University of Alberta

Phenotypic plasticity in sea urchin larvae: Environmental effects reveal true developmental potential

by

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Dedicated to my dad Jack (John), my brother Brendan, my husband Shawn and especially my mom, Anne Eaves (nee Joel) 1946-2007.

I could not have done this without them.

Abstract

Pelagic marine environments are highly dynamic and pose grave threats to small-bodied planktonic organisms. Nonetheless, more than 70% of benthic marine animals possess a planktonic larval stage that can last for days, weeks or years. Larvae experience unstable food supplies, threats from predator attack, and variable physical conditions. Using sea urchins — a model organism for developmental biologists since the mid-nineteenth century — I studied the effects of each of these three broad categories of environmental stress on larval development. The effect of food supply on larval development was investigated at two extremes: super abundant food and no food. When larvae of *S. purpuratus*, and *S. droebachiensis* were cultured with a dense, mixed-algal diet, larval cloning — a previously unrecognized phenomenon among echinoids — could be induced. Conversely, when *S. purpuratus* larvae were starved they appeared to re-absorb their guts to sustain the rest of the larval body until feeding resumed and they recovered. The effects of sub-lethal predator attack were investigated by fracturing the feeding arms of *S. droebachiensis* larvae. Fractured arms were repaired in three different ways: 1) degeneration followed by regeneration, 2) autotomy followed by regeneration, or 3) re-alignment and fusion of the fractured ends of the skeletal rod. All repairs were completed within 48 hours and neither the incidence, nor frequency of fractures affected the time to metamorphosis. Finally, the effects of variable oceanographic conditions on early development, the rates of fertilization, hatching, and gastrulation under different temperature and salinity combinations were studied in three congeneric species of sea urchins from a sympatric population. Embryos of *Strongylocentrotus franciscanus* tolerated a broader salinity range than embryos of *S. droebachiensis* and *S. purpuratus*,

and all embryos tolerated a broader salinity range at lower temperatures. Overall, the great regenerative capacity of echinoid larvae makes them extremely resilient to many forms of environmental stress. Moreover, the developmental plasticity of echinoid larvae is significantly greater than previously believed, and may have been key to their evolutionary persistence for half a billion years.

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Chapter I

General introduction

The global climate is changing, and the survival of species will depend on their ability to adapt fast enough (Visser 2008). Changes in species range, migration, reproduction and development have been correlated with rising temperatures for grasses, trees, invertebrates, and vertebrates (Root et al. 2003). Despite slower ocean warming, marine organisms may be responding to climate change faster than terrestrial counterparts (Richardson & Poloczanska 2008). Only a slight increase in temperature results in altered ocean currents, chemistry, nutrient cycling and phytoplankton blooms affecting species distribution ranges, reproductive cycles, and life cycles of marine organisms (Richardson & Poloczanska 2008). Changes in species traits in response to climate change are attributed to both heritable genetic changes in populations, and to phenotypic plasticity – the ability of individuals to modify their physiology, morphology or behavior in response to environmental conditions (Bradshaw & Holzapfel 2006). Phenotypic plasticity can enable an organism to respond quickly and effectively to environmental change, and can potentially facilitate species adaptation to variable conditions over several generations (Charmantier et al. 2008). Therefore, studies of phenotypic plasticity provide important insights into how organisms may adapt to a rapidly changing environment.

1.1 Relevance to marine larvae

Over 70% of benthic marine organisms have a pelagic, feeding larval stage (Thorson 1950). This larval stage can last for days, months, and even years (Scheltema

1986; Strathmann & Strathmann 2007). During this time larvae are vulnerable to many environmental perils such as unpredictable food supplies, predator attack and temperature and salinity variation (Thorson 1950). Because marine larvae are passively dispersed via oceanic currents they have little control over the types of conditions they encounter. Consequently, they must have some means of withstanding many forms of environmental stress. However, the marine environment also provides a unique habitat in which organisms can change more radically than those on land (Strathmann 1990). Marine invertebrate reproductive cycles, embryology, and timing and mode of development can all vary in response to temperature and food availability, and plasticity of these traits likely increases the probability of survival (Hadfield & Strathmann 1996). Although flexible reproductive and developmental characteristics are well known, they are also under-reported and under-appreciated (Hadfield & Strathmann 1996). Therefore, the objective of this thesis is to explore variation in developmental outcomes induced by a variety of environmental effects using the well-studied larvae of sea urchins.

1.2 Why study echinoid development?

Because they are so suitable for experimental studies, sea urchins have been studied by developmental biologists since the mid nineteenth century (Monroy 1986). Adults are readily accessible from any marine coast, large quantities of gametes can be obtained from artificially induced spawning, eggs have completed secondary meiosis prior to being released, and many echinoid embryos and larvae are transparent (Hinegardner 1969; Monroy 1986). Consequently, biologists have learned a great deal regarding the patterns and mechanisms of early embryogenesis, and the form and function of early larvae (Hyman 1955; Hinegardner 1969; Davidson 1989; Cameron et al.

1991). In addition, because of their phylogenic position within the deuterostomes — the lineage that includes hemichodates, urochordates, and chordates (Zeng & Swalla 2005) — echinoderms share several hallmark developmental features with other members of this group including: 1) a blastopore that does not form a mouth, 2) radial cleavage pattern, 3) regulative development, and 4) a tendency towards enteroceolous coelom formation (reviewed by Gilbert 1997). However, despite the suitability of echinoids as a model organism, their larvae are notoriously difficult to culture in the laboratory and consequently substantially less is known about later stages of development (Cameron & Hinegardner 1974; Minsuk & Raff 2002).

1.3 Overview of 'normal' echinoid development

Echinoid embryology and development has been described by many (see MacBride 1903; Hymann 1955; Hinegardner 1969; Czihak 1971; among others). Echinoids are usually dioecious, and gametes are released into the water column where fertilization occurs. Within minutes, a fertilization envelope elevates around the embryo (Fig. 1-1A) and embryogenesis proceeds until a motile, ciliated blastula is ready to hatch. Immediately after hatching, gastrulation begins with the thickening of the vegetal pate and invagination of the blastopore. This invagination forms the archenteron (Fig. 1-1B) and gives rise to the future endoderm and mesoderm of the larval body. The archenteron elongates through the blastocoel by convergence and extension, and at the anterior tip it connects to the ectoderm to form the larval mouth. Along the lateral sides of the archenteron two mesodermal outpockets form, and these differentiate into the larval coeloms including the anterior coelom on the right side of the larva, and the axocoel,

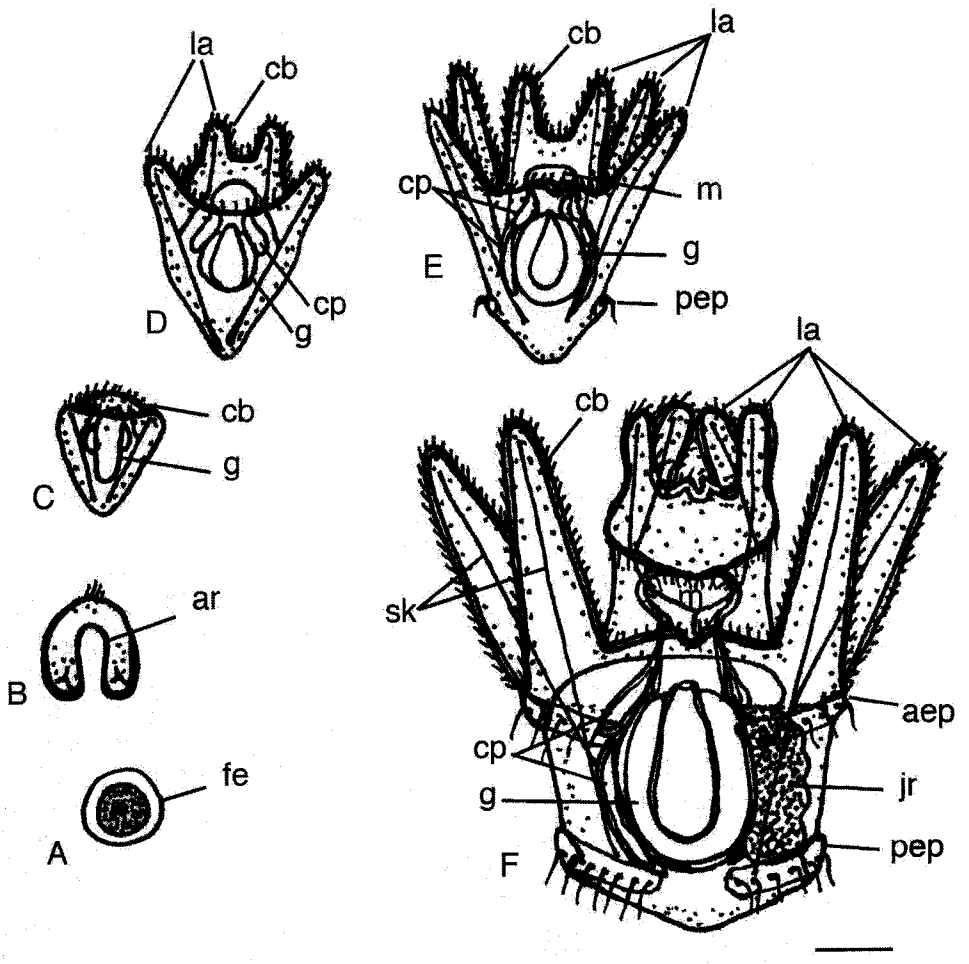

Figure 1-1. Diagram of sea urchin larval development (ventral view). A. Egg with fertilization envelope (fe), B. gastrula with archenteron (2 d)(ar), C. prism (4 d) with apical ciliary band (cb) and early gut (g), D. 4-arm pluteus larva (10 d) with larval arms (la), and coelomic pouches (cp), E. 6-arm pluteus larvae (21 d) with mouth (m) and posterior epaulettes forming (pep), F. 8-arm pluteus larva (28 d) with anterior (aep), juvenile rudiment (jr) and skeletal rods (sk). Scale bar approximately 150 μm.

hydrocoel and somatocoel on the left side of the larva. As the gastrula continues to grow a ciliary band differentiates along the apical margin of the ectoderm surrounding the oral ectoderm (Fig. 1-1C). This ciliary band generates swimming and feeding currents for the larva. As the ciliary band lengthens it is supported by projections of the larval body to create a directional feeding current towards the larval mouth (Fig. 1-1D)(Strathmann 1971; 1975). To maintain symmetry of this feeding structure the larval arms typically form in pairs and are supported by calcareous skeletal rods (Fig. 1-1E). Most echinoid larvae form a total of eight arms. As food particles arrive at the mouth they are ingested when the larva swallows them into the single cell-thick, tripartite digestive tract including: a ciliated and muscularized esophagus, ciliated gut and a ciliated and mucularized intestine and anus. The endodermal cells of the gut accumulate lipid during larval growth, and these same cells are reorganized into the adult digestive tract after metamorphosis (Burke 1981). Additional ciliated structures (epaulettes) form around the circumference of the larval body, and allow for bi-directional propulsion of the larva as it grows heavier with differentiated internal structures (Strathmann 1971; 1975).

1.4 Formation of the juvenile body

One of the more peculiar aspects of echinoid development is formation of the juvenile body almost as a parasite within the larval body (Fig. 1-1F). Normally, on the left side of the body, an ectodermal invagination extends a finger-like projection towards the expanding hydrocoel adjacent to the larval gut. When the ectodermal and mesodermal structures are in contact, they proliferate together and differentiate the pentaradially symmetric water-vascular system of the juvenile. Gradually juvenile skeletal structures

including the genital plates and adult spines differentiate around the juvenile water-vascular system and in many larvae juvenile spines or defensive pedicelaria differentiate in ectodermal pockets on the posterior and right side of the larval body, which will become the aboral surface of the juvenile after metamorphosis. The oral-aboral axis of the developing juvenile is established perpendicular to the anterior-posterior axis of the larval body (Hymann 1955). When the primary podia of the juvenile rudiment become motile, the larva is competent to settle and metamorphose.

Sea urchin metamorphosis is a truly radical event. A competent larva will settle on an appropriate substrate and partly evert its juvenile rudiment (Burke 1980; 1983; Cameron & Hinegardner 1978; Kitamura et al. 1993; Eaves 2005). The larva adheres to the substrate by extending its primary podia from inside the juvenile rudiment and by generating strong ciliary reversals with its epaulettes (Satterlie & Cameron 1985). Once attached, the ectoderm of the larva begins to collapse thereby exposing the skeletal rods that are later cast away (Burke 1983). The juvenile rudiment 'opens' into a radially symmetric juvenile with adult spines projecting horizontally around the oral surface, and the remaining larval ectoderm collapses and is destroyed (Chia & Burke 1978). Externally, metamorphosis takes less than an hour (Satterlie & Cameron 1985). However, internally metamorphosis proceeds for up to a week as the digestive tract of the larva is remodeled into that of the juvenile (Burke 1981). Afterwards the juvenile begins to graze on diatoms.

Echinoid development can take days to several months depending on the mode of larval development. The mode of development is determined by maternal allocation of yolk reserves during oogenesis. In general, larger eggs provisioned with ample yolk have a shorter non-feeding larval developmental period (lecithotrophy) (Strathmann et al. 1992). In contrast, small eggs with minimal yolk require a protracted larval feeding period to acquire sufficient resources to sustain development to metamorphosis (planktotrophy) (Strathmann et al. 1992). Each mode of larval development has advantages and disadvantages. Lecithotrophic larvae require substantially less time to settle in the benthic adult habitat, are not vulnerable to planktonic food-supply, occur in smaller cohorts because fewer large eggs can be produced, and have significantly reduced dispersal potential (Thorson 1950). Plantotrophic larvae, on the other hand, can be produced in large cohorts because the eggs are small and require minimal maternal investment, and the protracted feeding period allows increased dispersal potential. However, because the rate of planktotrophic development depends on food availability, planktotrophic larvae are exposed to diverse environmental hazards during protracted dispersal periods (Thorson 1950). Despite these evident disadvantages, planktotrophy persists as the dominant form of development of benthic marine invertebrates (Thorson 1950).

1.5 Environmental effects on larval development

The overall objective of this thesis is to explore the true developmental potential of echinoid larvae by exploiting their phenotypically plastic responses to various environmental effects. By comparing the effects of multiple forms of environmental influence during different periods of embryo and larval development among species of

the sea urchin *Strongylocentrotus* I discovered several novel forms of phenotypic plasticity. Originally, each of these factors was being examined for its effects on the recent discovery of larval cloning in echinoids (Eaves & Palmer 2003). However, additional curious observations led to entirely new morphogenetic phenomena being explored instead.

1.6 Effects of food abundance

Many planktotrophic larvae have specialized feeding structures that can change size relative to the rest of the larval body according to food abundance, including the diameter of velar lobes in bivalve and gastropod larvae (Strathmann et al. 1993; Klinzing & Pechenik 2000), and larval arm lengths of brittlestar (Podolsky & McAlister 2005) and echinoid larvae (Boidron-Metairon 1988; Fenaux et al. 1988, 1994; Hart & Scheibling 1988; Strathmann et al. 1992; Hart and Strathmann 1994; Sewell et al. 2004; Miner 2005, 2007; Reitzel & Heyland 2007). However, none of these studies examined phenotypically plastic responses to extreme food conditions, therefore the first and second studies of this thesis examined the effects of extreme food levels on larval morphogenesis in two parts: 1) supra-optimal food conditions, and 2) extreme food-limited conditions (starvation). In the first study (chapter II, appendices A-1, A-2) echinoid larvae were cultured with a high-quality and abundant food source to observe what the larval body did with an excess of food. Surprisingly, larvae began to form asexual clones by detaching parts of their bodies. These clones initiated a secondary, wholly novel developmental program and alternate life history pattern previously unrecognized for this group of animals despite having been extensively studied for well over a century. In the second study (chapter III),

larvae were cultured under variable food conditions and were either consistently starved, fed at low or high concentration of food, or subjected to a transient period of starvation after which the larvae were fed again. In response to starvation the diameter of the guts shrank significantly, not because of a lack of distention from gut contents, but rather because of a novel morphogenetic mechanism thought to represent a form of resource re-allocation during transient periods of extreme food limitation.

1.7 Effects of physical damage

Embryos and larvae of benthic marine invertebrates are preyed upon by carnivorous zooplankton, fish, benthic invertebrates and protozoans (reviewed by Rumrill et al. 1985). However, despite the abundance of predator-induced morphologies in plants and animals from terrestrial and aquatic environments, only recently have predator-induced morphological defenses been investigated in marine larvae. Gastropod larvae form rounder shells with smaller apertures in the presence of predator cues (Vaughn 2007), and echinoid larvae can clone themselves into smaller individuals in the presence of predator cues (Vaughn & Strathmann 2008). Furthermore, gastropod veliger larvae can regenerate damaged structures after surviving a predator attack (Hickman 2001). Clearly, not all larval predator attacks should be assumed to be fatal. Even fragile echinoplutei have a lower incidence of mortality among older larvae (Rumrill et al. 1985, Allen & McAlister 2007). Therefore, the third study of this thesis (chapter IV) examines how fragile microscopic organisms might be able to survive non-lethal predator attacks by investigating how damaged feeding structures are repaired, and the effect of physical trauma on the timing of development of the juvenile rudiment.

1.8 Effects of abiotic factors

Abiotic factors such as temperature and salinity affect the development, survival and distribution of marine invertebrates (Kinne 1964, Scheltema 1965). Temperature significantly affects the season and rate of marine invertebrate development (reviewed by Hoegh-Guldberg & Pearse 1995) and salinity also influences the rate of development (reviewed by Richmond & Woodin 1996). The key factor determining the range of abiotic conditions developing embryos can tolerate is determined by the habitat of the adult (Stickle & Diehl 1987). However the early developmental stages of most invertebrates have a much narrower tolerance range than later larval stages or adult organisms (Saranchova et al. 2006). How does abiotic stress tolerance during early development compare among closely related species? The final study (Chapter V) examined the effects of variable temperature and salinity combinations on the early development of three species of congeneric sea urchins to characterize the optimum abiotic environmental conditions for early development.

Collectively these studies provide a comprehensive survey of responses to unpredictable physical and biological factors experienced by pelagic larvae of a well-studied group of benthic invertebrates. They also provide some valuable insights into how organisms may adapt to changing global climate conditions, with some very unexpected results.

