of the lowest are recorded for species from warm tropical or subtropical waters (Boidron-Metairon 1988; Eckert 1995; Podolsky and McAlister 2005). This suggests that there may be a latitudinal gradient in phenotypic plasticity of larval feeding structures.

Similarly, the tropical Pacific species larvae in this system may not have evolved to express phenotypic plasticity because they may only experience low resource levels. Despite the well-documented annual heterogeneity of resource levels, some Pacific echinoids (e.g. *Diadema mexicanum* and *Echinometra vanbrunti*) do not release their eggs during the period of the year with peak phytoplankton production (Lessios 1981). Consequently, larval settlement tends to occur before the period of seasonal upwelling (Lessios 1981). Reproduction during the off-season, with respect to phytoplankton production, suggests that these species may not be taking advantage of the higher resource levels during upwelling. However, timing their reproduction to avoid upwelling may mitigate the effects on duration of the larval period that could result from the lower water temperatures during upwelling (Thorson 1950; Glynn 1972; Hinegardner 1975; Lessios 1981). Species evolving in this habitat may time their reproduction and the duration of larval development to guarantee that larvae reach metamorphosis before upwelling. Furthermore, the upwelling period is characterized not only by high nutrient levels and lower water temperatures, but also by strong offshore transport (Smayda 1966; D'Croz and Robertson 1997). Larvae that are transported offshore may not be able to find suitable sites for post-metamorphic settlement (Lessios 1981). Reaching metamorphosis before upwelling would increase the probability that larval settlement occurs near shore.

Alternatively, there may be finer-scale, localized heterogeneity in food levels within each respective ocean basin. For example, intensity of upwelling varies along the Pacific coast of Central America (Wyrtki 1967; Legeckis 1985; McCreary et al. 1989). The adult urchins collected from the Pacific in this study came from the Bay of Panama, which has localized high levels of nutrient upwelling (Kwiecinski and Chial 1983). Other areas along the Pacific coast of Panama have lower levels of nutrient upwelling, e.g. the Gulf of Chiriqui (Kwiecinski and Chial 1983). Within-ocean basin differences in the heterogeneity of food resources may affect the evolution of plasticity if there are high levels of larval exchange and genetic mixing among populations from different locales. Spatially heterogeneous environments with a high degree of patchiness are thought to select for the evolution of phenotypic plasticity (Levins 1968). However, in light of the timing of reproduction, selection for small egg size in Pacific species and the constraints that low endogenous energetic resources may have on the expression of plasticity (see below), and the possibility of high levels of larval exchange among Pacific locales, selection may have favored a generalist fixed arm length strategy for resource acquisition, instead of a phenotypically plastic one.

Selection on life history characters: The confounding aspect of egg size

A discussion of the evolution of phenotypic plasticity or phenotypic fixation of feeding structures in this system must consider the documented differences in egg size between Caribbean and Pacific species. Egg size has long been considered an important component of the life histories of marine organisms (Thorson 1950; Vance 1973; Christiansen and Fenchel 1979; Strathmann 1985; Jaeckle 1995; Levitan 2000; Moran 2004;

Allen 2005). In the Panamanian Isthmus system egg size is larger in many Caribbean species than in their Pacific geminates. Lessios (1990) has shown that members of geminate pair echinoids found in the western Caribbean have larger egg sizes than their eastern Pacific counterparts due to changes in productivity following the rise of the Isthmus of Panama. The results from the current study show the same pattern (see Table 3.1) in a subset of the species examined by Lessios (1990). A similar pattern has been demonstrated for bryozoans (Jackson and Herrera 1999) and arcid bivalves (Moran 2004). This pattern supports theoretical models that predict that the greater endogenous resources found in large eggs, which represent an increased maternal investment per ovum, evolve in response to a poor larval feeding environment, as found in the western Caribbean (Vance 1973; Lessios 1990; Levitan 2000). Conversely, small egg sizes in the eastern Pacific likely represent an evolutionary response to high levels of oceanic productivity (Lessios 1990, Moran 2004).

An investigation of the effects of egg size on the expression of phenotypic plasticity in the Panamanian echinoid system would be ideal. However, the arguments for the expression of plasticity as an evolutionary response to historical heterogeneity in food resource levels and to a reduction in egg size are confounded in the Panamanian system, i.e. within each geminate pair, the species with smaller egg size inhabits the heterogeneous environment of the eastern Pacific. Results from a recent study I conducted using echinoid species in the genus *Strongylocentrotus* that differ in egg size (see Chapter 4) indicate, however, that large egg size is associated with the expression of greater degrees of phenotypic plasticity and of longer arm relative to body lengths than small egg size. In light of the results obtained in the current study, the expression of longer arm relative to body lengths in the Caribbean species may reflect the fact that these species develop from a larger

egg than their Pacific counterparts. Caribbean species may have obtained a greater benefit, in terms of fitness, by having experienced selection for larger initial endogenous energetic reserves, i.e. larger egg size, than for phenotypic plasticity of exogenous food collection structures. The result of a greater degree of phenotypic plasticity in the larger-egged *Strongylocentrotus* species does not match the results obtained in the current study. This may be due to the fact that *Strongylocentrotus* is a temperate genus and the larger-egged species (*S. franciscanus*) examined in Chapter 4 develops from an egg that is larger in size than any of the tropical species examined in the Panamanian system. Further research on larval growth and egg composition/quality using different populations of each Panamanian system species evolving in areas of different productivity may help to elucidate the patterns found in this study.

CHAPTER FOUR

EGG SIZE AND THE EVOLUTION OF PHENOTYPIC PLASTICITY IN LARVAE OF THE ECHINOID GENUS *STRONGYLOCENTROTUS*.

Summary

Planktotrophic larvae grow by utilizing energy obtained from food gathered in the plankton. Morphological plasticity of feeding structures has been demonstrated in multiple phyla, in which food-limited larvae increase feeding structure size to increase feeding rates. However, before larvae can feed exogenously they depend largely on material contained within the egg to build larval structures and to fuel larval metabolism. Thus, the capacity for plasticity of feeding structures early in development may depend on egg size. Using the congeneric sea urchins *Strongylocentrotus franciscanus* and *S. purpuratus*, which differ in egg volume by 5-fold, I tested whether the degree of expression of feeding structure (larval arm length) plasticity is correlated with differences in the size of the egg. I experimentally manipulated egg size of *S. franciscanus* (the larger-egged species) by separating blastomeres at the 2-cell stage to produce half-sized larvae. I reared half-size and normal-size larvae under high and low food treatments for 20 days. I measured arm and body lengths at multiple ages during development and calculated the degree of plasticity expressed by larvae from all treatments. Control and unmanipulated *S. franciscanus* larvae (from ~1.0 nl eggs) had significantly longer arms relative to body size and a significantly greater degree of

plasticity than half-sized *S. franciscanus* larvae (from <0.18 nl eggs), which in turn expressed a significantly greater degree of plasticity than *S. purpuratus* larvae (from ~0.3 nl eggs). These results indicate that egg size affects larval arm length plasticity in the genus *Strongylocentrotus*; larger eggs produce more-plastic larvae both in an experimental and a comparative context. However, changes in egg size alone are not sufficient to account for evolved differences in the pattern of plasticity expressed by each species over time and may not be sufficient for the evolutionary transition from feeding to non-feeding.