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Chapter II

Larval cloning in echinoids and comparisons with other echinoderm classes[2-1]

2.1 Introduction

Nearly a century ago, Mortensen (1921, p.147-149) described asexually reproducing ophiuroid larvae from his plankton samples. Unfortunately his remarkable observation was dismissed as "in urgent need of confirmation", "totally opposed to what we know of the normal development of Ophiuroids" (MacBride 1921), and "audacious" (Bather 1921). Nearly seven decades later Bosch and colleagues (1989) reported larval cloning in field-collected asteroid larvae. Eventually, Mortensen's (1921) observations of ophiuroid larval cloning were confirmed (Balser 1998). These pioneering reports raised awareness that such a remarkable alteration in life history strategy was possible among echinoderms.

Several additional reports of echinoderm larval cloning have since followed (Bosch 1992; Rao et al. 1993; Jaeckle 1994; Vickery and McClintock 2000; Knott et al. 2003, Eaves and Palmer 2003, appendix 1, 2; Balser 2004). However, most of these reports describe asexual reproduction among field-collected larvae. Larval cloning has been much less well-studied in laboratory culture (Balser 1998; Vickery and McClintock 2000; Eaves and Palmer 2003), likely because culturing larvae with a protracted developmental period through to later stages is notoriously difficult (Minsuk and Raff 2002). As a result, little is known about the morphogenesis of echinoderm, and particularly echinoid, clones.

[2-1] A version of this chapter has been submitted for publication in the journal Evolution & Development.

Larvae may clone in three different ways: paratomy, autotomy and budding (Jaeckle 1994; Balser 2004). Paratomy is most commonly observed in asteroids, whereby the posterolateral arms develop into small bipinnaria larvae that separate from the primary larva while the remainder of the primary larval body is intact (Bosch et al. 1989; Bosch 1992; Rao et al. 1993; Jaeckle 1994: Vickery and McClintock 2000; Knott et al. 2003; Balser 2004). In contrast, autotomy involves the spontaneous separation of the larva into two relatively large pieces, each of which regenerates the missing portion of the larval body after detaching. Currently, larval cloning by autotomy has primarily been observed in asteroids (Jaeckle 1994; Vickery and McClintock 2000). In ophiuroids this process occurs late in development when the juvenile body is released from the remnants of the larval arms that regenerate a new ophiuroid larva (Balser 2004). Lastly, budding is the subtlest form of larval cloning. It involves the aggregation of mesenchymal cells in the blastocoelar space beneath a localized dedifferentiation of the larval ciliary band to form a ball of cells equivalent to a blastula that separates (Jaeckle 1994). Budding is the most ubiquitous form of cloning among echinoderm larvae and occurs in asteroids (Jaeckle 1994), echinoids (Eaves and Palmer 2003), and holothuroids (Eaves and Palmer 2003; Balser 2004).

I describe here the budding process in detail using light microscopy and histological examination of developing clones from three echinoid species: the regular urchins *Strongylocentrotus purpuratus* and *S. droebachiensis*, and the sand dollar *Dendraster excentricus*. Details of this process are compared to what is known about

cloning in other echinoderm classes and its significance to the evolution of deuterostome development.

2.2 Materials and methods

2.2.1 Gamete collection and larval culturing

Adult *Strongylocentrotus purpuratus* and *S. droebachiensis* were collected from low rocky intertidal and subtidal zones near Brady's Beach, near Bamfield, British Columbia, and spawned by intracoelomic injection of 0.55 M KCl. Larvae derived from multiple sets of parents (2 male/ female pairs of *S. droebachiensis* and 5 pairs of *S. purpuratus*) were maintained in a nineteen hour light cycle at 14°C in pasteurized seawater and fed 10^6 cells/ml of a 1:1:1 mix of *Isochrysis galbana*, *Rhodomonas salina*, and *Dunalliela tertiolecta*. Adult *Dendraster excentricus* were collected from intertidal mudflats (Patricia Bay, British Columbia) and spawned immediately in the lab as described for *S. purpuratus* and *S. droebachiensis*. Larvae were maintained in darkness at 13°C in filtered seawater and fed *Isochrysis galbana* at 10^6 cells/ml. Cloning larvae of each species were isolated in individual 8 ml glass vials and cultured as described above for serial observations.

2.2.2 Histological sections

Various stages of cloning *S. purpuratus* larvae were preserved in 2.5% glutaraldehyde in filtered seawater for one hour, rinsed 3 times in 2.5% sodium bicarbonate buffer (pH 7.2) and post-fixed for 1 hour in 2% osmium tetroxide in 1.25% sodium bicarbonate buffer. Specimens were dehydrated through a graded ethanol series

(30%, 50%, 70%, 90%, 95%, 100%), rinsed in propylene oxide and imbedded in Epon epoxy resin. Semi-thin (1μm) sections were cut using a diamond knife on a Reichert-Jung Ultracut E microtone and stained with Richardson's stain for contrast.

2.2.3 Microscopy

Larvae and histological sections were photographed on an Olympus CX40 compound microscope with a Nixon CoolPix 4500 digital camera with eyepiece adapter.

2.3 Results

2.3.1 Morphology of cloning in *Strongylocentrotus purpuratus*

Cloning was observed in approximately 5% of laboratory-reared *S. pupuratus* larvae (N= 4000). Cloning larvae were very distinct from non-cloning counterparts after five weeks of development (Fig. 2-1A,B). In the earliest stages, cloning larvae have a slightly elongated and rounded posterior end beneath the posterior fused epaulettes. Within the blastocoelar space of the larva a septum bearing many filopodial projections extended from the basal surface of the ectoderm and constricted towards the midline (Fig. 2-2A). As the ectoderm constricted, the posterior epaulettes appeared to de-differentiate and the long cilia were lost. Frequently the posterior end of a skeletal rod of the primary larva protruded into the blastocoel of the clone (Fig. 2-2B), and was absorbed by mesenchyme cells prior to separating from the primary larva (Fig. 2-2C). In rare instances an archenteron-like structure formed at the posterior-most end of the developing clone (Fig. 2-2D). Most *S. purpuratus* clones did not have an archenteron-like structure at the time of separation and consisted of a single-cell thick transparent

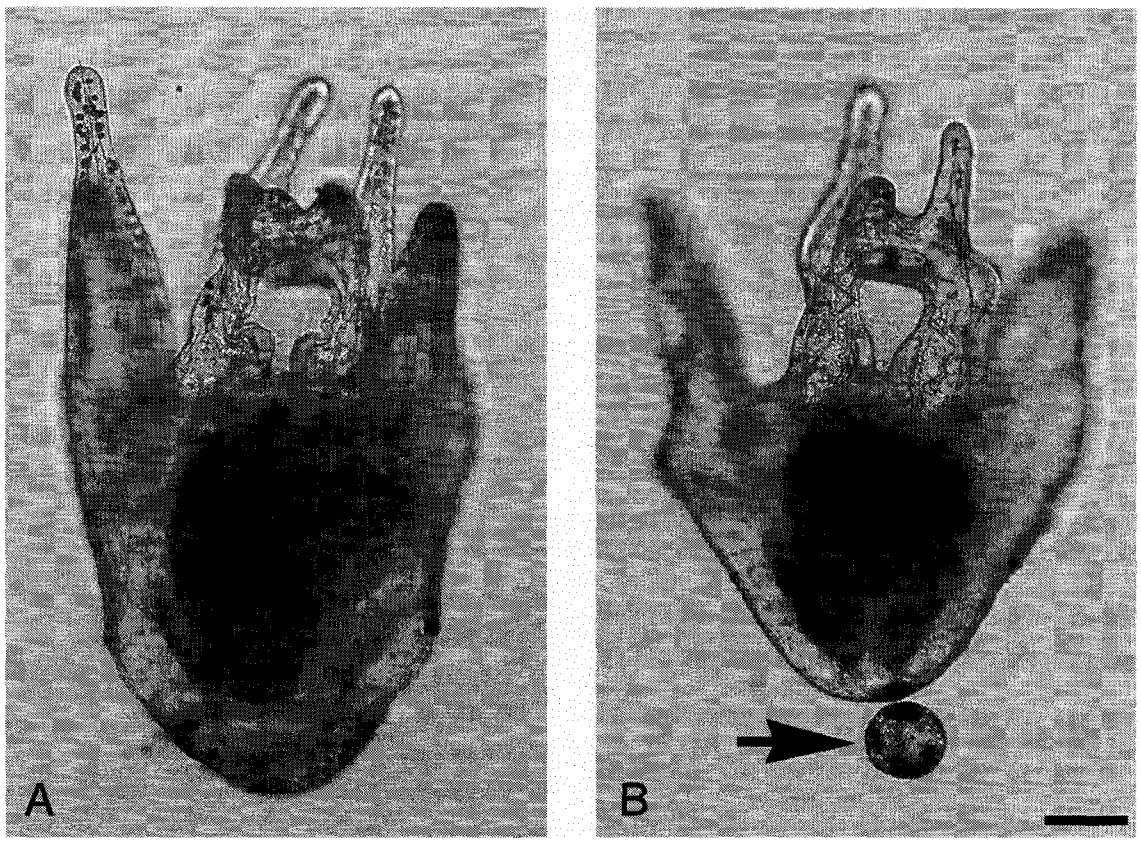
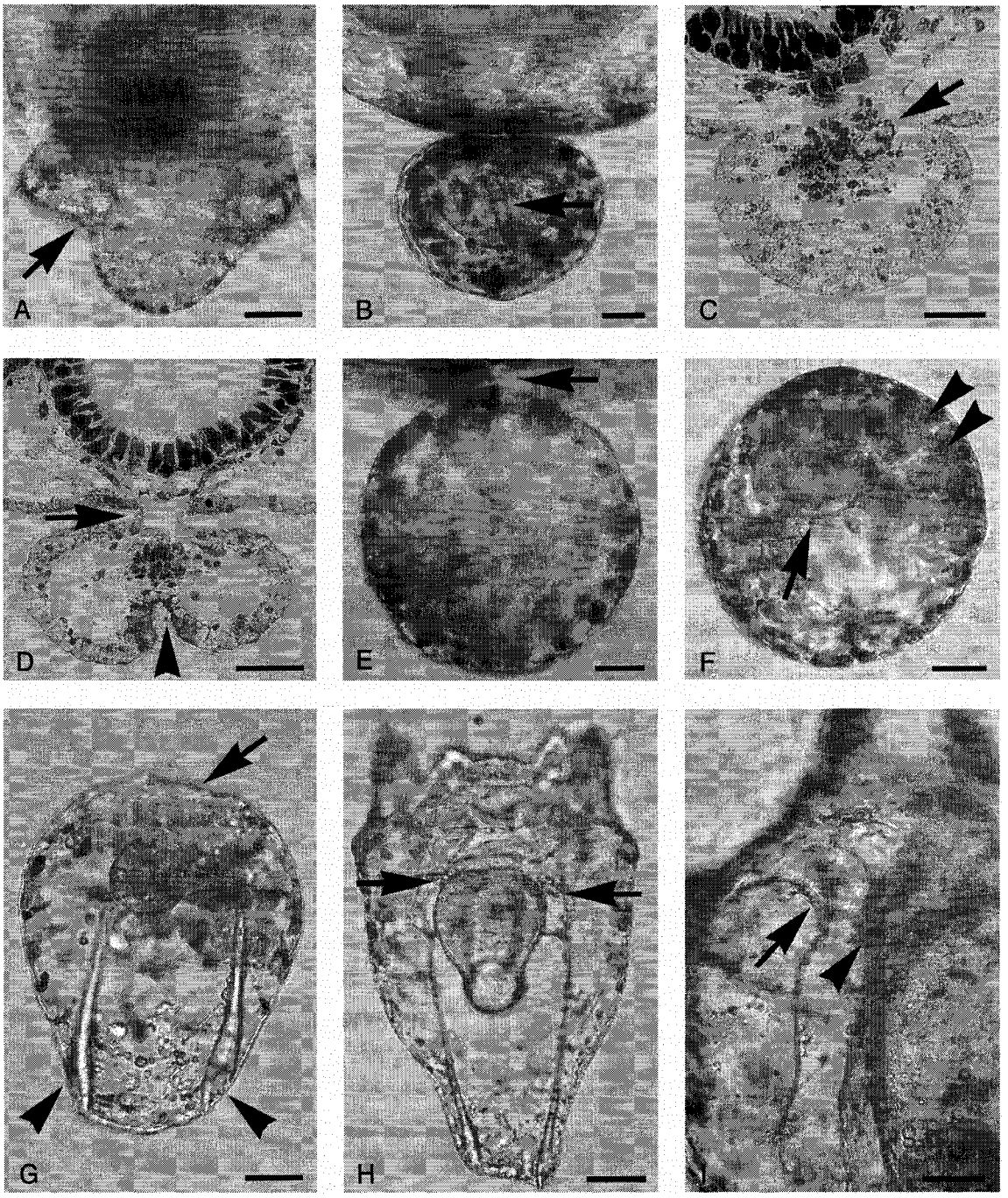


Figure 2-1. Non-cloning and cloning phenotypes of *Strongylocentrotus purpuratus* larvae. (A) 5 wk old, non-cloning larva compared to (B) sibling larvae with a motile clone (arrow) competent to separate from the posterior end of the primary larval body. Note the pigment cell aggregations in the ectoderm of the clone and attachment site on the primary larva. Scale bar- 115 μm.

Figure 2-2. Clone formation and development in *Strongylocentrotus purpuratus* larvae. (A) In the earliest stages of clone formation, the larval posterior end is slightly rounded compared to non-cloning larvae and a constriction circumscribing the posterior margin of the fused ciliary band starts to form (arrow). (B) 1 wk prior to detachment the blastocoel of the developing clone is separate from that of the primary larva. Note the ectoderm of the developing clone contains numerous pigment cells and the base of a skeletal rod protrudes from the blastocoel of the primary larva into the blastocoel of the clone (arrow). (C) Section of a developing clone at a comparable stage to (B). Several mesenchymal cells are shared between the blastocoel of the primary larva and that of the developing clone (arrow). (D) Section of a developing clone nearly completely constricted from the primary larval body (arrow) with an archenteron-like invagination at the posterior end (arrowhead). (E) Motile clone minutes prior to detachment from the primary larva; only a thin, transparent tether remains (arrow). (F) 4 d after detachment the blastocoel of the clone shown in (E) contains clusters of green-pigmented mesenchymal cells (arrowheads) and a gut (arrow). (G) 1 wk after detachment the apical ciliary band (arrow), and a bilateral set of skeletal rods (arrowheads) are formed on the clone. (H) 2 wk after detachment the larval arms , mouth and eosophagus have differentiated at the anterior end of the clone. The remaining green mesenchymal cells are arranged in aggregates (arrows) on either side of the larval digestive tract corresponding to the position of coelomic pouches. (I) 23 d post-detachment, an ectodermal vestibule (arrow) in contact with a hydrocoel (arrowhead) initiates juvenile rudiment formation in the clone. Scale bars: (A) 36 μm, (B) 27μm, (C) 35μm, (D) 38μm, (E) 20μm, (F) 30μm, (G) 45μm, (H) 46μm, (I) 13μm.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ectoderm with many transparent mesenchyme cells in the blastocoel (Fig. 2-2E). Each clone had approximately 150 pigment cells clustered together in patches in the ectoderm (Fig. 2-1A). The ectoderm of the clone was evenly ciliated and once the ectoderm of the clone was separate from that of the primary larva, the clone was independently motile and tethered by a thin, transparent attachment (Fig. 2-2E). To separate from its clone, the primary larva frequently constricted its posterior end near the point of clone attachment and dragged the clone against any available surface. After separation, the primary larva regenerated new posterior epaulettes and was indistinguishable from non-cloning siblings after metamorphosis.

2.3.2 *Strongylocentrotus purpuratus* clone morphogenesis

After detachment from the primary larva, several green mesenchyme-like cells were present in the blastocoel (Fig. 2-2F), the origin of which is unknown. However, they were not evident in clones when they first detached. A ciliated gut also formed among the mass of mesenchyme cells in the anterior half of the blastocoel, but typical echinoderm gastrulation was not observed in detached clones. An ectodermal thickening formed at the apical end of the clones at a point corresponding to the former attachment site to the primary larva. The mouth and oral ciliary band differentiated from the apical thickening thereby conserving the anterior-posterior axis of the primary larva (Fig 2-2G). Several transparent and green mesenchyme cells were present in the blastocoel, and bilateral skeletal rods began to form *de-novo*. Approximately two weeks after detachment, cloned larvae were largely indistinguishable from sexually derived larvae. The dorsal hood and mouth were proportionately smaller compared to those of sexually derived larvae, and

two clusters of green mesenchyme-like cells persisted in symmetrical positions alongside the gut (Fig. 2-2H). I could not be sure whether these were precursors to the coelomic sacs or not. However, three weeks post-separation green mesenchyme-like cells were no longer detected in the blastocoel, a third pair of larval arms began to form, and early juvenile rudiment formation was observed (Fig. 2-2I).

2.3.3 Larval cloning in *Strongylocentrotus droebachiensis*

Cloning among *S. droebachinesis* larvae was observed at much lower frequency (<1% of larvae, n= 2000), but the process was nearly identical to that of *S. purpuratus*. Cloning larvae were initially detected as having a protruding and slightly bulbous posterior end. A constriction formed around the posterior epaulettes and the blastocoel of the primary larva and clone were defined (Fig. 2-3A). After 5 - 8 days the clone became motile while tethered to the primary larval body. The blastocoel contained many transparent mesenchyme cells, and the ectoderm contained several pigment cells but they did not form aggregates as in *S. purpuratus* (Fig. 2-3B). Within 2 - 3 days of separation, the blastocoel contained several green mesenchymal cells (Fig. 2-3C), and within a week a digestive tract had formed although details of this process were not observed.

2.3.4 Larval cloning in *Dendraster excentricus*

Approximately 3.5% (N= 500) of *D. excentricus* larvae were observed to clone in laboratory cultures. Cloning larvae were detected by the appearance of an ectodermal outpocket adjacent to the juvenile podia and future mouth (Fig. 2-4A). These buds

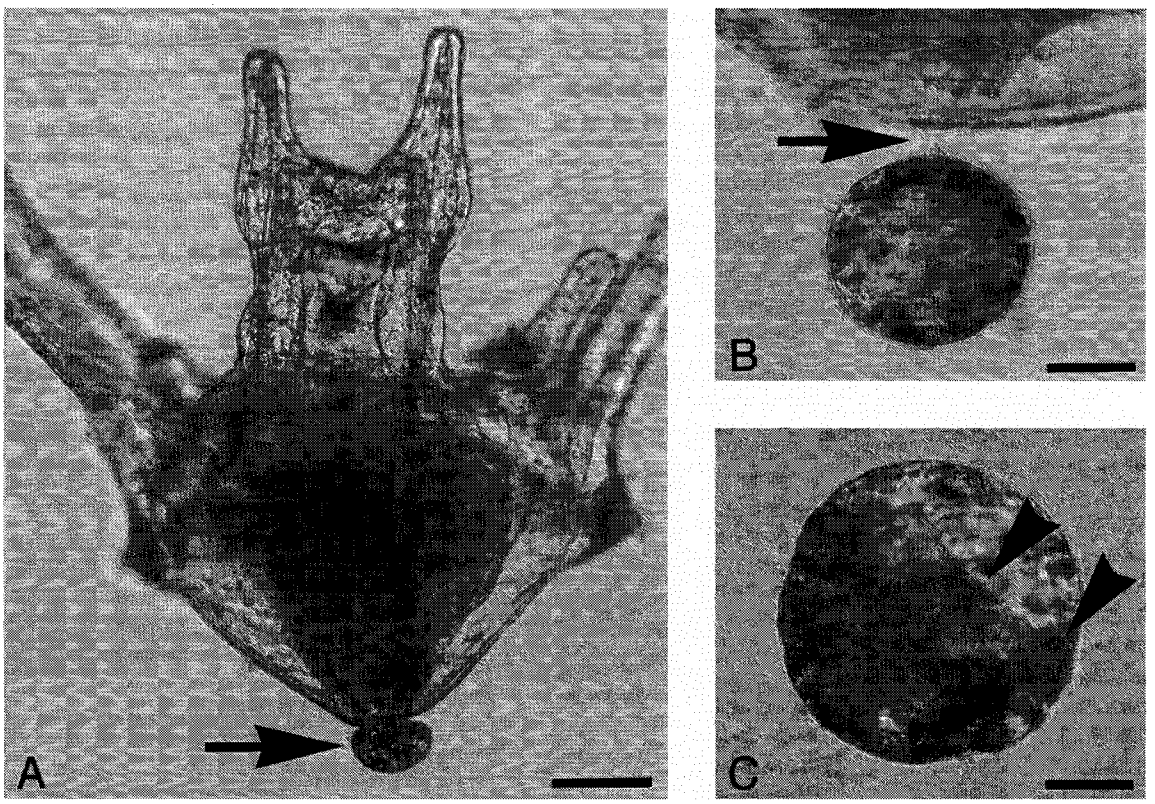

Figure 2-3. Clone formation in *Strongylocentrotus droebachiensis* larvae. (A) 3 wk old larvae with a developing clone at the posterior end (arrow). (B) Motile clone attached by a thin tether (arrow) to the primary larva. Note the density of pigment cells in the ectoderm of the clone. (C) Same specimen 2 d after detachment, the blastocoel of the clone contains several clusters of green mesenchymal cells (arrowheads). A digestive tract later formed in this specimen (not shown). Scale bars: (A) 120 μm, (B) 33 μm, (C) 23 μm.

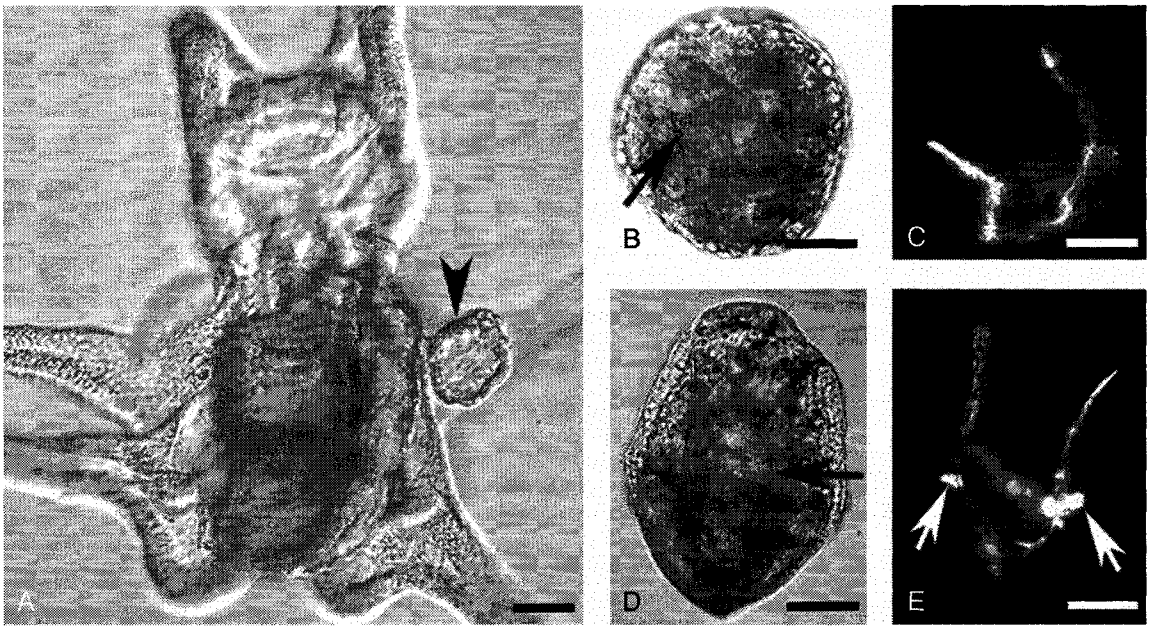

Figure 2-4. Cloning in *Dendraster excentricus* larvae. (A) Ventral view of a 7 wk old larva with a clone constricting from ectoderm on the larval left side (arrowhead). (B) Separated clone with an archenteron-like structure (arrow). (C) Same clone viewed under polarized light revealing a bilateral pair of spicules. (D) Clone, three days post-separation with a functional digestive tract. (E) Same clone as (D), viewed under polarized light and revealing a new pair of spicules (arrows). Scale bars: (A) 50 μm, (B, C) 15 μm, (D, E) 20μm.

initially had a single-cell thick ectoderm and contained several transparent mesenchyme cells. Once separated the blastocoel virtually filled with mesenchyme cells and an archenteron-like structure formed (Fig. 2-4B) as well as a bilateral pair of skeletal rods (Fig. 2-4C). The buds were evenly ciliated and rotated counter-clockwise around their longitudinal axis as they moved forward. Within two days the archenteron-like structure had constricted into a tripartite digestive tract (esophagus, gut and intestine)(Fig. 2-4D) and feeding began. An additional pair of skeletal rods formed, corresponding to the third pair of larval arm rods in sexually-derived larvae (Fig. 2-4E). Development was not observed beyond this point.

2.4 Discussion

2.4.1 General aspects of cloning in echinoid larvae

The prevalence of cloning among laboratory-reared larvae is significantly lower than frequencies in excess of 85% among field-collected asteroid larvae (N= 400-500 individuals m^{-3})(Balser 2004). This discrepancy is likely due to environmental conditions other than food availability that influence larval cloning induction in the field. However, larval cloning in echinoids has so far only been observed in laboratory cultures (Eaves and Palmer 2003; present study).

Clone formation generally begins after the juvenile rudiment starts to form. In all species examined, clone formation occurred along a ciliary band, and most commonly echinoid clones detached as buds. However, intraspecific variation was also observed; The location of clone formation differed between the irregular and regular echinoids, as

clones formed only on the lateral sides of *Dendraster excentricus*, and the posterior ends of *Strongylocentrotus purpuratus* and *S. droebachiensis*. A more subtle difference in clone formation between the two Strongylocentrotids was the clustered arrangement of the purple pigment cells, which were present in *S. purpuratus* clones but absent in *S. droebachiensis* clones.

2.4.2 Cell types in larval clones

With few exceptions, at the time of separation, echinoid larval clones consist of a spherical ectoderm and some mesenchymal cells in the blastocoel. The mesenchymal cells associated with spicule re-absorption are likely primary mesenchyme cells derived from the primary larva and sequestered into the developing clone. Several additional mesenchymal cells have been observed with multiple processes extending between different parts of a developing clone suspended in the blastocoel, as seen in budding asteroids (Jaeckle 1994). These could be blastocoelar cells (Tamboline and Burke 1992). However these cells could have migrated into the developing clone from the primary larva or ingressed from the ectoderm of the developing clone, so their origin remains unclear. The origin of the green mesenchymal-like cells that appear after detachment from the primary larva is also uncertain. These green cells are not observed in the blastocoel prior to clone detachment and either ingress from the ectoderm of the clone, or differentiate from the non-pigmented mesenchymal cells already present in the clone's blastocoel. However, they are potentially an invaluable characteristic for identifying newly cloned echinoid larvae collected in plankton samples as they are not present during normal larval ontogeny.

The abundance of mesenchymal cells associated with clone morphogenesis suggests a significant function. Mesenchyme-like cells also accumulate at the site of clone formation in asteroids. Jaeckle (1994) hypothesized these mesenchyme-like cells could potentially contribute to coelom formation in clones, as do secondary mesenchyme cells during normal embryonic development (Ettensohn and Ruffins 1993). In regenerating asteroid larvae mesenchymal cells with pseudopodia aggregate at the site of surgical bisection (Vickery et al. 2002). These cellular aggregations were interpreted as potential homologues to the mesenchymal blastemas that form in adult echinoderms when regenerating lost structures (Vickery et al. 2002). The role of mesenchymal cells in larval cloning (Jaeckle 1994; present study) and regeneration (Vickery et al. 2002), indicates these cells are pluripotent well into the later stages of larval development.

Pigment cells are also prominent during clone formation, particularly in *S. purpuratus*. During normal embryogenesis pigment cells are not prevalent in *S. purpuratus* until after gastrulation (Eaves 2005). In clones, however, pigment cells are abundant and arranged in clusters prior to any indication of gastrulation. Recently, Takata and Kominami (2004) identified pigment cells as the bottle cells that trigger the onset of gastrulation in the echinoid *Echinometra mathaei*, a previously unrecognized function for this cell-type. Additionally, both pigment cells and blastocoelar cells provide innate immunity to sea urchin larvae (reviewed by Smith et al. 2006). Given their many functions during development, secondary mesenchyme-derived cells may be conditionally specified to produce a clone from the larval body under the correct environmental conditions and could therefore be regarded as a candidate population of

larval stem cells. A key test of this hypothesis would be the allotransplantation of secondary mesenchyme-derived from the site of clone formation into a non-cloning larval counterpart to observe if these cells induce formation of a clone.

2.4.3 Archenteron formation without gastrulation?

Gastrulation, or the differentiation of endoderm from ectoderm, in echinoid clones has not been fully characterized. Remarkably this fundamental process appears to vary among all cloning echinoderm larvae. For example, formation of an archenteron–like structure has been observed in cloned larvae of *Dendraster excentricus* and some *S. purpuratus* (present study), holothuroids (Eaves and Palmer 2003; Balser 2004), ophiuroids (Balser 1998), and asteroids (Rao et al. 1993; Jaeckle 1994). Conversely, in most echinoid clones an archenteron-like structure has not been observed prior to gut formation in a dense mass of mesenchymal cells in the blastocoel. However, unusual modes of archenteron-like structure formation do occur in some developing echinoderm clones. Bosch and colleagues (1989) described the formation of a tube from a longitudinal in-folding along the aboral surface of asteroid clones that sinks into the blastocoel thereby forming an archenteron in a morphogenetic process otherwise unknown in echinoderms.

2.4.4 Comparisons with other echinoderm classes

Larval cloning can occur nearly anytime during echinoderm development (Table 2-1A). Cloning is observed as early as midway through larval development when several weeks or months remain until metamorphosis, as seen in asteroids (Bosch et al. 1989;

1992; Rao et al. 1993; Jaeckle 1994, Vickery and McClintock 2000; Knott et al. 2003), echinoids (Eaves and Palmer 2003) and holothuroids (Balser 2004). Cloning can also occur late in development when the larva is almost competent to metamorphose, as observed in asteroids (Jaeckle 1994; Knott et al. 2003; Balser 2004) and echinoids (Eaves and Palmer 2003). However, in some animals a whole-body reorganization, such as metamorphosis, is required to release 'expendable' body parts, as seen in ophiuroids (Mortensen 1921; Balser 1998; 2004) and holothuroids (Eaves and Palmer 2003).

The location of clone formation on the primary larval body varies considerably among echinoderm classes (Table 2-1B). Clones form from the larval arms in asteroids (Bosch et al 1989; 1992; Rao et al. 1993; Jaeckle 1994; Vickery and McClintock 2000; Knott et al. 2003; Balser 2004), ophiuroids (Mortensen 1921; Balser 1998) and holothuroids (Balser 2004). The oral hood can yield a clone in asteroids (Jaeckle 1994; Vickery and McClintock 2000), meanwhile the posterior end of the larval body can clone in every class capable of larval cloning (Balser 1998; Vickery and McClintock 2000; Eaves and Palmer 2003). Regardless of the site of formation on the larval body, the majority of echinoderm clones conserve the anterior posterior polarity of the primary larva (Table 2-1C); although exceptions exist in asteroids (Jaeckle 1994; Vickery and McClintock 2000). In very few cases has the conservation of the dorsal-ventral and left- right polarity been observed (Vickery and McClintock 2000), however vital marker tracing will be required to determine this.

Table 2-1. Summary of known cloning processes among echinoderm larvae; (+) indicates known occurrence, (-) indicates known counterpoint of a described trait, (?) indicates unknown (see text for references).

	Asteroids	Ophiuroids	Holothuroids	Echinoids
a) *Time of clone formation during larval development*				
Mid	+	?	?	+
Late	+	?	+	+
Metamorphosis	?	+	+	?
b) *Location of clone formation on the primary larval body*				
Arms	+	+	+	?
Oral hood	+	?	?	?
Posterior	+	?	+	+
Lateral	+	?	?	+
c) *Polarity of clone body axes conserved from those of the primary larva*				
Anterior-Posterior	+/-	+	+	+
Dorsal-Ventral	+	?	?	?
Left-Right	+	?	?	?

Larval cloning is a seemingly highly variable phenomenon among echinoderm classes. However, as more examples of larval cloning are discovered the extreme variability of this process seen in asteroids will likely be found to be the rule in other echinoderm groups as well (Eaves and Palmer 2003; Knott et al. 2003). Furthermore, the capacity for asexual reproduction clearly demonstrates that cells of the echinoderm larval body retain immense regenerative and re-specification potential (Jaeckle 1994; Vickery and McClintock 1998; Eaves and Palmer 2003), likely mediated by genetic programs similar to those necessary for asexual budding in colonial tunicates, and regeneration and stem cell renewal across the deuterostomes (Swalla 2006).

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Chapter III

Having the guts to survive:

A novel phenotypically plastic response to starvation in sea urchin larvae[3-1]

3.1 Introduction

Marine invertebrate larvae must withstand many challenges while adrift in the plankton for days, months, and possibly years (Scheltema 1971*a*,*b*, 1986). Factors such as temperature and salinity variation, predator attack, and food limitation all threaten the development and survival to metamorphosis of free-swimming, feeding (planktotrophic) larvae (Thorson 1950, Scheltema 1971*a*). Nevertheless, larval planktotrophy is ancestral (Strathmann 1978) and widespread, occurring in more than 70% of extant marine invertebrates (Thorson 1950).

Planktotrophic larvae can adapt morphologically to variable environmental conditions during development, and echinoids provide a dramatic example: larvae produce different size feeding structures (larval arms) depending on the availability of food (Boidron-Metairon 1988; Fenaux et al. 1988, 1994; Hart and Scheibling 1988; Strathmann et al. 1992; Fenaux et al. 1994; Hart and Strathmann 1994; Sewell et al. 2004; Miner 2005, 2007; Reitzel and Heyland 2007). Larvae with longer arms collect food more efficiently than shorter-armed counterparts (Hart and Strathmann 1994). Moreover, differences in food ration result in a heterochronic shift in the development of larval structures relative to post-larval ones. When food is scarce resources are allocated

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[3-1] This chapter was co-authored, with CA Bergstrom as second author. A version of this chapter was submitted for publication in the Journal of Experimental Marine Biology & Ecology.

to larval feeding structures. However when food is abundant resources are allocated earlier in development to structures conserved through metamorphosis, including the larval gut and juvenile rudiment (Strathmann et al. 1992; Fenaux et al. 1994; Miner 2005).

Food-limited development is potentially common for larvae of benthic invertebrates (Paulay et al. 1985). Thorson (1950) declared "the significance of starvation for the total waste of pelagic larvae [...] one of the most urgent problems in marine and larval ecology". Paleontological evidence suggests food-limitation has also had a profound effect on the evolution of echinoid life histories: nonplanktotrophic development evolved independently in nine clades of echinoids from five orders during the last 10m.y. of the Cretaceous, likely in response to an unstable food supply (Jeffery 1997). However, remaining clades of echinoids with planktotrophic larval development were no more likely to go extinct than clades with nonplanktotrophic larval development during that same period (Smith and Jeffery 1998). Clearly planktotrophic larvae have some capacity to withstand periods of extreme food-limitation. Yet rather few studies have examined the effects of starvation on echinoid larval phenotypic plasticity. Larvae of the sand dollar *Dendraster excentricus* can sustain up to 19 days of starvation without reaching a "point of no return" and resume juvenile rudiment development when feeding is recommenced (Boidron-Metairon 1988). Fenaux and others (1988) describe starved larvae of *Paracentrotus lividus* cease growing shortly after the onset of the feeding phase of development, and similar observations were reported for larvae of the urchin *Evechinus chloroticus* (Sewell et al. 2004). In addition, larvae subjected to transient

periods of starvation take longer to develop (Boidron-Metairon 1988; Fenaux et al. 1988; Sewell et al. 2004), and produce smaller juveniles after metamorphosis (Fenaux et al. 1988). Furthermore, anecdotal reports suggest echinoderm larvae left unfed for weeks remain reasonably healthy regardless (Olson and Olson 1989). Surely planktotrophic larvae must have a means to survive the absence of an exogenous food source. Yet, surprisingly, no starvation survival mechanism has yet been described.

Here we examine a novel aspect of echinoid larval plasticity in response to starvation: changes in gut size in *Strongylocentrotus purpuratus*. Using conventional light microscopy and basic statistical analysis we examined two main questions: 1) Does the larval gut shrink in food-limited conditions? If so, 2) how much can gut size be reduced and still permit recovery of the larva? The results are easy to digest.