Introduction

During larval development, organisms typically encounter unpredictable feeding environments (Conover, 1968). Consequently, planktonic larvae have the potential to be food limited (Olson and Olson, 1993) and experience high rates of mortality due to the indirect effect of a prolonged period that exposes larvae to greater levels of predation (Rumrill, 1990). Because of these harsh circumstances, there is strong selection for traits that ameliorate the effects of adverse feeding conditions (Doughty, 2002). Selection can act on phenotypic variation in traits associated with the utilization of two different energetic resources available to the larva: 1) the endogenous energetic reserves obtained from the parent or 2) the exogenous food resources acquired from the larval feeding environment.

The degree of parental investment in an individual offspring is reflected in the size of the egg from which that individual develops (Jaeckle, 1995). Egg size is correlated with initial size of the larva, larval habitat, the duration and rate of larval development, and the mode of larval nutrition (McEdward, 1986a, b). Egg size can affect an individual offspring's fitness (Vance, 1973; Christiansen and Fenchel, 1979; Strathmann, 1985; Sinervo and

McEdward, 1988; Hart, 1995; reviewed by Havenhand, 1995; McEdward, 1996; Emlet and Hoegh-Guldberg, 1997) and consequently is an important trait in life history studies of many organisms (Emlet et al., 1987). Egg size is a trait that can change in response to selection (Lessios, 1990; Jackson and Herrera, 1999; Moran, 2004). However, closely related species that inhabit the same larval environment, and likely experience similar selective pressures for resource acquisition, can have very different egg sizes (Wray and Raff, 1991; Herrera et al., 1996; Allen and Podolsky, 2007) suggesting that egg size may respond indirectly to selection on other species-specific life-history characteristics.

Phenotypic plasticity allows organisms to match trait expression to environmental heterogeneity (West-Eberhard, 2003). Morphological phenotypic plasticity in response to food resource level has been demonstrated in planktotrophic (feeding) larvae from multiple species in different phyla (Echinoderms: Boidron-Metairon, 1988; Strathmann et al., 1992, 1993; George, 1994, 1999; Hart and Strathmann, 1994; Sewell et al., 2004; Miner, 2005, Podolsky and McAlister, 2005; Reitzel and Heyland, 2007; Molluscs: Klinzing and Pechenik, 2000). Under low food availability, larvae increase the length of a food-collecting ciliated band, a response that is correlated with lengthening of skeletal arm rods in pluteus larvae. Longer arms increase the rate at which larvae clear food from suspension (Hart and Strathmann, 1994) and could increase the uptake of dissolved organic matter by changing larval surface area (Manahan et al., 1983). For these reasons, arm length has been used as an indicator of larval nutritional history in the field (Strathmann et al., 1992).

Plasticity of larval feeding structures hinges on an energetic investment trade-off between larval and juvenile structures; increased investment in arms can result in decreased or delayed investment in other structures, such as the juvenile rudiment (Strathmann et al.,

1992; Heyland and Hodin, 2004). Plasticity of arm length is expressed during early larval development (approximately 1 to 2 weeks post-fertilization for most species: see Echinoderm references listed above), suggesting that planktotrophic larvae may utilize endogenous resources for the initial production of food collecting structures, then move to exogenous resources for the development of other, later-appearing structures. Endogenous resources are provided to individual offspring within the egg and egg size is positively correlated with the level of investment (Jaeckle, 1995). The capacity for plasticity of arm length early in development may therefore depend on the amount of maternally provisioned energetic reserves, and thus on egg size (Herrera et al., 1996).

There are two alternative hypotheses of the effect of egg size on plasticity. The first is an argument based on energy and materials in the egg: larvae from larger eggs may have a greater capacity for the expression of plasticity because they have access to and make use of a larger store of endogenous energy and materials. The second is an evolutionary argument: larvae that develop from smaller eggs may have been selected for a greater scope for plasticity to take better advantage of scarce exogenous food. Herrera et al. (1996) predicted that plasticity may be more important, but more difficult to express, in larvae that develop from smaller eggs. One recent study (Podolsky and McAlister, 2005) found support for this prediction among ophiuroid pluteus larvae. Ophiuroids possess a pluteus larval form that is similar in structure and function to the echinoid pluteus larva, and is thought to have evolved independently. Their study was not an explicit test of the hypotheses presented here; however, the authors found that smaller-egged species in the genus *Macrophiothrix* expressed plasticity of larval arm length, whereas larger-egged species did not. Another recent study (Reitzel and Heyland, 2007) specifically tested for an effect of egg size on the

expression of phenotypic plasticity in echinoid pluteus larvae. Using subtropical irregular echinoid species from multiple genera, the results also indicate that plasticity of larval arm length was exhibited by smaller egged species and not by larger egged species.

Both Podolsky and McAlister's (2005) and Reitzel and Heyland's (2007) studies provide solid comparative datasets of plastic responses to decreased food levels in species (and genera) that develop from differently sized eggs, although Reitzel and Heyland's (2007) approach does not control for phylogeny or for the different environments that their species have evolved in. My study complements the results of these previous studies because I investigated within a species the effects of experimental manipulations of egg size on the expression of plasticity. By physically manipulating egg size, I am able to separate the two arguments (energy/materials and evolutionary) for why egg size may play a role in the expression of plasticity of feeding structures. In addition, I control for phylogeny and evolutionary environment by comparing plastic expression between two species in the same genus that co-occur in the same habitat.

In this study, I investigated whether egg size affects the expression of larval arm length plasticity by experimentally halving egg size of *Strongylocentrotus franciscanus*. I experimentally reduced the amount of available endogenous material available to developing larvae, using blastomere separation at the 2-cell stage to produce viable offspring that were one-half normal size (Driesch, 1892; Okazaki and Dan, 1954; Horstadius, 1973). This protocol provides a rigorous within-species test of the effect of egg size on the expression of plasticity. If *S. franciscanus* larvae from half-size eggs have a decreased capacity for plasticity early in development compared to *S. franciscanus* larvae from normal eggs, this would support the hypothesis that the amount of endogenous material in the egg can affect

morphological plasticity. Alternatively, if larvae from half-size eggs show no difference in the capacity for plasticity early in development compared to larvae from normal eggs, this would support the hypothesis that egg size is linked to plasticity not through direct effects of the amount of material in the egg, but indirectly through the effects of natural selection acting simultaneously on multiple life-history characteristics.