3.2 Materials and methods

3.2.1 Larval culturing

Adult *Strongylocentrotus purpuratus* were collected subtidally adjacent to Brady's Beach, near Bamfield, British Columbia, Canada. Spawning was induced by intracoelomic injection of 0.55 M KCl. Embryos derived from one male/ female pair were cultured in pasteurized seawater and incubated at 14°C. Immediately after hatching, blastulae were divided into three replicates of each experimental treatment (summarized in Figure 1). Fed embryos were cultured with a 1:1:1 mix of *Rhodomonas salina*, *Isochryisis galbana*, and *Dunaliella tertiolecta*. High food cultures received 50,000

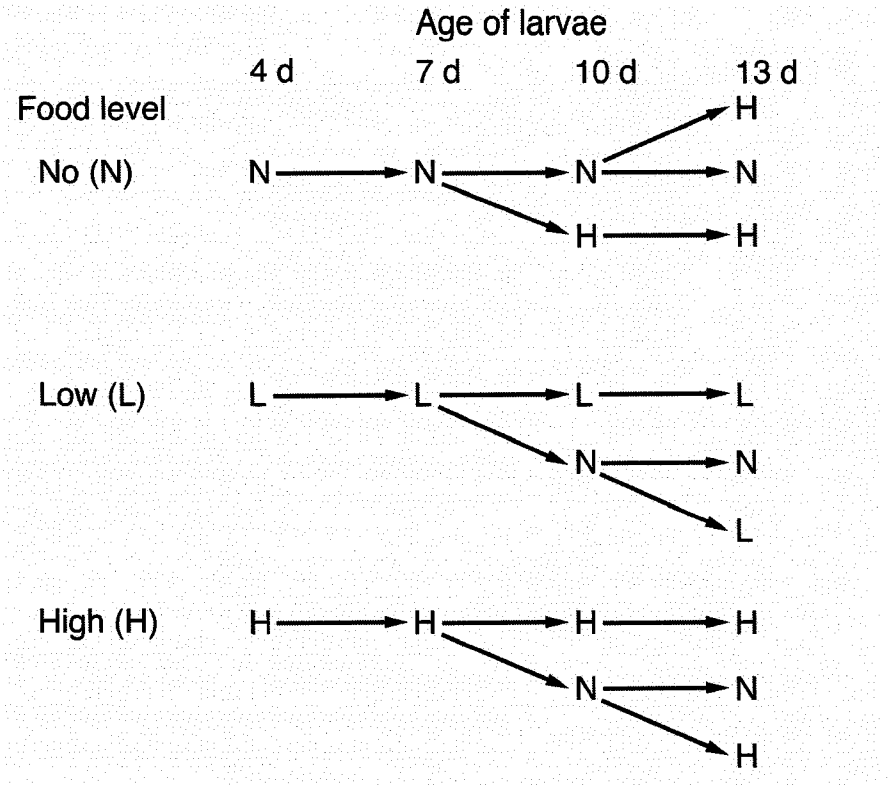


Figure 3-1. Feeding and starvation treatments for larvae of *Strongylocentrotus purpuratus*. Horizontal arrows indicate consistent food conditions between sampling days, diagonal arrows indicate a change in food conditions for larvae.

cells/ml, low food cultures received 500 cells/ml, and the remaining treatments received no food. The high food treatment was used to determine the maximum gut and body width for the duration of the experiment. The low food treatment was used to determine the gut and body width without any possibility of the gut being filled to capacity and distended by ingested algal particles. Initially, 100 blastulae were transferred into each 500 ml beaker for a concentration of 1 blastula/ 5 ml. The volume of larval culture containers was reduced at each sampling interval to compensate for larvae that were removed for analysis. Beginning at day 4 larvae were sampled every 72 hrs when culture suspensions were replaced. All cultures were stirred manually twice a day to maintain even distribution of food particles. Some larvae were also transferred into different treatments at different times, as outlined in Figure 3-1. The duration of the experiment was determined by the time it took the high food treated larvae to begin to grow the third pair of larval arms, thereby drastically altering the shape of the larval body at the body width measurement points.

3.2.2 Larval measurements

Using a 53 μm Nitex sieve, larvae were concentrated into a minimal volume of water during culture changes, at which point approximately 10 larvae were removed from each of 3 replicate treatments for a total of N = 30 per treatment, and preserved and mounted on slides following McEdward (1984). Larvae were photographed on an Olympus CX40 compound microscope with a Nixon CoolPix 4500 digital camera. Because of the cone-shape morphology of 4-arm *S. purpuratus,* specimens could not readily be positioned with the anterior-posterior axis parallel to the plane of focus for

accurate arm and body length measurements, instead body width and gut diameter were recorded. The width of the larval body was measured between the lowest points of the oral ciliary band on the lateral sides of the larval body (Fig. 3-2A). The width of the gut was measured at the widest part of the stomach parallel to the body-width measurement (Fig. 3-2A). The thickness of the gut endoderm could not be accurately resolved. Measurements were done using calibrated Adobe Photoshop 7.0 software. Body width and gut diameter were log10 transformed to standardize variances and normalize distributions.

3.2.3 Statistical analyses

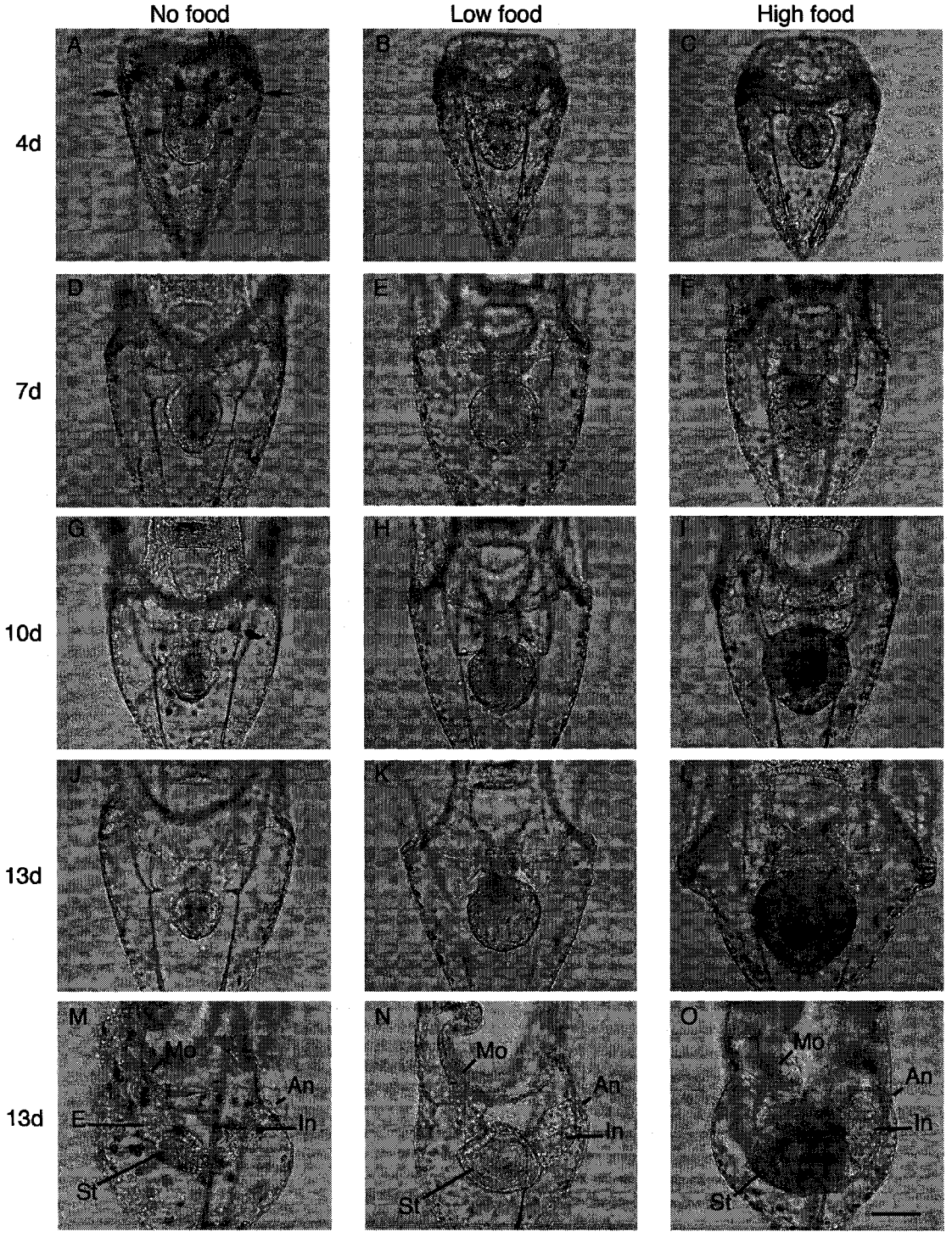
Larvae that were kept in constant food levels (no food, low, or high food) throughout the experiment (from day 4 to day 13) were considered controls (Fig. 3-1). The slopes between gut diameter and body size were compared among food level controls with analysis of covariance (ANCOVA), and if significant, two-way ANOVA's were run separately for both gut diameter and body size as dependants, using food level and time (day) as fixed factors. Gut diameters of larvae were compared among variable food treatments using ANCOVA to take into account variation due to body size. Separate ANCOVAs were run for variable food treatments with different initial food levels at days 4 and 7. Comparisons of slopes between gut diameter and body size were done for each ANCOVA, and if non-significant, the ANCOVA was re-run with the interaction term removed. All further comparisons of gut diameter were done using their estimated marginal means to control for variation in body size.

3.3 Results

3.3.1 Effects of different food levels on morphogenesis of *Strongylocentrotus purpuratus*

At the end of the pre-feeding period (day 4) gut and body width did not differ significantly among treatments (Fig. 3-2A-C). After three days of feeding treatments (day 7), unfed larval stomachs appeared to be slightly narrower, and were surrounded by a few pigment cells (Fig. 3-2D). The stomachs of low and high food treated larvae had a comparatively similar morphology. However, the endoderm (stomach wall) of high food treated larvae had darkened slightly from accumulating lipid derived from digested algae (Fig. 3-2F). After six days of feeding treatments (day 10), unfed larval stomachs appeared much narrower than those of fed siblings, gut endoderm was transparent and associated with several transparent mesenchyme cells. In addition, around the digestive tract and suspended in the surrounding blastocoel were many large, globular pigmented (purple) mesenchyme cells, some with short filopodial projections, (Fig. 3-2G). In contrast, only a few transparent mesenchyme cells, and rarely any pigmented mesenchyme cells, surrounded the digestive tract of low food treated larvae, and gut endoderm was only slightly stained from digested algal lipids (Fig. 3-2H). Only a few transparent mesenchyme cells, and no pigmented mesenchyme cells, surrounded the stomachs of high food treated larvae and the gut endoderm was considerably darkened from algal lipid staining (Fig. 3-2I). After 9 days of feeding treatments (day 13) the gut of unfed larvae appeared considerably narrowed, and fewer pigmented and transparent mesenchyme cells surrounded the digestive tract (Fig. 3-2J). Low food cultured larvae appeared relatively unchanged compared to the same treatment at day 10, and showed

Figure 3-2. Forms of *Strongylocentrotus purpuratus* larvae reared under different food levels. *A-C,* larvae at the end of pre-feeding development. *D-F*, 7 d larvae illustrating the effects of no, low and high food levels after 72 hr. *G-I*, 10 d larvae with pronounced gut size differences among no, low and high food treatments. *J-L*, 13 d larvae having very reduced stomachs in the no food compared to stomach sizes in the low and high food treatments. *M-O*, lateral views of the same 13 d larvae as in *J-L*. The guts of the no food treated larvae have a reduced, tubular morphology compared to the low and high food digestive tracts. Arrows in *A* indicate measurement points at the base of the oral ciliary band, arrow heads indicate where gut width was measured. Abbreviations: An, anus; E, esophagus; In, intestine; Mo, mouth; and St, stomach. Scale bar 50 μm.


No food
Low food
High food
4d
7d
10d
13d
13d
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
Mo
An
In
St
E

only a few pigmented mesenchyme cells around the apical region of the digestive tract (Fig. 3-2K). Highly fed larvae were considerably larger, and developmentally more advanced than food-limited sibling larvae (Fig. 3-2L). The endoderm of day 13 high food treated larvae was darkly stained with algal lipids, particularly when compared to the endoderm of food-limited larvae (compare figures 3-2J-L). Viewed laterally, the effect of gut narrowing was very distinct, among starved larvae the digestive tract was reduced to a narrow tube (Fig. 3-2M). Whereas in low food treated larvae the tri-partite differentiation of the digestive tract (esophagus, gut, and intestine) remained intact (Fig. 3-2N). All sections of the digestive tracts among high food treated larvae appeared enlarged (Fig. 3-2O). In addition, the abundance and distribution of pigmented mesenchyme cells varied considerably among the three food treatments. In starved larvae the globular, pigmented mesenchyme cells were distributed about the periphery of the bastocoel (Fig. 3-2M), compared to a diffuse arrangement throughout the ectoderm of fed larvae (Fig. 3-2N,O). Larvae sampled from both low and high food treatments had algal cells in their guts at the time of preservation, indicating food levels had not been depleted in either treatment.

3.3.2 Gut diameter and body width in constant feeding regimens

Among those larvae kept at constant food levels between day 4 and day 13 (see Fig. 3-1), gut diameter increased significantly with body width for all three food treatments (no food: R^2= 0.06; df= 119; P= 0.009, low food: R^2= 0.49; df= 119; P< 0.001, high food: R^2= 0.86; df= 119; P< 0.001). However, the strength of the relationship between gut diameter and body width increased significantly from no to low to high food

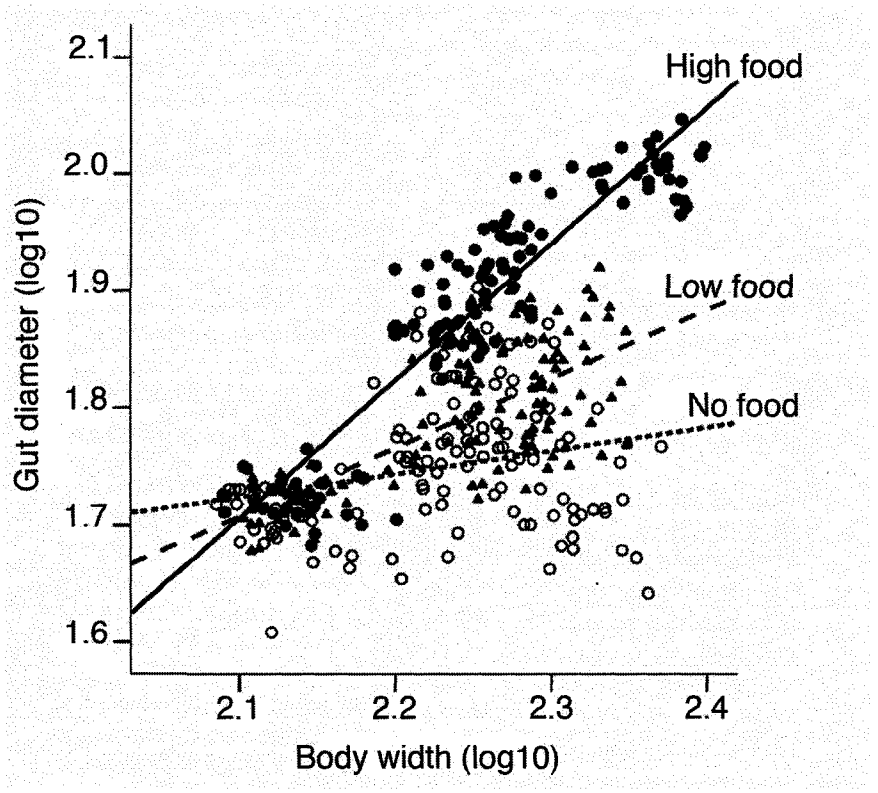


Figure 3-3. Gut diameter (micrometers) as a function of larval body width (both log10 transformed) for three constant food levels that were kept constant from day 3 through day 13. Solid line and closed circles represent high food, dashed line and closed triangles represent low food, and dotted line and open circles represent no food.

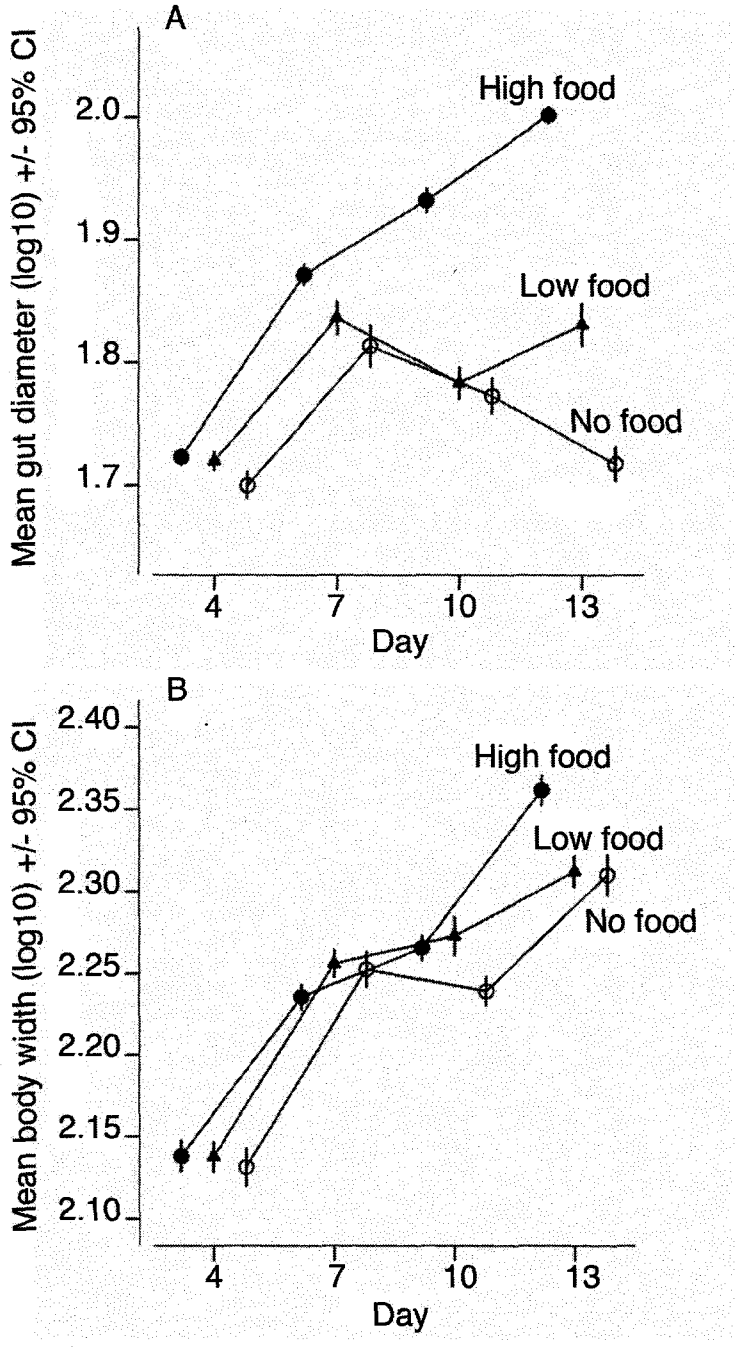


Figure 3-4. Mean gut diameter (A) (micrometers), and mean larval body width (B) (micrometers), as a function of time for larvae maintained under constant food levels. Error bars are 95% confidence intervals. N= 30 (3 replicates of 10) for each mean. In this and subsequent figures, points for the same day have been shifted slightly to one side or the other to avoid overlap, but larvae were all sampled on the same day.