In addition, I investigated the expression of larval arm length plasticity between species by examining larval development in two congeneric sea urchins in the genus *Strongylocentrotus* that have substantially different egg sizes. Adult animals in this genus are found in temperate coastal habitats off the Pacific coast of North America, have similar morphology and ecology, and develop via planktotrophic pluteus larvae (McEdward, 1986; Strathmann, 1987). I examined larval growth and morphological plasticity in *S. franciscanus* and *S. purpuratus*, which co-occur and have egg diameters (volumes) of roughly 135 (1.29) and 80 (0.27) μm (nl), respectively (Emlet et al., 1987). I used these two species to elucidate the role of egg size on the evolved capacity for plasticity in this genus because they inhabit the same larval feeding environment and yet have naturally occurring variation in egg size.

Materials and Methods

Adult *Strongylocentrotus franciscanus* and *Strongylocentrotus purpuratus* sea urchins were collected from a sub-tidal population located off the coast of Carlsbad, CA, by employees of Marine Research and Educational Products (M-REP, Inc.) in February 2006. The urchins were packed in moist paper towels and shipped overnight to Chapel Hill, NC, where they were maintained in a recirculating artificial seawater aquarium held at 15°C and 33.5‰ salinity.

Larval culture

Upon receipt, adult urchins were induced to spawn gametes by peristomial injection into the body cavity of approximately 1-ml of 0.5M KCl. Eggs were collected and washed once in artificial seawater (ASW: Instant Ocean, Aquarium Systems; 33.5‰ salinity), and sperm were collected by mouth pipette and kept on ice until use. Larval cultures of *S. franciscanus* were established by fertilizing eggs from 2 females with sperm from 5 males. Larval cultures of *S. purpuratus* were established by fertilizing eggs from 2 females with sperm from 7 males. Initial mean ($\pm$ 1 S.E.) egg diameters (means of 10 eggs each) for the two *S. franciscanus* females were 122.7 $\pm$ 0.35 µm and 124.7 $\pm$ 0.17 µm and for the two *S. purpuratus* females were 81.8 $\pm$ 0.00 µm and 85.2 $\pm$ 1.12 µm. Egg volumes (assuming a sphere) were 0.967, 1.015, 0.287, and 0.324 nl respectively.

Fertilized embryos and larvae were reared in one of two replicated food environments (5 and 0.5 algal cells/µl). Each food level was then replicated among either three (*S. franciscanus*) or four (*S. purpuratus*) cultures. Each larval culture was fed the unicellular alga *Dunaliella tertiolecta* (UTEX Algal Supply, Austin, TX) daily, starting at 48h (all ages reported are post-fertilization). All cultures were reared in ASW in 1-l plastic tri-pour beakers at densities of 1 larva ml^{-1} and water was changed every other day. The cultures were maintained in an environmental chamber held at 17°C and were continually stirred at approximately 10 strokes min^{-1} with acrylic paddles to homogenize food and to keep larvae in suspension (Strathmann, 1987). *D. tertiolecta* was cultured at room temperature in autoclaved ASW enriched with a modified Guillard's f/2 medium (Florida Aqua Farms, Inc.). Algae were separated from the growth medium by centrifugation and then re-suspended in fresh ASW before use.

Blastomere Separation

Blastomere separation at the 2-cell stage produces viable offspring that are one-half normal size (Okazaki and Dan, 1954). Individual blastomeres were isolated at the 2-cell stage from a sub-set of the fertilized *S. franciscanus* embryos using a modification of a common *S. purpuratus* blastomere separation protocol (Harkey and Whiteley, 1980; Allen, 2005). To remove the fertilization envelope (FE), eggs were passed repeatedly through a 100 µm nitex mesh within one minute post-fertilization. Upon removal of the FE, fertilized eggs were kept cool in glass dishes of ASW held on ice and monitored for signs of cleavage. The glass dishes were coated with a thin layer of 2% agar in ASW to prevent the fertilized eggs from sticking to the sides. After approximately 2 hours in chilled ASW, embryos underwent first cleavage and were washed 4 times with an isosmotic solution of calcium- and magnesium-free seawater (CaMgFSW; recipe in Strathmann, 1987). Brief exposure (less than 30 minutes) to CaMgFSW dissolved the hyaline layer; blastomeres were easily separated upon gentle stirring. Embryos were returned to chilled ASW after separation to continue development.

The blastomere separation protocol routinely produces two different size classes of embryos: 'half' size embryos that develop from dissociated blastomeres and 'whole' size embryos that develop from non-dissociated blastomeres. Following the separation protocol, embryos were sorted into whole- and half-size classes by pouring through a 70 µm nitex mesh. Half-size embryos passed through the mesh and whole-size embryos did not. In addition to the larval cultures established for larvae developing from untreated, 'full' size eggs (detailed above), larval cultures of whole- and half-size *S. franciscanus* were established from the embryos subjected to the blastomere separation protocol. Fertilized whole- and

half-size embryos and larvae were reared in one of two different replicated food environments. Each food level was then replicated among three (whole-size *S. franciscanus*) or four (half-size *S. franciscanus*) cultures. Cultures of larvae that were subjected to the blastomere separation protocol were reared in the same manner as previously described for normally developing, untreated, full-size *S. franciscanus* and *S. purpuratus* larvae. The only difference in culture set-up was that half-size larvae were reared in smaller plastic tri-pour beakers (400-ml instead of 1-l), albeit at the same density as larvae in the other treatments.

Measures of Phenotype

On days 3, 5, 7, 10, 13, and 16, approximately 10 larvae were removed from each culture. *S. purpuratus* larvae were also removed on day 20. The larvae were placed on a glass slide, immobilized with a dilute (<10%) solution of buffered formalin in ASW, and covered with a glass cover slip raised on clay feet. Three-dimensional Cartesian coordinates were recorded of multiple morphological features for 5 larvae from each culture (Figure 4.1). These landmarks included the tip and base of each anterolateral and postoral arm rod, the posterior tip of the larva, the tip of the oral hood (i.e. the mid-point of the soft-tissue that stretches between the pair of anterolateral arms), and points at the anterior and posterior ends of the stomach. To collect data from each larva, I used a digitizing tablet (Hyperpen 12000U, Aiptek Inc.) to capture x and y coordinates of morphological landmarks. Simultaneously, I obtained z coordinates from a rotary encoder (U.S. Digital) coupled to the fine focus knob of a Wild M-20 compound microscope (McEdward, 1985). Using these 3-D Cartesian coordinates, I geometrically reconstructed individual arm, body, and stomach lengths for each larva. Because the postoral and anterolateral arms were the most prominent