Table 3-1. Statistical significance of variation in gut diameter and body width as a function of time and constant food level.

	Factor	MS	df	F
Gut diameter	Food	0.542	2	482.087**
	Day	0.362	3	321.889**
	Food x day	0.115	6	102.558**
	Error	0.001	348	-
Body size	Food	0.009	2	12.199**
	Day	0.571	2	777.997**
	Food x day	0.010	6	13.637**
	Error	0.001	348	-

** $P<0.01$.

treatments (ANCOVA: food by body size interaction term F= 76.37; MS= 0.17; df= 2, 354; P< 0.001; Fig. 3-3).

Both absolute gut diameter and absolute body size changed significantly with time, but this depended on food treatment (Fig. 3-4; Table 3-1). Gut diameter increased drastically with time at high food levels. However, at low food levels gut diameter increased from day 4 to day 7 after which it decreased slightly, and with no food, it increased from day 4 to day 7 but then steadily decreased to day 13. Body size increased with time in a more concordant way among food treatments (Fig. 3-4B; see lower F-value relative to gut size in table 1, although significant interaction among food levels was still present). Both gut diameter and body size exhibited greatest differences among food levels at day 13 while the smallest differences were seen at day 4 (Fig. 3-4), although this was most striking for gut diameter.

3.3.3 Gut diameter variation in response to changing food levels

The relationship between gut diameter and body width varied significantly among treatments (ANCOVA: treatment x body size interaction F= 6.315; MS= 0.008; df= 6; P< 0.001). Therefore, for comparisons we made between mean gut diameters of specific treatment groups, we compared slopes of gut diameter to body size pair-wise, adjusting for multiple comparisons with sequential Bonferonni corrections. If these post-hoc pair-wise comparison were non-significant, we dropped the interaction term between mean gut diameter and body size from the model. None of the following pair-wise

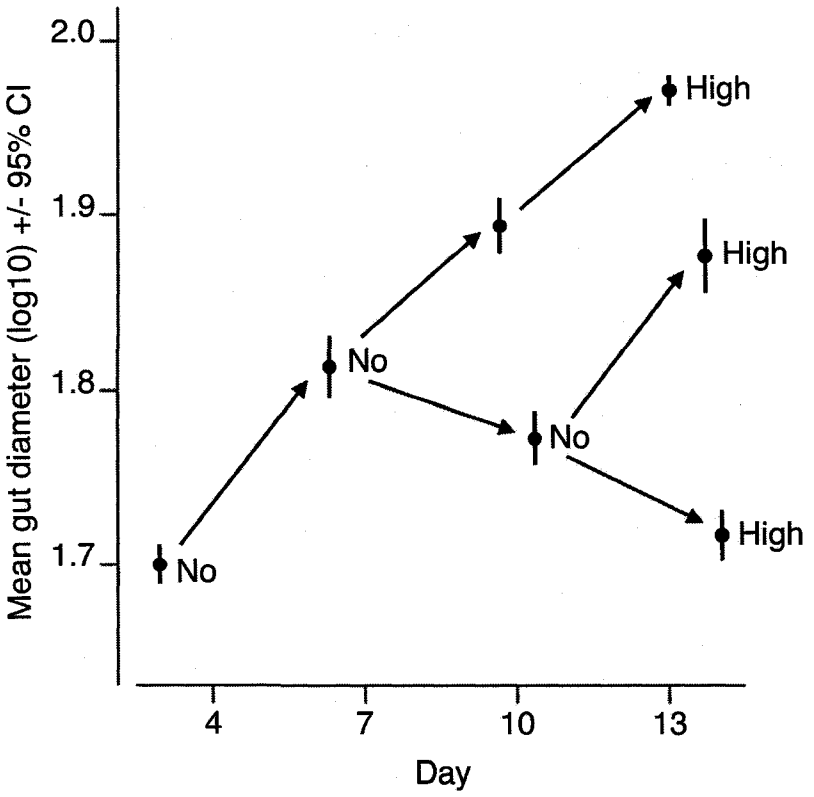


Figure 3-5. Mean gut diameter (micrometers) as a function of time for larvae started with no food (No) and subsequently exposed to different food regimes (No food, High food). Error bars are 95% confidence intervals. N= 30 (3 replicates of 10) for each mean. Arrows show sequence of food level adjustments with time.

comparisons of estimated marginal means demonstrated significant interaction terms between gut diameter and body size after Bonferroni corrections for multiple comparisons (6 comparisons in total; all $P > 0.05$).

3.3.3.1 No food initially

Among starved larvae, mean gut diameter (MGD) increased between day 4 and day 7 (pair-wise comparison of estimated marginal means, with Bonferroni corrections for multiple comparisons; $P < 0.001$ (Fig. 3-5), after which MGD declined significantly by day 10 ($P = 0.015$ after corrections) and day 13 ($P < 0.001$ after corrections). Starved larvae fed a high food ration after day 7 had significantly larger MGD at day 10 ($P < 0.001$ after corrections) and again at day 13 ($P < 0.05$ after corrections). MGD also increased significantly when starved larvae received a high food ration starting on day 10 ($P < 0.05$ after corrections).

3.3.3.2 Low food initially

The relationship between gut diameter and body size did not differ significantly among treatments (ANCOVA: treatment x body size interaction $F = 1.990$; $MS = 0.003$; df= 6; $P = 0.069$), therefore, slopes between gut and body size were kept constant by removing this interaction term from the ANCOVA. Gut diameter differed significantly among treatments ($F = 21.409$; $MS = 0.030$; df= 6; $P < 0.001$) when using a common slope for all treatments.

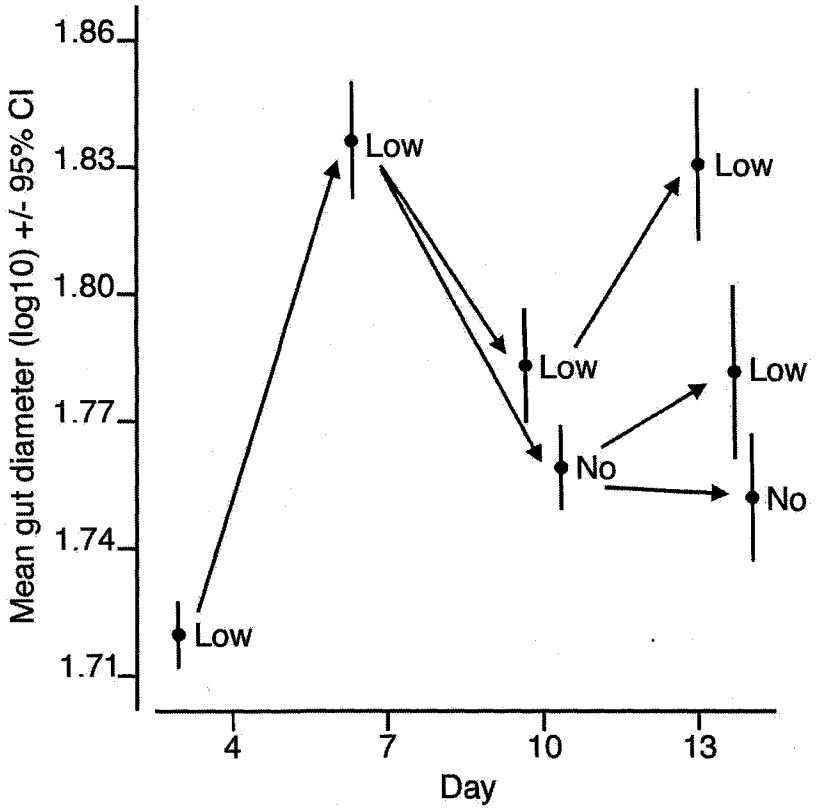


Figure 3-6. Mean gut diameter (micrometers) as a function of time for larvae started with low food (Low) and subsequently exposed to different food regimes (No food, Low food). Error bars are 95% confidence intervals. N= 30 (3 replicates of 10) for each mean. Arrows show sequence of food level adjustments with time.

MGD increased between day 4 and day 7 among low food treated larvae (Fig. 3-6), decreased between day 7 and day 10, then increased again by day 13. Low food treated larvae starved between day 7 and day 10 exhibited a significant reduction in MGD (pair-wise comparison of estimated marginal means, with Bonferroni corrections for multiple comparisons; $P< 0.001$), and had a greater reduction in MGD compared to that of the continual low food treated group although the difference was not significant after Bonferroni corrections ($P> 0.05$). When the low food treatment was resumed from day 10 to day 13, MGD increased to a value similar to the MGD of continually fed group at day 10.

3.3.3.3 High food initially

The relationship between gut diameter and body size did not differ significantly among treatments (ANCOVA: treatment x body size interaction $F= 1.615$; $MS= 0.001$; df= 6; $P= 0.145$), therefore, slopes between gut and body size were kept constant by removing this interaction term. Gut diameter differed significantly among treatments ($F= 107.567$; $MS= 0.092$; df= 6; $P< 0.001$) when using a common slope for all treatments.

MGD increased most rapidly among larvae fed a high food ration for the duration of the experiment (Fig. 3-7). Larvae that were starved between day 7 and day 10 had a significantly smaller MGD (pair-wise comparison of estimated marginal means, with Bonferroni corrections for multiple comparisons; $P< 0.001$), when high food treatment was resumed MGD increased significantly and nearly to the same MGD as the continually fed treatment (pair-wise comparison of estimated marginal means, with

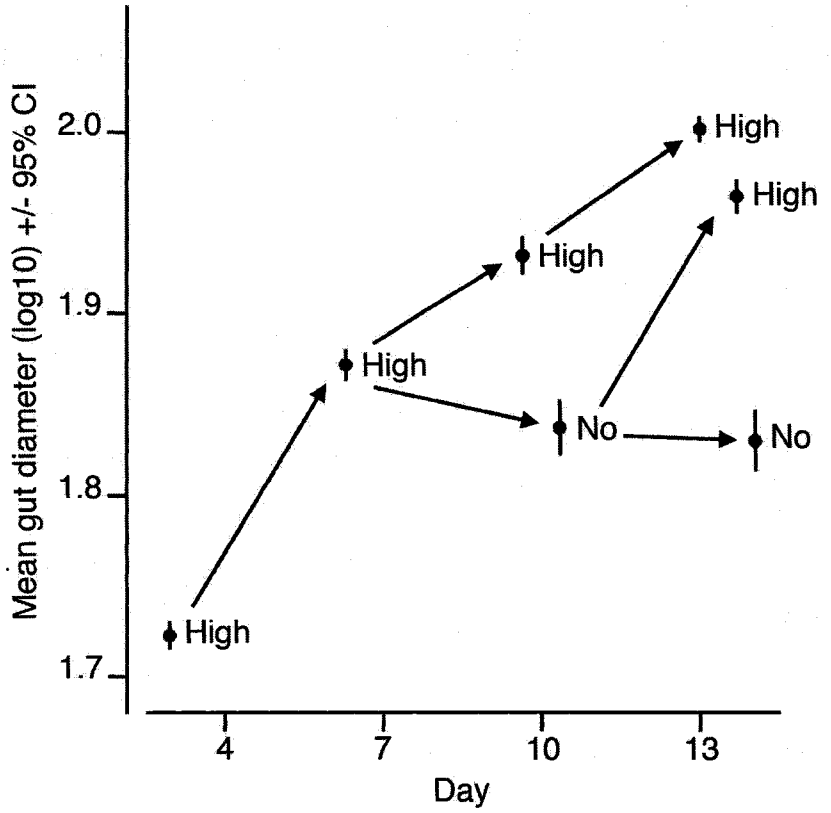
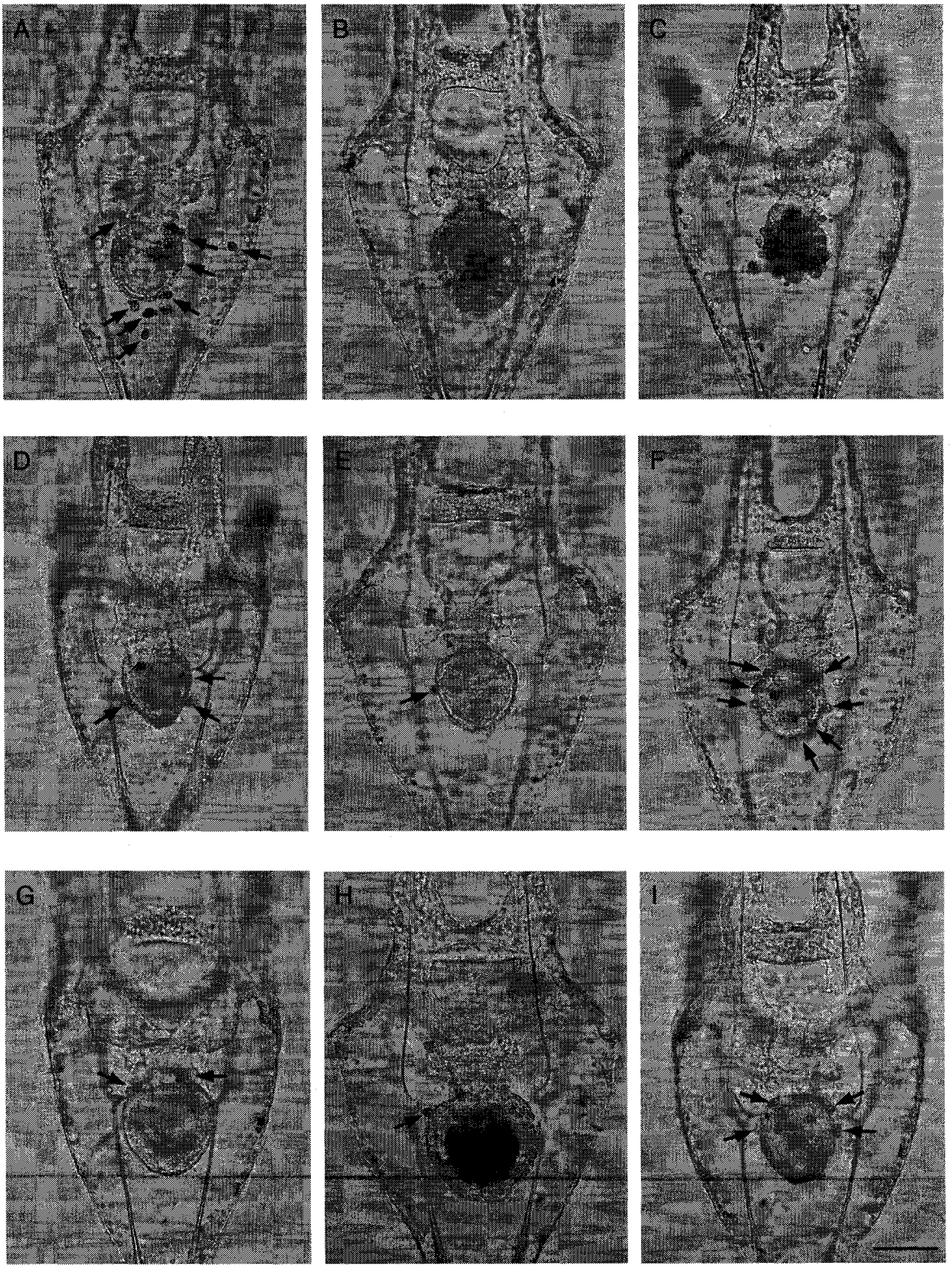


Figure 3-7. Mean gut diameter (micrometers) as a function of time for larvae started with high food (High) and subsequently exposed to different food regimes (No food, High food). Error bars are 95% confidence intervals. N= 30 (3 replicates of 10) for each mean. Arrows show sequence of food level adjustments with time.

Figure 3-8. Morphogenesis of *Strongylocentrotus purpuratus* larvae under varying food and starvation regimens. *A*, 10 d larva that has never been fed, note the abundance of pigment cells (arrows) migrating toward the gut. *B*, 13 d larva, starved until day 10, then fed for 3 days. The gut is expanded and filled with food, and pigment cells are localized to the anterior region of the gut. *C*, larva from the same treatment as *B*, however, the gut is too reduced to digest food. *D*, a 10 d larva that experienced low food treatment until day 7 and was then starved for three days. Note the pigment cells around the gut. *E*, 13 d larva, same treatment as *D*, followed by low food levels for three days. *F*, 13 d same treatment as *D*, followed by an additional three days of starvation, note the abundance of pigment cells that have migrated towards the gut. *G*,10 d larva, high food treatment until 7d, followed by three days of no food. *H*,13 d larva, same treatment as *G*, followed by three days of high food. *I*, 13 d larva, same treatment as *G*, followed by additional 3 days of no food, note the pigment cells around the gut. Scale bar 50 μm.


A
B
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Bonferroni corrections for multiple comparisons; $P< 0.001$). In contrast, the MGD of larvae that were continually starved starting at day 7 decreased significantly from day 7 to day 10 (pair-wise comparison of estimated marginal means, with Bonferroni corrections for multiple comparisons; $P< 0.001$), but then leveled off from day 10 to day 13.