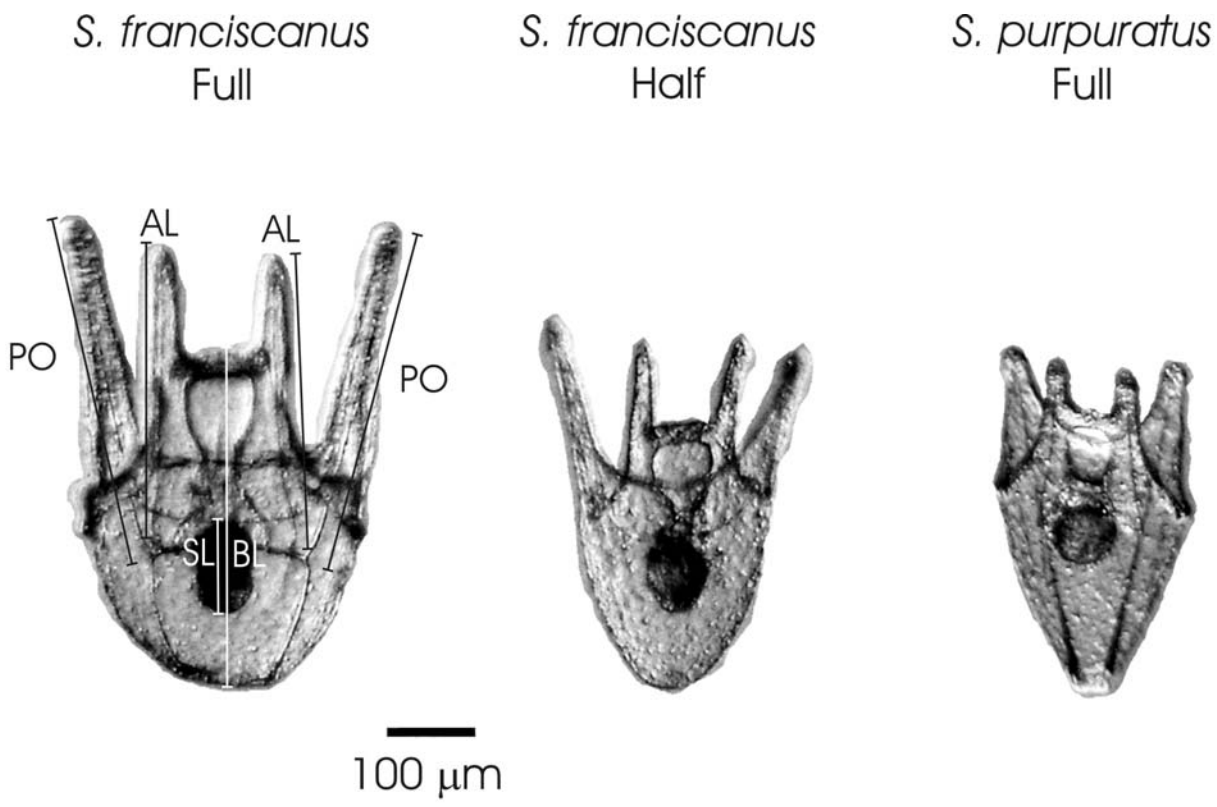


Figure 4.1: Low-fed *Strongylocentrotus franciscanus* larvae from full- and half-size eggs and low-fed *S. purpuratus* larvae from a full-size egg. All larvae are from day 10 of development post-fertilization. Morphological characters that I measured on days 3, 5, 7, 10, 13, 16, and 20 (*S. purpuratus* only): AL = Anterolateral arm, PO = Postoral arm, BL = Body length at midline, SL = Stomach length. All larvae are displayed at the same magnification; scale bar represents 100 microns.

arms at the stages when I collected measurements, my analysis focuses on plasticity in their summed length ("total arm length").

Statistical Analysis

Two analysis of variance (PROC MIXED: SAS Institute, Cary, NC) tests were conducted using the results obtained in this study. For the comparison among normally developing (full-size eggs), treatment control (whole-size eggs), and treatment (half-size eggs) *S. franciscanus* larvae, I tested for the effect of variation among treatment, day of development (day), food level (food), and culture replicate on total arm length. The statistical model included terms to account for variation due to the interactions of treatment with food, treatment with day, day with food, and the three-way interaction of treatment by day by food. Treatment, food, day, and the interaction terms were coded as fixed effects and culture as a random effect. Day was coded as a repeated measure with culture as the subject; the type of covariance structure of the R matrix was specified as Compound Symmetry (CS). The factor culture was nested within treatment and food. Degrees of freedom were calculated using the DDFM=BW (Between-Within) option in PROC MIXED. Body length was included in the model as a quantitative covariate. I compared models both with and without the body length interaction terms and used the model (no interaction terms) that provided the better fit to the data using Akaike's information criteria (AIC) (Littell et al., 1996). The specific comparisons of effects due to treatment and treatment with food interaction between larvae developing from half versus whole, half versus normal, and normal versus whole size eggs were tested using the CONTRAST statement in PROC MIXED (SAS Institute, Cary, NC). For the comparison between normally developing (full-

size eggs) *S. franciscanus* larvae with normally developing *S. purpuratus* larvae, I tested for the effect of variation among species (instead of treatment) and all of the factors described above. The 'treatment' and 'species' models were the same except for the terms used to account for the effects due to either treatment or species and their interactions with the other factors (the interaction terms). Arm and body length values for individual larvae were natural log transformed prior to analysis for both statistical tests to meet the assumptions of normality.

To investigate differences in the degree of plasticity expressed by individuals from each species and/or treatment over time, I calculated the absolute and percentage differences in mean total arm length between food treatments on each measurement day. I also calculated these values for mean relative arm length (arm length: body length ratio). Positive deviations from zero indicate that low fed larvae had longer arms, either absolutely or relative to body length, than high fed larvae. Lastly, I calculated the average percent difference across days in relative arm length expressed by larvae within each species and treatment.

Results

Modification of the blastomere separation procedure used for *Strongylocentrotus purpuratus* larvae by Allen (2005) was successful; separation of *S. franciscanus* blastomeres at the two cell stage produced embryos and larvae that were approximately half-sized (Figure 4.1). However, yield of half-size larvae was low, necessitating the use of smaller beakers for larval culture. There were no half-size larvae available for measurement after day 13.

ANOVA among full-, whole-, and half-size *S. franciscanus* larvae detected significant effects of treatment, day, food, and the interactions of treatment with day, day with food, and the three way interaction of treatment by day by food (Table 4.1). ANOVA also detected a significant effect due to body length. There was no effect due to the interaction of treatment with food. The results for the specific contrasts are also presented in Table 4.1: the specific contrasts of a treatment effect between half- versus whole- and half- versus full-size larvae were significant, indicating that larvae from full- and whole- size eggs developed longer arms when controlling for body size than larvae from half-size eggs. The specific contrast of a treatment effect between full- and whole-size larvae was not significant. The specific contrasts of a treatment by food interaction effect between half- versus whole-, half- versus full-, and full- versus whole-size larvae were not significant, indicating that there was no difference in the effect of the interaction of treatment with food (i.e. the degree of plasticity) on the expression of arm length when controlling for body size. ANOVA between untreated full-size *S. franciscanus* and *S. purpuratus* larvae detected significant effects of body length, species, day, food, and the interactions of species with day, species with food, and day with food (Table 4.2). There was no significant effect of the three way interaction of species by day by food.