3.3.4 Effects of changing food levels on morphogenesis of *Strongylocentrotus purpuratus*

3.3.4.1 No food

After day 10, starved larvae had many large, globular, purple-pigmented mesenchyme cells around the gut and suspended in the blastocoel (Fig. 3-8A). Starved larvae that had received a high food ration for three days had fewer pigmented mesenchyme cells, all localized around the apical region of the digestive tract (Fig. 3-8B). In contrast, larvae whose guts had reduced to approximately 55 μm or less were no longer of breaking down ingested algal particles (Fig. 3-8C), instead algal cells passed through the digestive tube intact (not shown).

3.3.4.2 Low food

Low-food cultured larvae also had globular, purple-pigmented mesenchyme cells around the gut (Fig. 3-8D), but many fewer than completely starved larvae (compare Fig. 3-8A with 3-8D). When low food levels were resumed, very few pigment cells were associated with the gut (Fig. 3-8E). Among larvae continually starved from day 7

onwards several purple-pigmented mesenchyme cells surrounded the gut (Fig. 3-8F), similar to starved larvae at day 10 (Fig. 3-8A).

3.3.4.3 High food

After three days of starvation, high-food treated larvae had a few pigmented mesenchyme cells, particularly around the apical region of the gut, and the endoderm had lost all staining from algal derived lipids (Fig. 3-8G). After high-food treatment was resumed the larval gut was filled with algal cells (Fig. 3-8H). If high-food conditions were not restored, by day 13 the gut was surrounded by pigmented mesenchyme cells (Fig. 3-8I), similar to low food treated and starved larvae (Fig. 3-8D).

3.4 Discussion

Previously, gut size has been used as a measure of larval body size in studies of larval growth (Strathmann et al. 1992; Fenaux et al. 1994). Our report here is the first to describe significant larval gut reduction in the absence of food corresponding with a novel cellular migration pattern not previously described in echinoid larvae. Particularly among starved larvae, absolute gut size was reduced to a dimension similar to that prior to the initiation of feeding, at which point the larvae would no longer digest ingested food. Moreover, body width expansion among starved larvae was correlated with a proportionally narrower mean gut diameter compared to fed sibling larvae (Fig. 3-3). Absolute gut size was also reduced in larvae that were initially fed and later starved. The transient reduction in gut width among day 10 low and high food treated larvae was more drastic during the first interval of starvation for larvae that were initially fed (Fig.3- 6, 3-

7). This could be attributed to a diversion of energy towards producing longer feeding arms in the presence of low food conditions (Boidron-Metairon 1988; Fenaux et al. 1988, 1994; Hart and Scheibling 1988; Strathmann et al. 1992; Fenaux et al. 1994; Hart and Strathmann 1994; Sewell et al. 2004; Miner 2005, 2007; Reitzel and Heyland 2007).

Conversely, the largest increase in gut diameter among all three food treatments occurred between day 4 and 7 (see Fig. 3-5, 3-6, 3-7). This observation cannot be attributed to distention of the gut by algal cells as none of the larval stomachs surveyed at day 7 were filled to capacity. Rather, only a few cells were observed suspended in the gastric cavity. Similarly, after a transient period of starvation the gut diameter of low food treated larvae increased although larval stomachs surveyed appeared to be nearly empty (Fig. 3-8*E*). However, physical distention was likely a factor among treatments that were transferred from starvation to high food treatments (Fig. 3-8*B*, *I*). With the exception of larvae that were too emaciated to recover in the no food treatment (Fig. 3-8*C*), all gut diameters increased when larvae resumed feeding. This is the first example of an adaptive reversal in developmental growth trajectory in echinoid larvae.

3.4.1 Possible mechanism of gut reduction

Gut reduction began with the loss of algal lipid pigment in the endoderm, followed by the accumulation of purple-pigmented mesenchyme cells around the digestive tract and significant reduction in the diameter of the gut. The accumulation of pigment cells around the gut has not been previously reported. Either these cells ingress from the larval ectoderm and migrate towards the endoderm of the gut to function in

coordinating a reduction in endodermal tissue, or additional pigment cells differentiate from the endoderm in response to starvation. Both transparent mesenchyme cells that migrate through the blastocoel (blastocoelar cells), and pigment cells, are derived from secondary mesenchyme during gastrulation in *Strongylocentrotus purpuratus* (Gibson and Burke 1985; Tamboline and Burke 1992). The pigmented mesenchyme cells described here were first observed associated with the basal lamina of the gut endoderm (Fig.3- 2*D*, Fig. 3-8*D*, *F*, *G*, *I*). As starvation progressed, the pigmented mesenchyme cells were suspended in the blastocoel of the larval body (Fig. 3-2*G*, *M*, Fig. 3-8*A*), but did not appear to enter the ectoderm (fig. 8*A*) as pigment cells normally would in *S. purpuratus* (Gibson and Burke 1985). Takata and Kominami (2003) described a similar pigmented cell type, with short filopodial projections that remained in the blastocoel and did not enter the ectoderm in larvae of the echinoid *Echinometra mathaei.*

If a tertiary surge of mesenchyme cell ingression from the endoderm of the gut is indeed taking place, it is a potential mechanism for energy re-distribution throughout the larval body in the absence of a circulatory system in the larval blastocoel. However, when food is limited, ingression of pigmented mesenchyme cells from the gut endoderm results in a reduction of endodermal mass, thereby the gut is reduced. During high food conditions, pigment cells did not accumulate around the gut, only transparent mesenchyme cells did. Further study is needed to quantify endodermal and pigment cell types during starvation to confirm this hypothesis.

A surge in differentiation of additional pigment cells may indicate a transition to an atypical development process. Pigment cells have diverse functions in echinoid larval development including: initiating gastrulation (Takata and Kominami 2004), innate immunity (Smith et al. 2006), and a prominent association with larval clone formation in *S. purpuratus* (Eaves & Palmer 2003; Chapter II). The role of pigment cells in gut reduction lends further support to the hypothesis that certain secondary mesenchyme-derived cells (including the pigment and blastocoelar cells) are a candidate population of conditionally-specified larval stem cells because they are prominently associated with a variety of major morphogenetic remodeling events (Chapter II).

3.4.2 Re-allocation of larval resources

Reduced gut size could be a survival mechanism for planktotrophic larvae that depend entirely on exogenous food to sustain a basal level of metabolic activity under food –limited conditions. The endodermal cells of the gut accumulate lipid during larval growth, and are reorganized into the adult digestive tract after metamorphosis (Burke 1981). In addition to a significant reduction in gut size, the most notable difference among larvae that received a high food ration prior to starvation was the loss of colored lipid from the endoderm. At a later interval the gut was also surrounded by pigment cells. In contrast, among food-limited larvae (both low and no food ration) that were starved continually the gut endoderm lacked any lipid –pigmentation, instead gut reduction and pigment cell accumulation happened immediately. Planktotrophic larvae may resort to a developmental stasis under low food conditions (Scheltema 1971*b*). Indeed, "growth stasis" occurs in larvae of a gastropod *Cymatium parthenopeum* capable of trans-Atlantic

dispersal (Pechenik et al.1984). Consistent with Scheltema's (1971*b*) hypothesis, we observed that the body width of larvae plateaued in food-limited conditions, while internally the gut was shrinking significantly, perhaps as a means of re-allocating energetic resources within the larval body.

Larval arms are the most metabolically active component of the larval body (McEdward 1984). Miner (2005) proposed a trade-off in larval arm length versus gut size in two *Strongylocentrotus* species prior to the onset of feeding. However, in the complete absence of food, planktotrophic larvae may have an all-or-nothing approach, where all available resources are put into feeding structures if the alternative is not to survive (Shilling 1995). The ability to use gut endoderm as a stored energy resource may serve this very function in starved planktotrophic larvae.

3.4.3 Rapid evolution of echionoid developmental modes

Particularly among echinoderms, planktotrophic larval development has given way repeatedly to non-feeding, yolk-provisioned development (lecitrotrophy), and this transition is unlikely to reverse (Strathmann1978). The key benefit of lecithotrophy is faster development independent of an exogenous food source, thereby reducing the amount of time larvae are exposed to predator attack and variable environmental conditions in the plankton (Thorson 1950; Strathmann 1978).

With such obvious adaptive benefits to lecithotrophic development, why hasn't it evolved even more frequently among species of echinoderms with protracted

planktotrophic dispersal periods? If planktotrophic larvae indeed have the ability to re-allocate tissue in their larval bodies this would serve as an invaluable survival mechanism for teleplanic (far-wandering) larvae in particular (Scheltema 1971*b*). Furthermore, the capacity to re-allocate endogenous resources shows how larvae could readily assimilate a 'new' endogenous energy source — namely increased maternal investment in the egg — thereby becoming less dependant on exogenous food levels (Herrera et al. 1996). If true, echinoid larvae in particular may be pre-adapted towards an evolutionary transition favoring lecithotrophy (Strathmann et al. 1992). Consistent with this, Reitzel and Heyland (2007) correlated a lower phenotypic plasticity response to exogenous food levels among larvae derived from larger yolky eggs.

Ultimately, gut reduction is a reversible process. Therefore, the ability to metabolize internal energy reserves may be a very versatile mechanism in planktotrophic larvae, consistent with a frequent, independent evolutionary switch to lecithotrophy (Strathmann 1978). However, the observation of gut reduction somewhat confounds our ability to estimate the dispersal potential of larvae based on developmental times determined in the laboratory (Emlet 1995; Paulay and Meyer 2006). Not only can larval growth slow down, but to some extent it can also be reversed, resulting in unpredictable but potentially even longer periods of planktonic larval dispersal. Regardless, gut reduction and recovery is another example of how remarkably versatile planktotrophic larval development can be. Such versatility may be a key reason for why it has persisted for over half a billion years.

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Chapter IV

Effects of limb damage on developing sea urchin larvae: Repair, regeneration and time to metamorphosis[4-1]

4.1 Introduction

More than 70% of marine invertebrates have a pelagic larval period of development (Thorson 1950). Although planktonic dispersal has significant ecological advantages, it also entails substantial disadvantages such as exposure to unfavorable environmental conditions and a diverse array of predators (Thorson 1950). Marine planktonic environments are highly dynamic, and the composition of zooplankton predators and prey changes constantly (Vaughn 2007). Consequently, larvae are naturally selected to survive in an environment wholly different from that of the adult (Hickman 2001). Moreover, the toll levied by larval predators is likely the most significant, yet understudied, factor affecting larval survival (Thorson 1950). How do fragile, microscopic marine larvae survive in such a diverse, multi-predator environment?

Because the primary cause of larval mortality is generally presumed to be whole ingestion by predators (Jägersten 1972; Vermeij 1993), the repair capability of larvae has been greatly under-investigated. In gastropods, veliger larvae of *Nassarius paupertus* have a 'beak' projecting from the margin of the larval shell aperature (Hickman 2001). This shell structure is the first line of mechanical defense protecting the soft cephalic region of the larval body during predator attack (Hickman 2001). Veliger larvae of

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[4-1] A version of this chapter will be submitted for publication to the journal Invertebrate Biology.

another gastropod, *Littorina scutulata* develop rounder shells with smaller aperatures when exposed to chemical cues from predatory zoea larvae of *Cancer* spp. (Vaughn 2007). However, an entirely different phenotypic result is induced in echinoid larvae of the sand dollar *Dendraster excentricus* when cultured in the presence of chemical cues from a predatory fish: remarkably, they spontaneously clone themselves into smaller individuals (Vaughn & Strathmann 2008).

Predators of developing echinoids in the plankton include: larval crustaceans, adult chaetognaths, and fish (Rumrill et al. 1985; Pennington et al. 1986; Allen 2008). Rates of predation on developing echinoids are highest until they develop larval arms, at which point rates steadily decline (Pennington et al. 1986; Rumrill et al. 1985; Allen 2008). Therefore, as echinoid larvae continue to grow predator attacks should be less successful. The only vulnerable body parts with dimensions comparable to that of early developmental stages for these same predators are the larval arms. The larval arms are projections of the larval body supported by skeletal rods and are bordered by a ciliary band that generates swimming and feeding currents (Strathmann 1971). Echinoid larval arms change form in response to variable food conditions (Boidron-Metairon 1988; Fenaux et al. 1988; Hart & Scheibling 1988; Strathmann et al. 1992; Fenaux et al. 1994; Hart & Strathmann 1994; Sewell et al. 2004; Miner 2005, 2007; Reitzel & Heyland 2007), and longer larval arms collect food particles more efficiently than shorter larval arms (Hart & Strathmann 1994). So, given the apparent importance of functional arms to larval growth and survival, how do echinoid larvae survive a rapid, non-lethal predator attack?

Surveys of field-collected specimens of later stage echinoid larvae for damaged arms would likely attribute damage to collection instead of predator attack. However, field-collected veliger larvae with damaged shells are hypothesized to be survivors of predator attacks (Hickman 2001). When these damaged larvae are cultured in the laboratory the broken aperatural margins are rapidly restored. The capacity to repair and regenerate damaged larval structures efficiently is likely an integral adaptation for surviving the larval dispersal phase (Hickman 2001). Because of their small size, delicate structure, and limited defensive behaviour (Rumrill et al. 1985), echinoid larvae must also have a means of surviving non-lethal predator attacks, particularly to their arms. Using a microsurgical technique and conventional light microscopy I examined several questions: 1) How do larval arms heal? 2) How long does larval arm repair take? 3) Do repaired arms have a different form compared to intact controls? and 4) Does damage to the larval arms affect development rate or time to metamorphosis?

4.2 Materials and methods

4.2.1 Gamete collection and larval cultivation

Gametes were collected from a naturally spawning male and female adult *Strongylocentrotus droebachiensis* maintained in a display tank at Bamfield Marine Sciences Centre connected to a free-flowing seawater system and fed *Macrocystis integrifolia*. Gametes were rinsed three times in pasteurized seawater (PSW) and initially incubated at 11°C until the larvae were large enough for surgery, when they were cultured on the bench top in the lab at 12-14.5 °C. Larval cultures were aerated twice daily and fed 10^4 cells per ml of *Isochrysis galbana* (CCMP1323 Provasoli-Guillard

National Centre for Culture of Marine Phytoplankton, West Boothbay Harbor, Main U.S.A.).

4.2.2 Surgical procedure

Early six-armed larvae with primary vestibule invaginations were isolated in a drop of water in a depression slide. Each larva was oriented on its ventral surface and an even downward stroke of the tip of a scalpel blade (#11 Sterisharps, Greenwood, SC USA) was applied to fracture the skeletal rod without severing it from the larval body. Each larva was photographed and then cultured individually in an 8 ml glass vial (FBG Anchor, London UK). PSW and food was changed every 48 hours.

4.2.3 Observations of larval limb healing

All larvae were examined daily under a dissecting microscope to confirm swimming and feeding were ongoing and to monitor for signs of infection. Larvae subjected to a single arm fracture (N = 10) were photographed immediately after the procedure, again 48 hrs later, and when rudiment motility was observed. A single arm fractured was administered to a second sample of larvae (N = 5) for image documentation of wound repair at 2 h intervals. Serial-fractured larvae (N = 10) were photographed after each procedure was administered at 24 h intervals and when rudiment motility was observed. Multiple-fractured larvae were photographed prior to and immediately following all arms being fractured (N= 10). Non-surgically modified controls were photographed at the same intervals as surgically modified larvae. Whole larvae were photographed at approximately 100 X magnification and larval arms were photographed at approximately 400X magnification using a Nikon CoolPix 4500 camera mounted on an

Olympus CX40 microscope with an eyepiece adapter. Images were transferred using Nikon View 5 software into Adobe Photoshop CS2 where exact sizing was calibrated from stage micrometer images. Distribution of skeletal mesenchyme, blastocoelar, and pigment cells was compared between surgically-modified and non-modified postoral arms of the same larvae, and to control larvae with intact arms.

4.3 Results

4.3.1 Degeneration and regeneration

In all cases arm repair began with constriction of the ectoderm around the fracture location thereby closing off the larval blastocoel in less than an hour (Fig. 4-1A). If a compound fracture occurred, and the integrity of the blastocoel was compromised on the fractured section of the arm, the blastocoel of the distal portion of the arm rapidly lost volume within 2 h (Fig. 4-1B). The ectoderm surrounding the location of the fracture healed into a continuous epithelium within 6 hours, meanwhile the fractured portion of the larval arm became increasingly shriveled in appearance as the skeletal rod began to be reabsorbed from the distal end towards the location of the fracture (Fig. 4-1C). The fractured portion of the larval arm shrank significantly within 12-20 hours and the ciliary band was lost (compare Fig. 4-1C to 4-1D). The degenerating arm section was heavily pigmented as the pigment cells appeared to be concentrated in the shriveling ectoderm. The fractured arm 'waved' in and out of the oral field of the larva depending on the

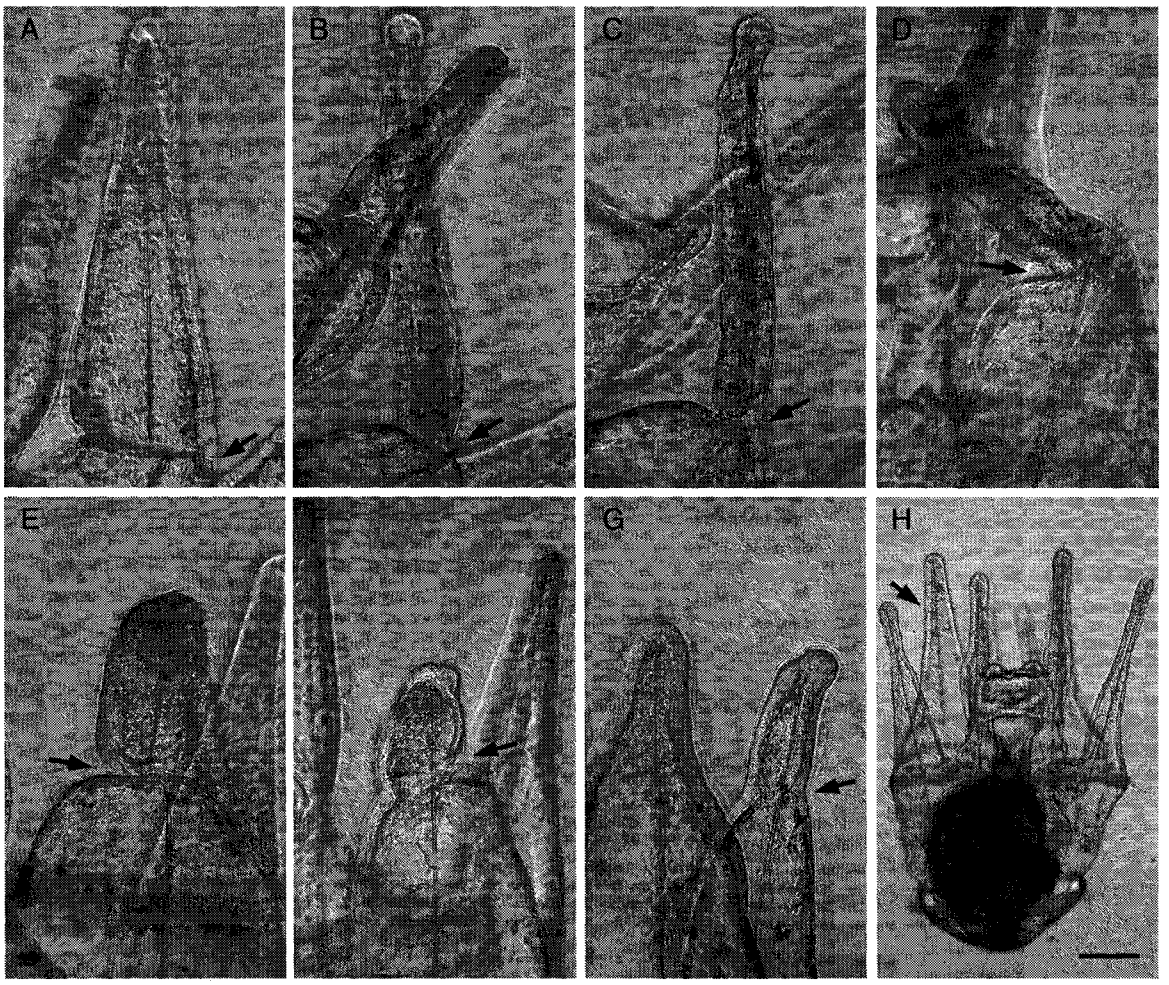


Figure 4-1. Sequential observations of arm degeneration and regeneration following the microsurgical fracturing of the left postoral arm of an individual 19 d *Strongylocentrotus droebachiensis* larva. A. Immediately post-fracture (arrow), B. 2 h post-fracture ectoderm has constricted surrounding the fracture and blastocoel volume has been lost in the fractured arm section. C. 6 h post-fracture the fractured arm section is shortening. D. 20 h post-fracture the same section of arm is less than half its original length. E. 24 h post-fracture the degenerating section of arm is stabilized. F. 28 h post-fracture the blastocoel of the basal portion of the arm has re-opened with that of the fractured section. G. 48 h post-fracture the formerly fractured section has regenerated. H. Whole larva 10 d post-fracture, arrow indicating regenerating fractured arm. Scale bar = 50 μm for A-G, same scale bar = 350 μm for H.

direction of the ciliary current being generated by the larva. At the distal end of the skeletal rod in the basal portion of the larval arm the skeletal mesenchyme cells generated at least two projections that extended into the degenerated section of the larval arm, and eventually stabilized it within 24 h (Fig. 4-1E). Once the fractured and degenerated arm segment had stabilized, the blastocoel between the larval body and the fractured distal arm segment re-opened (Fig. 4-1F). When blastocoel continuity was restored the dense aggregation of dark mesenchyme cells at the distal end of the blastocoel in the broken segment became less apparent, and transparent mesenchyme cells migrated into the distal region a day after the initial fracture (Fig. 4-1F). The fractured section of the larval arm regenerated a ciliary band and re-extended the skeletal rod (Fig. 4-1G). Within 2 days pigment cells were evenly distributed throughout the ectoderm in the regenerated segment and the fractured arm was indistinguishable from intact larval arms (Fig. 4-1H).

4.3.2 Autotomy and regeneration

If the ectoderm surrounding the fracture location was severely twisted or torn around the broken end of the skeletal rod, the fractured portion of the larval arm fell off within 2 hours (compare Fig. 4-2A and 4-2B). In 24 h the ectoderm surrounding the fractured skeletal rod healed, and regenerated a new ciliary band at the tip of the larval arm abundant with dark pigment cells (Fig. 4-2C). Two days post-injury, regenerated larval arms appeared normal, and pigment cells had dissipated from the regenerating distal arm tip (Fig. 4-2D).

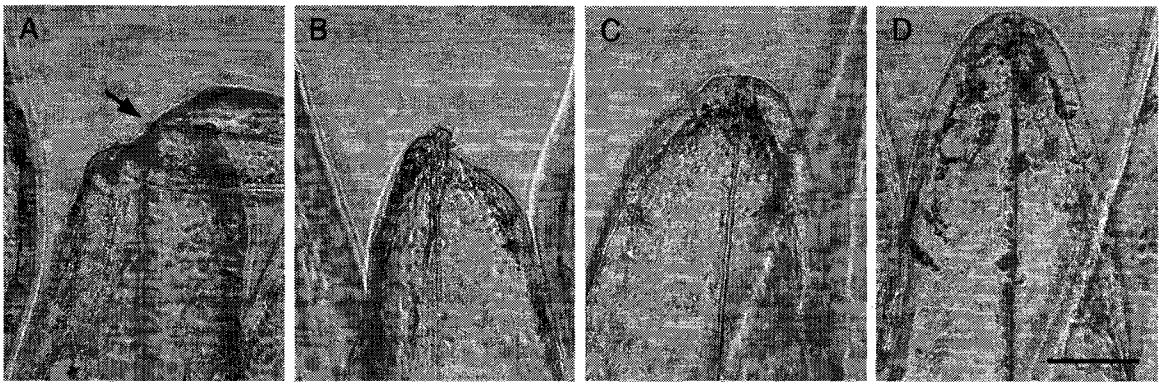


Figure 4-2. Sequential observations of arm autotomy and regeneration following the microsurgical fracturing of the left postoral arm of an individual 19 d *Strongylocentrotus droebachiensis* larva. A. Immediately post-fracture (arrow), B. 2h post fracture- the ectoderm surrounding the location of the fracture has constricted, and the distal section of the arm has detached. C. 24 h post-fracture- the ciliary band has regenerated at the fracture site and the distal-most tip of the regenerating arm is crowded with dark pigment cells. D. 48 h post-fracture- the regenerated arm appears normal, and pigment cells have re-distributed throughout the ectoderm of the larval arm. Scale bar = 50 μm.

4.3.3 Skeletal rod re-alignment

The 'waving' motion of the fractured arm section appeared to produce the fastest healing process. When the fractured ends were shifted enough that they overlapped with one another, the overlapping fractured ends fused together within 12 hours and blastocoel continuity was maintained between the larval body and the fractured arm section (compare Fig. 4-3A and 4-3B). The fused section of skeletal rod appeared to be remodeled into a single continuous rod without any variation in diameter from intact sections, and the larval arm appeared normal in less than a day (Fig. 4-3C).

4.3.4 Effects of incidence and frequency of arm fracture on the rate of development

All larvae subjected to a single fracture (Fig. 4-4A) looked normal after two days, and all had motile juvenile podia by day 29 (14 days post-procedure) (Fig 4-4B). Among larvae subjected to five consecutive days of fractures (Fig. 4-4C), each arm appeared normal within 1-2 days, depending on the mechanism of arm repair, and when re-fractured the arms again appeared normal within 2 days. In less than a week after the last fracture was administered all larvae had active juvenile podia by day 29 (Fig. 4-4D), and were indistinguishable from control larvae (Fig. 4-4E, F). Larvae subjected to multiple traumas, by having all arms fractured at once (Fig. 4-4G), also healed within 2 days, and displayed all forms of arm repair (degeneration, autotomization, and skeletal rod re-alignment). These larvae also all had active podia in the juvenile rudiment by day 29 (Fig. 4-4H).

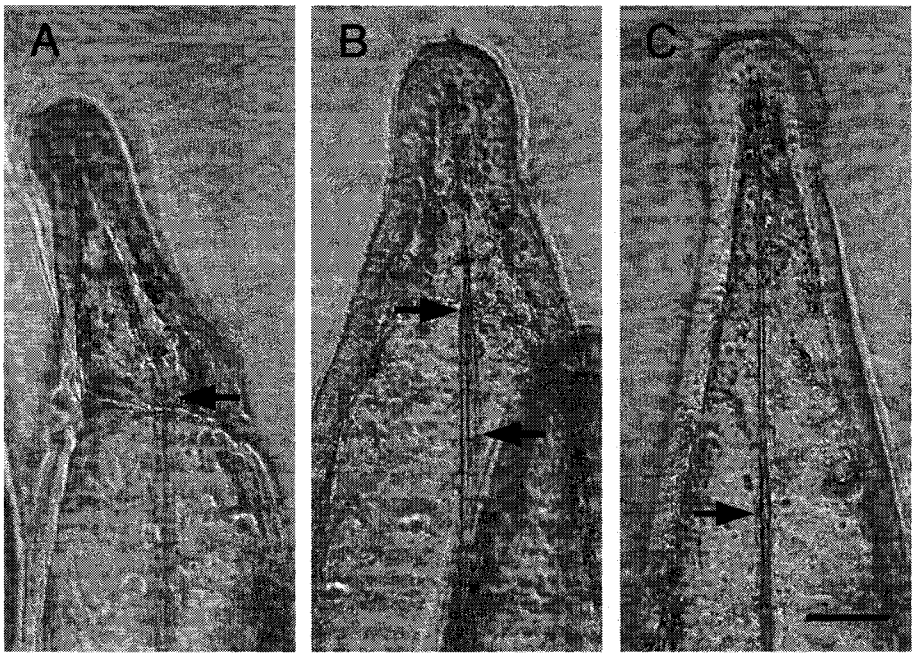
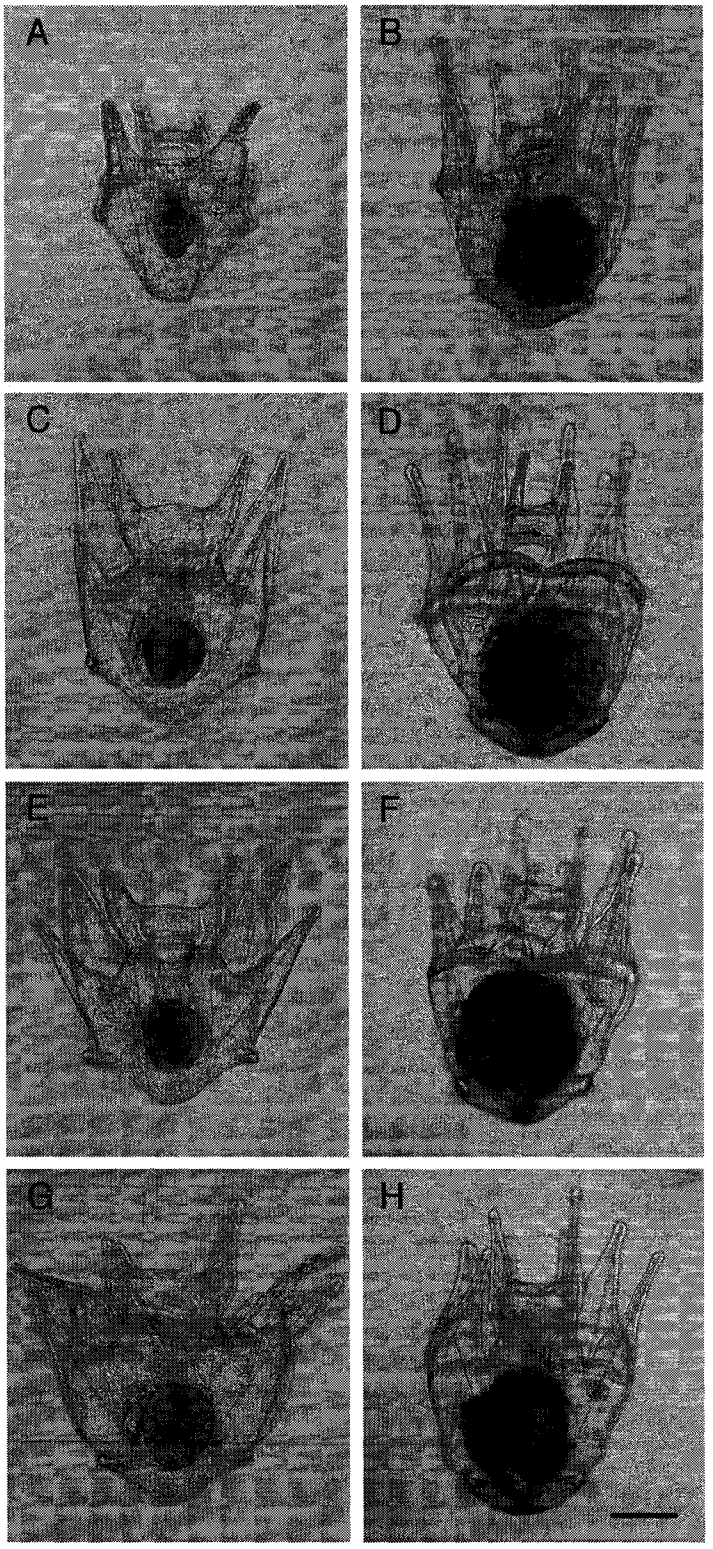


Figure 4-3. Sequential observations of skeletal rod repair following the microsurgical fracturing of the right postoral arm of an individual 17 d *Strongylocentrotus droebachiensis* larva. A. Immediately post-fracture (arrow), B. 10 h post-fracture- the broken end of the skeletal rod has migrated posteriorly and aligned parallel (arrows) to the rest of the rod that extends into the larval body. C. 24 h post-fracture- the arm appears normal with only a small node on the skeletal rod remaining where the two broken ends had fused (arrow). Scale bar = 30 μm.

Figure 4-4. Comparison of developmental time to metamorphic competence between intact, single and multiple arm fractured *Strongylocentrotus droebachiensis* larvae. A. Immediately post-fracture of the right postoral arm in a 15 d larva, B. same specimen 14 d later. C. Intact 17 d larva (control). D. Same intact larva with motile rudiment (29 d). E. 18 d larva immediately after second of five consecutive daily arm fractures. F. Same specimen at 29 d with motile juvenile rudiment. G.19 d larva post-multiple fracture procedure (all arms broken). H. Same specimen at 29 d with motile juvenile rudiment. Scale bar = 200 µm.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4.4 Discussion

Larval wound repair was first examined in Metchnikoff's (1893) classic study of asteroid larval recovery from a rose-thorn puncture of their single-cell thick ectoderm — a study that ultimately lead to the discovery of innate immunity. From the molecular to the organismal level, animals must be able to survive environmental threats. One of the most understudied threats to marine larvae is predator attack (Thorson 1950). However, predator attack is not always fatal and larvae have mechanisms to recover from such attacks (Hickman 2001; Vaughn 2007). The present study supports this observation, as all larvae completely recovered from artificial arm fracture. However, the range of mechanisms used to heal the same injury, and the minimal impact of injury on time to metamorphic competence, were both surprising. The three types of arm repair — degeneration and regeneration, autotomy and regeneration, and skeletal rod re-alignment — differed mainly in the degree of degeneration required prior to regenerating the damaged section of larval arm.

4.4.1 Arm degeneration

The most commonly observed process of degeneration involved the broken section of the limb reducing into an undifferentiated tissue mass at the fracture point prior to regenerating a new arm tip. The rate-limiting step in this process appeared to be reabsorption of the fractured section of the skeletal rod from the distal tip. Echinoid larvae can rapidly shorten and lengthen skeletal rods to modify the length of the oral ciliary band depending on food abundance (Boidron-Metairon 1988; Fenaux et al. 1988; Hart & Scheibling 1988; Strathmann et al. 1992; Fenaux et al. 1994; Hart & Strathmann 1994; Sewell et al. 2004; Miner 2005, 2007; Reitzel & Heyland 2007). This same

mechanism likely accounts for skeletal rod reduction during damaged arm degeneration shown here. Adult cidaroid pencil urchins exhibit a similar example of skeletal element reabsorption near the wound location: When the distal end of a spine is damaged, only that section of the spine is discarded, and the intact proximal section of the spine is retained (Wilkie 2001). However, this process requires the calcite-resorbing activity of a phagocytic syncytium (Prouho's membrane) to 'dissolve' the spine skeleton prior to detaching the damaged section (Märkel & Röser, 1983; Wilkie 2001). This process requires several days in adult Cidaroids compared to 24 hrs reported here for larval arms. However, the adult spines of Cidaroids are extremely robust (up to 1 cm in diameter) compared to skeletal rods in echinoid larvae studied here (approximately 2-3 μm). Skeletal reabsorption has been described in larvae, but not in the context of wound repair: Larvae of *Strongylocentrotus purpuratus* reabsorb the basal section of the first pair of skeletal arm rods as a normal part of development (Eaves 2005). In addition, during larval clone formation, the same basal section of skeletal rods often extends into the developing clone and must be 'dissolved' prior to clone separation (chapter II). In both these examples the section of skeletal rod being reabsorbed is surrounded by transparent mesenchyme cells (Eaves 2005, chapter II).

During degeneration of the fractured arm tip, dark mesenchyme cells were also abundant at the distal tip of the arm, and these were likely phagocytic blastocoelar cells (Smith et al. 2006). Blastocoelar and pigment cells both migrate throughout the larval body and function as innate immune cells (Smith et al. 2006). Similarly in adult echinoderms, when pedicellaria heads are released while defending against an attack, the

remaining stalk is shortened by tissue resorption and damaged tissues are endocytosed by phagocytic cells (Holland & Holland 1975; Dubois & Ghyoot 1995; reviewed by Wilkie 2001). The innate immune cells in larvae possibly serve a similar role by removing cell debris during arm degeneration.

4.4.2 Arm autotomy

The extent of tissue degeneration at fracture sites was greatly reduced when the fractured section of the arm was released from the larval body. Autotomization of body parts is a defensive adaptation and the most pervasive cause of structural loss in echinoderms (Wilkie 2001). Autotomy usually occurs at a pre-formed breakage plane that dissociates quickly (within minutes). The dominant tissue type at these sites is mutable collagenous tissue that can rapidly and irreversibly lose tensile strength at the site of autotomy in response to neural signaling (Wilkie 2001). However, autotomy may also occur in echinoderms at sites that lack obvious autotomy-related features (Wilkie 2001). For example, when adult asteroids, holothuroids, and ophiuroids are wounded, the damaged body region can be detached (Mladenov & Burke 1994). This process requires several minutes or days, similar to the damaged spine autotomy mechanism of Cidaroids (described above). However, in the larval echinoids studied here the fractured section of the larval arm remained connected to the larval body solely by the ectoderm, which the larval body appeared to sever at the fracture location within minutes or hours. Although this occurred less frequently than did degeneration of the fractured section of larval arm, autotomizing the fractured section likely accelerated the wound repair process, by

generating less cell debris and reducing the need for histolysis (Candia Carnevali & Bonasoro 1995; Wilkie 2001).