Calculation of the mean percent difference between low and high fed larvae across all days within each treatment indicated that low fed larvae had arms that were absolutely longer than high fed larvae (see Figure 4.2 and Table 4.3): full size *S. franciscanus* (mean 5.51%: range -6.81% to 15.41%); whole size *S. franciscanus* (mean 10.12%; range -7.13% to 20.40%); half size *S. franciscanus* (mean 13.25%; range -0.70% to 22.91%); full size *S. purpuratus* (mean 2.34%; range -3.37% to 5.58%). A similar calculation indicated that low

fed larvae had arms that were longer relative to body size than high fed larvae: full size *S. franciscanus* (mean 11.88%: range 2.64% to 17.45%); whole size *S. franciscanus* (mean 10.37%; range 0.45% to 16.05%); half size *S. franciscanus* (mean 8.40%; range -0.28% to 15.46%); full size *S. purpuratus* (mean 4.53%; range 0.19% to 9.77%). High fed larvae had arms that were longer relative to body size than low fed larvae (negative value) only on day 13 for half size *S. franciscanus* (Table 4.3).

Differences in degree of plasticity of arm: body length ratios (using natural log-transformed values) among treatments over time are depicted graphically in Figure 4.4. Positive deviations from zero indicate low fed larvae had longer arms, relative to body length, than high fed larvae. *S. franciscanus* larvae from the full-, whole-, and half-size treatments all exhibited a similar pattern in the trajectory of their degree of plasticity curves: there was no significant effect due to the interaction of treatment with food (Table 4.1). Degree of plasticity increased rapidly through day 10 (excluding day 7 for the whole-size treatment) and plateaus at approximately 0.15 (all treatments) before decreasing slowly by day 16 (full- and whole-size) or decreasing rapidly to zero (half-size) by day 13. Considered collectively, the pattern exhibited by the trajectories of the *S. franciscanus* treatments differ from the pattern exhibited by *S. purpuratus*: there was a significant effect due to the interaction of species with food (Table 4.2). For *S. purpuratus*, degree of plasticity increased slowly over time, fluctuating with each measurement around approximately 0.05, before decreasing slowly after day 13.

Table 4.1: Analysis of Variance (ANOVA) results for full versus whole versus half sized *Strongylocentrotus franciscanus* larvae. Dependent variable is total arm length with body length as a quantitative covariate. Listed also are the results of the specific contrasts of an effect due to treatment and to treatment by food interaction between whole versus half size larvae.

Source	**df**	**F value**	**Pr > F**
Treatment	2, 14	25.99	<.0001
Day	4, 44	246.45	<.0001
Food	1, 14	80.96	<.0001
Treatment*Day	8, 44	9.24	<.0001
Treatment*Food	2, 14	0.46	0.6418
Day*Food	4, 44	10.92	<.0001
Treatment*Day*Food	8, 44	2.68	0.0171
Body Length	1, 389	510.63	<.0001
Contrasts: Treatment	**df**	**F value**	**Pr > F**
Half vs. Whole	1, 14	36.26	<.0001
Half vs. Full	1, 14	46.99	<.0001
Full vs. Whole	1, 14	1.32	0.2704
Contrasts: Treatment by Food	**df**	**F value**	**Pr > F**
Half vs. Whole	1, 14	0.38	0.5450
Half vs. Full	1, 14	0.88	0.3639
Full vs. Whole	1, 14	0.10	0.7533

Table 4.2: Analysis of Variance (ANOVA) results for full size *Strongylocentrotus franciscanus* versus full size *S. purpuratus*. Dependent variable is total arm length with body length as a quantitative covariate.

Effect	**df**	**F value**	**Pr > F**
Species	1, 10	1352.13	<.0001
Day	6, 51	87.67	<.0001
Food	1, 10	73.20	<.0001
Species*Day	5, 51	26.80	<.0001
Species*Food	1, 10	20.06	0.0012
Day*Food	6, 51	4.11	0.0020
Species*Day*Food	5, 51	1.36	0.2545
Body Length	1, 397	498.89	<.0001

Figure 4.2: Mean summed length of Postoral and Anterolateral arms (± 1SE) for High-fed (*filled* symbols) and Low-fed (*open* symbols) larvae over time. Arm length values for individual larvae were natural log transformed before means were calculated. **A:** *Strongylocentrotus franciscanus* larvae from full-size eggs. **B:** *S. purpuratus* larvae from full-size eggs. **C:** *S. franciscanus* larvae from whole-size eggs: individual blastomeres did not dissociate during the blastomere separation treatment. **D:** *S. franciscanus* larvae from half-size eggs: individual blastomeres dissociated into half-size "eggs" during the blastomere separation treatment.

Figure 4.2

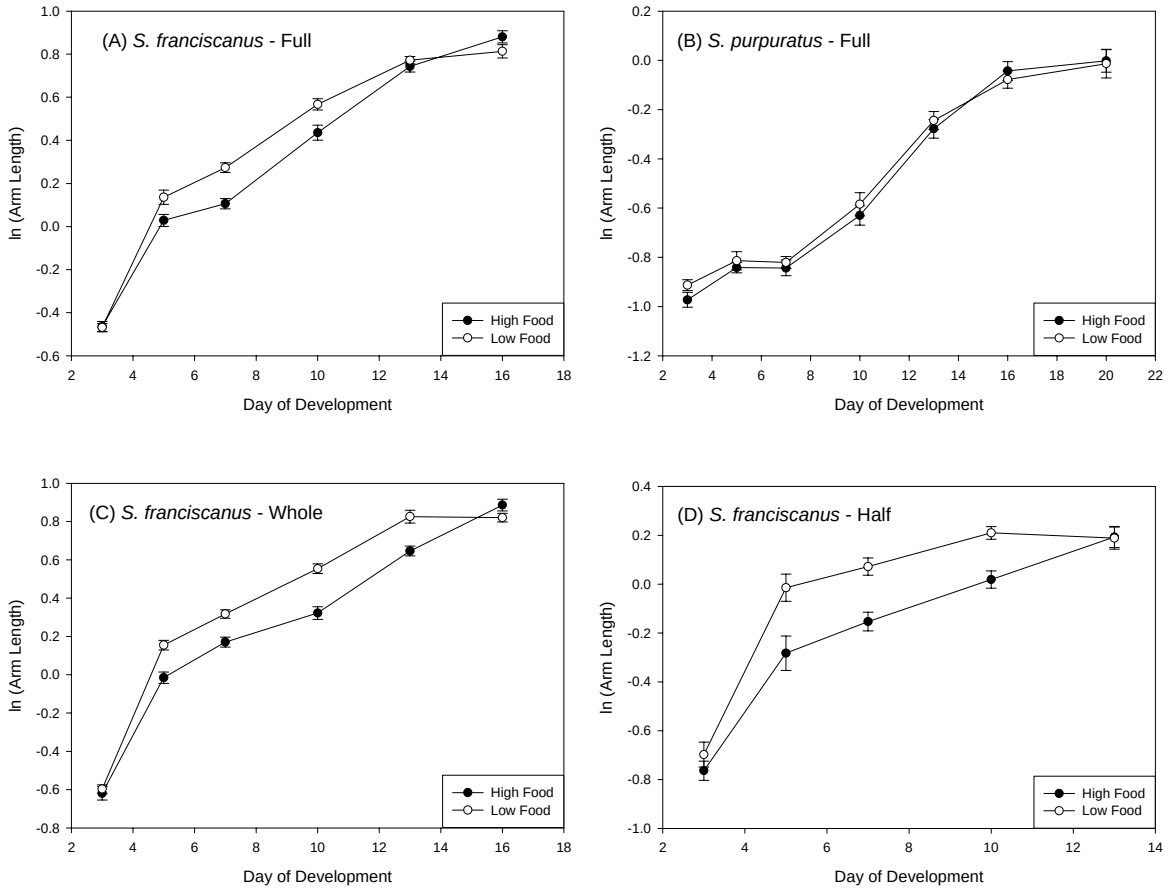
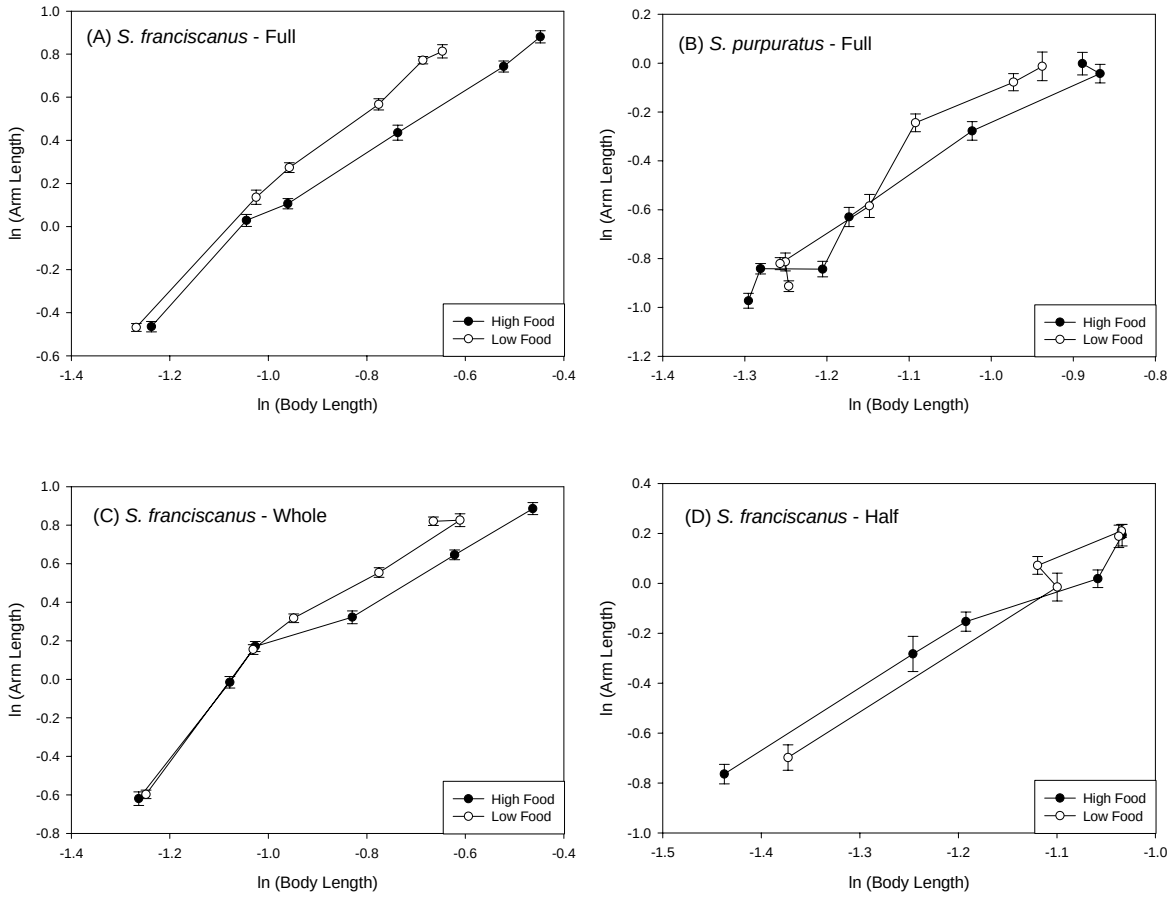

(A) S. franciscanus - Full
(B) S. purpuratus - Full
(C) S. franciscanus - Whole
(D) S. franciscanus - Half
ln (Arm Length)
Day of Development
High Food
Low Food

Table 4.3: Mean percent difference in absolute total arm length (normal text) and relative arm length (total arm to body length ratio: **bold text**) by species, treatment, and day. Positive values indicate that low fed larvae had either absolutely or relatively longer arms than high fed larvae. Note: values of arm and body length were not natural log-transformed for this analysis.

	S. franciscanus			**S. purpuratus**
Day	**Full**	**Whole**	**Half**	**Full**
3	-0.54	1.65	6.83	5.43
	2.64	**0.45**	**1.25**	**1.11**
5	10.39	15.51	22.91	3.57
	8.47	**11.31**	**11.65**	**0.19**
7	15.41	13.53	20.11	1.82
	15.04	**6.43**	**13.93**	**6.91**
10	11.98	20.40	17.08	5.58
	15.43	**16.05**	**15.46**	**2.85**
13	2.61	16.73	-0.70	3.52
	17.45	**15.42**	**-0.28**	**9.77**
16	-6.81	-7.13		-3.37
	12.24	**12.54**		**6.76**
20				-0.16
				4.12

Figure 4.3: Mean summed length of Postoral and Anterolateral arms (± 1SE) for High-fed (*filled* symbols) and Low-fed (*open* symbols) larvae versus mean summed Body Length at midline (± 1SE). Arm and Body length values for individual larvae were natural log transformed before means were calculated. **A:** *Strongylocentrotus franciscanus* larvae from full-size eggs. **B:** *S. purpuratus* larvae from full-size eggs. **C:** *S. franciscanus* larvae from whole-size eggs: individual blastomeres did not dissociate during the blastomere separation treatment. **D:** *S. franciscanus* larvae from half-size eggs: individual blastomeres dissociated into half-size "eggs" during the blastomere separation treatment.

Figure 4.3


(A) S. franciscanus - Full
(B) S. purpuratus - Full
(C) S. franciscanus - Whole
(D) S. franciscanus - Half
ln (Arm Length)
ln (Body Length)
High Food
Low Food

Discussion

In the Introduction, I have suggested two hypotheses regarding the effect of egg size on plasticity. The first was an energy/materials argument: larvae from larger eggs may have a greater scope for the expression of plasticity because they have access to and make use of the materials in a larger egg. The second was an evolutionary argument: larvae that develop from smaller eggs may have been selected for a greater capacity for plasticity to energetically discount their earlier dependence on exogenous food. The results presented here suggest that for *Strongylocentrotus*, the role of egg size in plasticity may derive from a combination of both hypotheses.

First, in response to low food, whole-size *S. franciscanus* larvae had relatively longer arms than half-size *S. franciscanus* larvae. The results of the contrast statements in the ANOVA indicate a significant difference between these two treatments (Table 4.1). Similarly, averaged over time, the mean percent difference in relative arm length between low and high fed larvae was 10.4% for whole- compared to 8.4% for half-size larvae. These results support the energy/materials argument because halving egg size via blastomere separation decreased the degree of plasticity that larvae expressed.

Second, in response to low food, untreated full-size *S. franciscanus* larvae (egg diameters from two adult females: 122.7 $\pm$ 0.35 μm and 124.7 $\pm$ 0.17 μm) had relatively longer arms than untreated full-size *S. purpuratus* larvae (egg diameters from two adult females: 81.8 $\pm$ 0.00 μm and 85.2 $\pm$ 1.12 μm). The result of the ANOVA indicates a significant difference between these two treatments (Table 4.2). Averaged over time, the mean percent difference in arm length relative to body length between low and high fed larvae was 11.88% for *S. franciscanus* and 4.53% for *S. purpuratus* larvae. These results do

not support the evolutionary argument of the second hypothesis that larvae from a species that has evolved smaller eggs express a greater degree of plasticity than larvae from a species that has evolved larger eggs.

Interestingly, experimentally reducing the amount of energy available to *S. franciscanus* larvae via blastomere separation did not result in the production of larvae that expressed a degree of plasticity comparable to *S. purpuratus* larvae. Half-size *S. franciscanus* larvae developed from individual blastomeres that were smaller than full-size *S. purpuratus* eggs (<70 um vs 81.8 - 85.2 um). Although half-size *S. franciscanus* larvae developed from comparable, yet slightly smaller eggs than full-size *S. purpuratus* larvae, they expressed a greater mean percent difference in relative arm length between low and high fed larvae when averaged over time (8.40% versus 4.53%, respectively).

Furthermore, halving the amount of energy available to a developing larva did not alter the pattern of plastic expression over time. Certainly there was variation in the degree of plasticity that was expressed by the different species and treatments over time (Fig. 4.4), but *S. franciscanus* larvae from the full-, whole-, and half-size treatments all exhibited a similar pattern as shown by the comparable trajectory of the degree of plasticity curves in Figure 4.4. Although it is not surprising for full- and whole- size larvae to develop along a similar trajectory, it is interesting that half size larvae followed a similar pattern and expressed degrees of plasticity comparable to larvae developing from 'normal' (full or whole) size eggs of the same species. Full-size *S. purpuratus* larvae, which develop from eggs that are comparable in size to half-size *S. franciscanus* larvae, exhibit a different pattern in degree of plasticity over time. These differences in response of arm length to food are captured in the results of the two ANOVAs: there was a significant species with food

interaction term (p = 0.0012) in the comparison between full-size *S. franciscanus* and *S. purpuratus* larvae, and a non-significant treatment with food interaction term (p = 0.6418) in the comparison among *S. franciscanus* larvae from the three treatments.

The similarity in developmental pattern among full-, whole-, and half-size *S. franciscanus* larvae, and their collective difference from the pattern expressed by *S. purpuratus* larvae, suggest that the evolutionary history and/or genetic predisposition of a species is, at least in this instance, more important than endogenous resource availability to the expression of plasticity. The trajectories displayed in Figure 4.4 suggest that half-size *S. franciscanus* larvae may be genetically programmed to express a pattern of plasticity unique to *S. franciscanus*. Half-size larvae are able to maintain a level of plasticity that is comparable to normal-size larvae through day 10. At this time, lack of endogenous resources may limit the degree of plasticity (of arm length relative to body length) that can be expressed by low fed, low endogenous energy, half-size larvae. Alternatively, *S. purpuratus* larvae, which develop from eggs that are comparable to half-size *S. franciscanus* 'eggs', exhibit minimal difference in relative arm length between low and high fed larvae over time, suggesting evolved differences between the two species in degree of plasticity.

Qualitative observation of Figures 4.2 and 4.3 reinforces the notion that there is an interplay between the energy/materials and evolutionary history for the effect of egg size on the expression of plasticity in *Strongylocentrotus*. In Figures 4.2 and 4.3, *S. franciscanus* larvae from all treatments exhibit a dramatic increase in arm length between days 3 and 5. The rapid increase is likely fueled by the large endogenous resources contained in the egg of this species because this pattern is apparent in both low and high fed larvae. Although large endogenous resources may fuel this pattern, it is clearly a pattern evolved by *S. franciscanus*

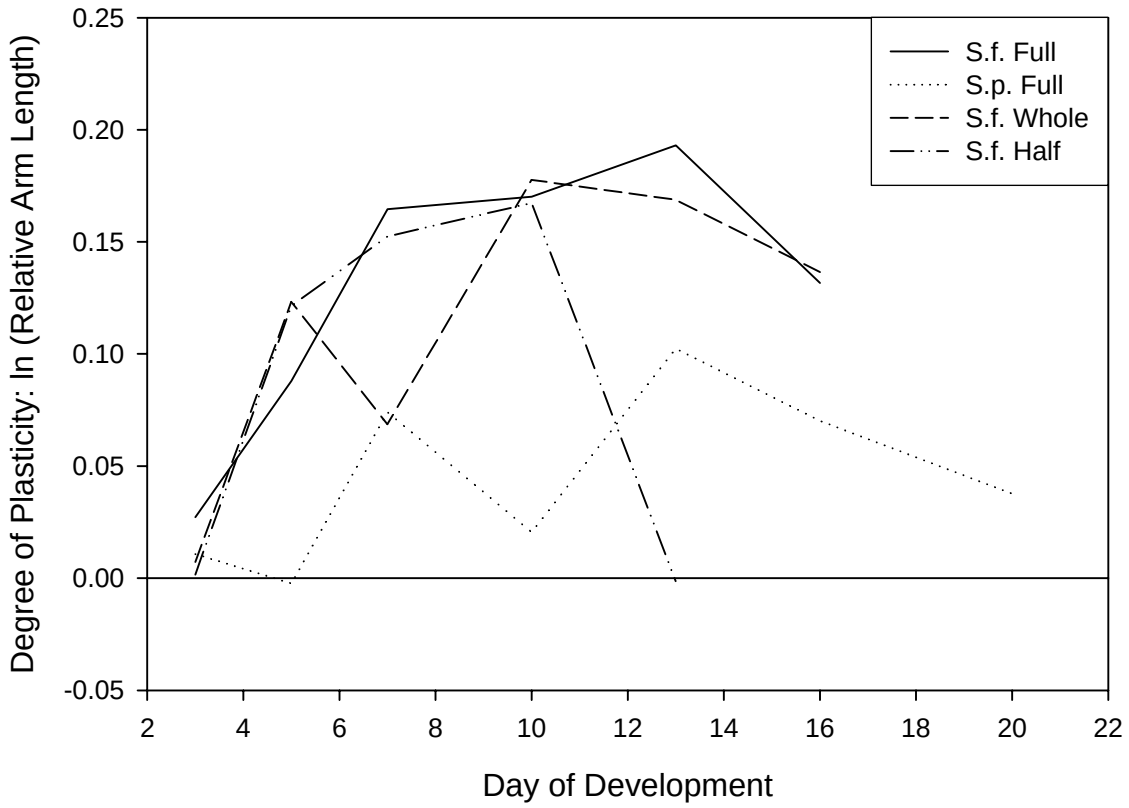


Figure 4.4: Degree of plasticity of Relative Arm Length for full-size *Strongylocentrotus purpuratus* and full-, whole-, and half-size *S. franciscanus* larvae over time (dotted, solid, dashed, dash-dotted lines, respectively). Degree of plasticity was calculated by subtracting the mean natural log-transformed Arm: Body Length ratios expressed by larvae reared in the high food environment from the mean natural log-transformed Arm: Body Length ratios expressed by larvae reared in the low food environment. Positive deviations from zero indicate low-fed larvae have longer arms, relative to body length, than high-fed larvae.

because half-size *S. franciscanus* larvae exhibit the same pattern. Half-size *S. franciscanus* larvae do not display a pattern similar to *S. purpuratus*, which are comparable in egg size. *S. purpuratus* larvae display a more gradual increase in arm length during early development, reflecting the smaller amount of egg-bound energy, and an evolved pattern of plasticity that is different than the pattern exhibited by *S. franciscanus*.

Full-size *S. franciscanus* and *S. purpuratus* larvae exhibit clear differences in the pattern of plastic expression of relative arm length over time. However, adults from these two species were collected from the same location and their larvae co-occur in the same planktonic habitat; most environmental characteristics, e.g. food availability, levels of predation, etc. may be expected to exert similar selective pressures on larvae from either species. What can explain the difference in the patterns of plasticity adopted by each species?

To minimize mortality, larvae are presumed to be under strong selection to decrease development time spent in the plankton by increasing food assimilation (Rumrill, 1990; Lamare and Barker, 1999). If this is indeed the case, then strategies that increase food capture under low-food conditions, e.g. expressing a high degree of arm length plasticity, would support this hypothesis. However, life in the plankton may not be as dangerous as previously thought, as rates of larval predation may be lower than recognized (Allen and McAlister, 2007). Using tethered crab megalopae and flavored agarose pellets as baits, these authors found bait loss rates (due to predation) that were 12-25 times greater on the benthos than the plankton. If this result is representative, then selection to increase number of progeny, consequently decreasing the amount of endogenous materials provided to a given egg, may be stronger than selection to decrease development time in the plankton. The results of my study suggest that this may be the strategy adopted by *S. purpuratus*.

Emlet et al. (1987) reviewed size at settlement data for echinoid species with planktotrophic larvae and found that over a wide range of egg sizes, size at settlement was relatively constant. Furthermore, Doughty (2002) found that plasticity and maternal provisioning strategies can coevolve to help larvae cope with unpredictable larval environments. There may be multiple viable strategies to increase food consumption to attain settlement competency, if the rates of planktonic predation are relatively low. Evolving a larger egg size, trading-off progeny number, and increasing food assimilation by expressing a higher degree of plasticity, which results in a decrease of development time may be one strategy, as exhibited by *S. franciscanus*. Alternatively, evolving a smaller egg size, increasing progeny number, and expressing a lower degree of plasticity, which results in an increase in development time may be another, as exhibited by *S. purpuratus*.

Egg size, plasticity, and life history evolution

The results of the present study differ with those of Podolsky and McAlister (2005) and Reitzel and Heyland (2007). Podolsky and McAlister's (2005) study of ophiuroid pluteus larvae indicated that the two smaller egged species exhibited plasticity of larval arm length and the two larger egged species did not. Reitzel and Heyland (2007) also found that larvae of the two smaller egged species (*Mellita tenuis* and *Clypeaster subdepressus*) exhibited a significantly higher plastic response to low food conditions than the larger egged species (*Leodia sexiesperforata*).

Initial egg size may account for the differing results among the present study and those of Podolsky and McAlister (2005) and Reitzel and Heyland (2007). Mean egg diameters of the four species of ophiuroids in the genus *Macrophiothrix* used by Podolsky

and McAlister (2005) were 147, 155, 166, and 230 µm. In Reitzel and Heyland's (2007) study, mean egg diameters were as follows: *M. tenuis* 99, *C. subdrepressus* 150, and *L. sexiesperforata* 191 µm. Mean egg diameters of the two *S. franciscanus* (122.7 and 124.7) and two *S. purpuratus* (81.8 and 85.2) individuals used in the present study are most comparable in size to *M. tenuis* and smaller than all of the other species in the two studies. Furthermore, only the present study quantifies the degree or level of plasticity expressed by species with relatively small egg sizes.

Reported mean egg diameters of other species in which plasticity has been demonstrated are generally less than approximately 170 microns (Strathmann et al. 1992; Hart and Strathmann, 1994; Eckert, 1995; Bertram and Strathmann, 1999). Reitzel and Heyland (2007) suggest that planktotrophic species with a very high degree of maternal provisioning (*L. sexiesperforata* in their study; *Encope michelini*, Eckert, 1995) have decreased plastic expression. However, among planktotrophic species with 'smaller' egg diameters that fall within the range presented above, increased maternal provisioning may confer increased capacity for plastic expression. Furthermore, the degree and pattern of plastic expression may well be closely associated with the developmental strategy (within planktotrophy) of a given species (as discussed above), the amount of endogenous energetic reserves (egg size), and selection from environmental variables that are unique for a population from a given location. Other life-history parameters such as longevity, maximum adult size, age at 1st reproduction, etc. must also be taken into account.

Strathmann et al. (1992) proposed that plasticity of exogenous feeding structures may be associated with the evolution of large egg size, the loss of feeding structures, and the adoption of non-feeding development in some species. Bertram and Strathmann (1998)

investigated whether endo- and exogenous food resources provide the same stimuli to developing *Strongylocentrotus droebachiensis* larvae. They found that larvae developing from the smaller eggs (153µm) of food-limited mothers did not produce larger feeding structures than larvae developing from the larger eggs (159µm) of food-satiated mothers. The authors suggested that changes in egg size alone may not lead to the loss of feeding structures, but that preexisting developmental plasticity may provide a mechanism for a coordinated suite of morphogenetic changes that leads to the evolution of non-feeding. In addition, unpublished work by Strathmann and Bertram (R.R. Strathmann, pers. comm.) in which egg volume of *S. purpuratus* was doubled by egg fusion to compare development with *S. droebachiensis* revealed that interactions between egg size and food supply did not override inter-specific differences in development of larval and juvenile structures. Their results suggested that an evolutionary increase in egg size alone does not result in the acceleration of the formation of juvenile structures and that other genetic changes are responsible for the evolution of non-feeding. In light of the differences in the patterns of plasticity exhibited by *S. franciscanus* and *S. purpuratus* in the present study, manipulations of egg size alone are clearly not sufficient to supercede genetic differences among species. Exogenous treatment with hormones (e.g. thyroxine), which has been shown to accelerate larval development (Heyland and Hodin, 2004; Heyland et al. 2004), coupled with egg size manipulations and/or genetic modification, may help to elucidate the mechanisms responsible for an evolutionary transition from feeding to non-feeding.

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