Particularly during asexual reproduction of larvae and adults, the plane of bisection is usually not morphologically defined prior to separation. Echinoderm larvae can release asexually developed clones from nearly every part of their larval body (reviewed by Eaves chapter II). During clone separation, autotomy of the developing clone begins with a constriction in the larval ectoderm that segregates the clone blastocoel from that of the primary larva (chapter II). However, this process requires days whereas autotomy of the damaged section of arm occurred within hours in the present study. The larval body can therefore coordinate rapid detachment of damaged sections, although the mechanism by which it does so is unknown.

4.4.3 Arm regeneration

Most living organisms have the ability to regenerate to some degree (Ghiselin 1987). Although adult echinoderms are renowned for their regenerative abilities, larval regeneration has received surprisingly little attention (Vickery & McClintock 1998). Czihak (1965) described regeneration of coeloms and juvenile-rudiment structures destroyed by laser ablation in echinoid larvae. Even more dramatically, both sections of experimentally bisected asteroid larvae, and the posterior section of bisected echinoid larvae could regenerate the missing half of the larval body (Vickery & McClintock 1998; Vickery et al. 2002). The ability to regenerate damaged or missing body parts likely

enhances larval survival rates in the planktonic environment (Vickery & McClintock 1998;Vickery et al. 2001)

4.4.4 Cell types and regeneration

A common feature of all three types of arm fracture repair was abundant mesenchyme cells at the wound site. Aggregations of mesenchyme cells often appear at the site of surgical bisection or wound repair in larval and adult echinoderms (Thorndyke et al. 1999; Vickery et al. 2002). They are also prominently associated with the site of bud formation during larval cloning (Jeackle 1994; chapter II). Although the transparent mesenchyme cells associated with skeletal rod remodeling were likely primary mesenchyme cells, secondary mesenchyme cells have been identified as the cells responsible for late larval (at or beyond the six-armed larval stage) and adult skeletal formation in *Pseudocentrotus depressus* and *Hemicentrotus pulcherrimus* sea urchins (Yajima 2007). Similarly, pigment cells, also derived from secondary mesenchyme cells, appeared to be conserved in degenerating arm tips, and concentrated at the site of wound repair and arm autotomization. In addition to their innate immune function (Smith et al. 2006), pigment cells accompany any major morphological remodeling event in the larval body such as clone formation (Eaves chapter II), gut reduction (Eaves & Bergstrom chapter III), and innate immune functions (Smith et al. 2006).

4.4.5 Rate of repair and juvenile rudiment development

The rate of wound repair was perhaps the most surprising outcome of this study. Previous investigations of larval regeneration observed a much slower rate of wound repair, albeit the procedures inflicted on larvae were much more extensive. Echinoid

larvae of *Lytechinus variegatus* surgically bisected into anterior and posterior halves required several days to regenerate missing structures, and anterior portions of bisected larvae regenerated only partially after two-weeks (Vickery et al. 2002). Curiously, the same study reported that a new juvenile rudiment developed in the regenerating anterior half of bisected sea star (*Pisaster ochraceus*) larvae in half the time observed in intact control larvae (Vickery et al. 2002). Does regeneration induce an accelerated developmental program of the juvenile rudiment in damaged larvae? I originally hypothesized that larval arm damage would produce a significant delay in the time required to reach metamorphic competence, particularly in larvae subjected to serial arm fractures over a five day period. Yet regardless of the extent or frequency of arm fractures that were induced, all larvae attained metamorphic competence at the same time as controls, consistent with the observations of Vickery and others (2002). Although no morphological difference was apparent among larvae competent to metamorphose, I cannot say whether larval injury had any impact on newly metamorphosed juveniles.

Morphological evidence of regeneration in marine larvae is scarce (Hickman 2001). In the laboratory, field-collected mollusc larvae repaired sub-lethal shell damage thought to have been inflicted by planktonic crustacean predators and repaired shells differed in form from intact shells (Hickman 2001). In contrast, the sea urchin larvae in the present study showed no obvious post-repair phenotype. Similar to adult echinoderms, the rapid regenerative capability of echinoderm larvae is likely crucial to larval survival.

Particularly for soft-bodied planktonic larvae, defensive structures are unlikely to be effective against the diversity of predators in the pelagic marine environment (Vaughn 2007). Furthermore, marine predators are thought to be abundant for brief periods or in patchy distribution patterns (Vaughn 2007). Therefore, larvae may be under strong selection pressure to recover quickly from injuries to avoid prolonged exposure to planktonic predators. Because echinoid larvae can repair limb damage so rapidly and without evidence of physical trauma, it would be impossible to identify field-collected larva that had survived a non-lethal predator attack. However, because of their impressive efficiency at repairing damaged structures, rapid regeneration should greatly increase the probability of echinoid larval survival following injury.

4.5 References

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Chapter V

Variation in environmental stress tolerance during the early development of congeneric sea urchins[5-1]

5.1 Introduction

Many benthic marine invertebrates release their gametes into the water column where fertilization occurs. But most embryos have limited motility and are at the mercy of whatever environmental conditions they encounter (Kinne 1970; 1971; Verween et al. 2007). Environmental parameters such as temperature and salinity affect the development, survival and distribution of marine invertebrates (Kinne 1964, Scheltema 1965). However, these parameters are changing as oceans are warming and marine organisms may be responding to climate change faster than terrestrial counterparts (Richardson & Poloczanska 2008). Only a slight increase in temperature can alter ocean currents, chemistry, nutrient cycling and phytoplankton blooms affecting species distribution ranges, reproductive cycles, and life cycles of marine organisms (Richardson & Poloczanska 2008). To predict how biodiversity will be affected by a changing global climate we need to characterize the environmental conditions species can tolerate, and compare tolerance ranges among sympatric and closely species.

5.1.1 Temperature and salinity

Temperature has many effects on marine invertebrate larvae including rate of development, duration of the pelagic period, and amount of larval mortality (Scheltema 1967). Generally, embryos and larvae develop faster at higher temperatures in their

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natural environment (reviewed by Hoegh-Guldberg and Pearse 1995), and echinoids are no exception (Stephens 1972). In fact, temperature has a greater influence on the rate of development than does food abundance (Scheltema 1965; Hart 1988). The earliest stages of development are likely the most sensitive because embryos require narrower temperature ranges than older larvae and benthic stages (Pelseneer 1901; Thorson 1950).

Salinity also affects survival of developing marine organisms. Salinity stress affects growth rate, incidence of mortality, and duration of the larval pelagic period (reviewed by Richmond and Woodin 1996). It is a key ecological factor in coastal zones subjected to high variability in this parameter (Anger 2003; Diele and Simith 2006). Even a transient, stochastic occurrence such as a large freshwater runoff event can significantly affect the growth and survival of coastal organisms (Richmond and Woodin 1996).

5.1.2 Ecology of adults

Having no osmoregulatory organ (Binyon 1966), and a permeable body wall (Drouin et al. 1985), the Echinodermata is the only major animal phylum that is stenohaline (Stickle and Diehl 1987). Regardless, adult echinoderms live in habitats subject to tidal and seasonal fluctuations (reviewed by Roller and Stickle 1994), with salinities sometimes as low as 5 parts per thousand (ppt), to as high as 60 ppt (Stickle and Diehl 1987). The distribution of adult sea urchins within salinity gradients may be determined by the osmotolerance of the larvae (Stickle and Diehl 1987). However, the lower salinity limit for most sea urchin larvae that have been examined is 20-25 ppt (reviewed by Metaxas 1998).

Within echinoderms, species of the genus *Strongylocentrotus* are thought to be the most studied sea urchins on earth (Rodgers-Bennett 2007; Scheibling and Hatcher 2007). In the North Pacific most strongylocentrotid species arose from a recent burst of speciation during the late Miocene and Pliocene resulting in ecological diversification of this group (Biermann et al. 2003). The red sea urchin, *Strongylocentrotus franciscanus,* occurs only in the northeastern Pacific from Baja California to Kodiak Alaska (Ebert et al. 1999; reviewed by Rodgers-Bennett 2007), and lives subtidally to depths of 90 meters (Morris et al 1980; reviewed by Rodgers-Bennett 2007). The purple sea urchin, *Strongylocentrotus purpuratus,* has a slightly narrower latitudinal range within that of *S. franciscanus*, but a greater distribution of depths down to 160 meters (Morris et al. 1980; reviewed by Rodgers-Bennett 2007). The green sea urchin, *Strongylocentrotus droebachiensis,* is circumpolar and can be found in both estuarine and coastal environments; it can tolerate a great range of temperatures (from –1 to 20°C), salinities (from 13 to 30 ppt), and lives at depths up to 300 meters (reviewed by Roller and Stickle 1994; Scheibling and Hatcher 2007). Despite overlapping spawning seasons, similar habitats (Strathmann 1987; Rodgers-Bennett 2007; Scheibling and Hatcher 2007) and close phylogenic proximity (Biermann et al. 2003; Lee 2003), how similar are the environmental parameters conducive for embryogenesis among these three species?

Previous studies have largely examined the effects of temperature and salinity as separate variables, and few studies have compared intraspecific variation in environmental parameters. We examined the impact of various temperature and salinity combinations on early development of three strongylocentrotid sea urchins from a single

sympatric population in the northeastern Pacific to address two main questions: 1) how does temperature affect salinity tolerance for each species, and 2) how do the temperature and salinity survival parameters compare among species? Our observations provide insight as to how speciation could occur by acclimation to new environmental parameters in an apparently brief amount of time (Biermann et al 2003; Lee 2003).

5.2 Materials and methods

5.2.1 Collection and spawning of adults

Adult *Strongylocentotus droebachiensis*, *S. franciscanus*, and *S. purpuratus* were hand-collected 7-10 m subtidally near Aguilar Point, Vancouver Island British Columbia (48° 50' 03", 125° 08' 05"). Animals were maintained in flow-though seawater tanks at the Bamfield Marine Sciences Centre for 1-3 months prior to spawning and fed *Macrocystis itegrifolia* or *Nereocystis luetkeana* daily. Spawning was induced by intracoelomic injection of 0.55 M KCl. Sperm was collected 'dry' from the aboral surface of spawning males, and females were inverted over bowls containing each salinity solution (described below) at 10°C, each replicate consisted of full-sib embryos, and adults were spawned only once. Prior to fertilization, eggs collected into each salinity were aliquotted into 125 ml jars and allowed to acclimate to each temperature range (15 minutes). Sperm suspensions were prepared for each salinity (approximately 1 in 10,000 dilution) and added to egg suspensions. All cultures were gently agitated after 5 minutes.

5.2.2 Temperature and salinity treatments

According to data collected from Sarita Hole Station, near Barkely Sound BC, the annual salinity and temperature at 10 m depth is between 22-34 ppt, at 7-14 °C (University of British Columbia Ocean Dynamics Laboratory). Three temperature treatments were established from this range: 7-8 °C, 10-11°C, and 13-14°C. For each of these temperature treatments, embryos were cultured at five different salinities: 22, 25, 28, 31, and 34 ppt. Hypotonic solutions (22, 25, 28 ppt) were prepare by diluting pasteurized seawater (PSW) (31 ppt) with deionized freshwater, and the hypertonic solution (34 ppt) was prepared by evaporating PSW in 4 L Pyrex flasks on a hot plate. Solutions were stored at 10°C.

5.2.3 Assays of temperature and salinity effects on embryogenesis

The survival of each species in all temperature and salinity combinations was quantified at fertilization, hatched blastulae, and gastrulation. For each species, three replicates (N= 20 embryos per replicate) were assayed at each temperature and salinity combination (total of 15 treatments). Morphogenesis of each species in all conditions was monitored and compared to normal development at 28 – 31 ppt. Observations were made at 100X magnification on an Olympus CX40 microscope. Images were obtained using a Nikon Coolpix 4500 camera mounted on an eyepiece adapter.

5.2.4 Statistical analysis

A total of three, 3-way contingency tables using a log-linear analysis were calculated using urchin species, temperature, and salinity as the variables. Individual

contingency tables were calculated for each life-history phase (fertilization, hatching, and gastrulation). The contingency tables compared the three variables together and then subsequent two-factor comparisons (similar to post-hoc t-tests for ANOVAs). Contingency tables were analyzed using the program available at http://faculty.vassar.edu/lowry/VassarStats.html.

5.3 Results

5.3.1 Fertilization

No obvious morphological differences were evident in eggs immersed in different salinities, and all eggs fertilized within ten minutes following addition of the sperm suspension. Rates of fertilization were consistently high among all species in all temperature and salinity combinations and no significant variation was detected (G^2= 3.58, df= 36, p= >0.99. Table 5-1A).

The rate of embryonic cleavage varied substantially among treatments, development proceeded faster and synchronously in cultures between 28 and 31 ppt for *S. franciscanus* and *S. droebachiensis*, and between 28 and 34 ppt for *S. purpuratus*. In sub-optimal salinities, particularly hypotonic conditions, the rate of cell cleavage was asynchronous and cleavage patterns were highly irregular with the majority of embryos having an odd number of asymmetric and loosely arranged cells (Fig. 5-1A) compared to control embryos (Fig. 5-1B). Normal developmental stages such as 8, 16, and 32 cell stages were unrecognizable. Comparatively, in hypertonic conditions (34 ppt for *S. franciscanus* and *S. droebachiensis*), blastomeres were tightly associated with one

Table 5-1.

Results of three-way contingency table comparing the effects of temperature, salinity and species on survivorship during fertilization, hatching, and gastrulation. Bold values indicate statistical significance at the level of P=0.05.

A. Fertilization

	G^2	df	p
Temp X Salinity X Species	3.58	36	>0.99
Temp X Salinity	0.58	8	0.99
Temp X Species	0.28	4	0.99
Salinity X Species	1.8	8	0.98

B. Hatching

	G^2	df	p
Temp X Salinity X Species	152.52	36	**0.0001**
Temp X Salinity	17.6	8	**0.024**
Temp X Species	3.16	4	0.5
Salinity X Species	90.74	8	**<0.0001**

C. Gastrulation

	G^2	df	p
Temp X Salinity X Species	222.3	36	**<0.0001**
Temp X Salinity	17.72	8	**0.02**
Temp X Species	4.28	4	0.36
Salinity X Species	152.1	8	**<0.0001**

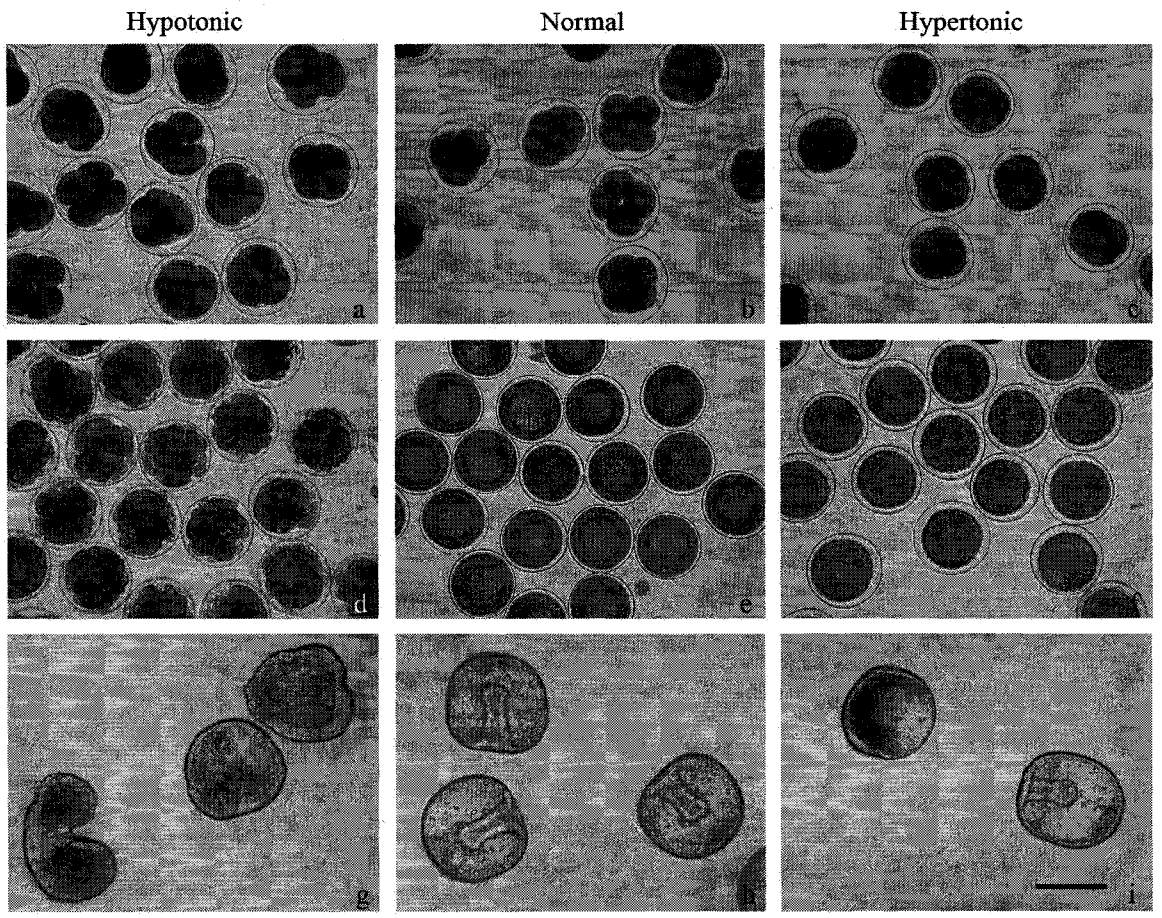


Figure 5-1. Effect of salinity on the morphogenesis of *Strongylocentrotus droebachiensis* cultured at 13-14°C. Embryogenesis in salinities of a) 22 ppt, b) 28 ppt, c) 34 ppt. Pre-hatching blastulae in d) 22 ppt, e) 28 ppt, f) 34 ppt. Gastrulae in g) 22 ppt, h) 28 ppt, i) 34 ppt, scale bar = 160 μm.

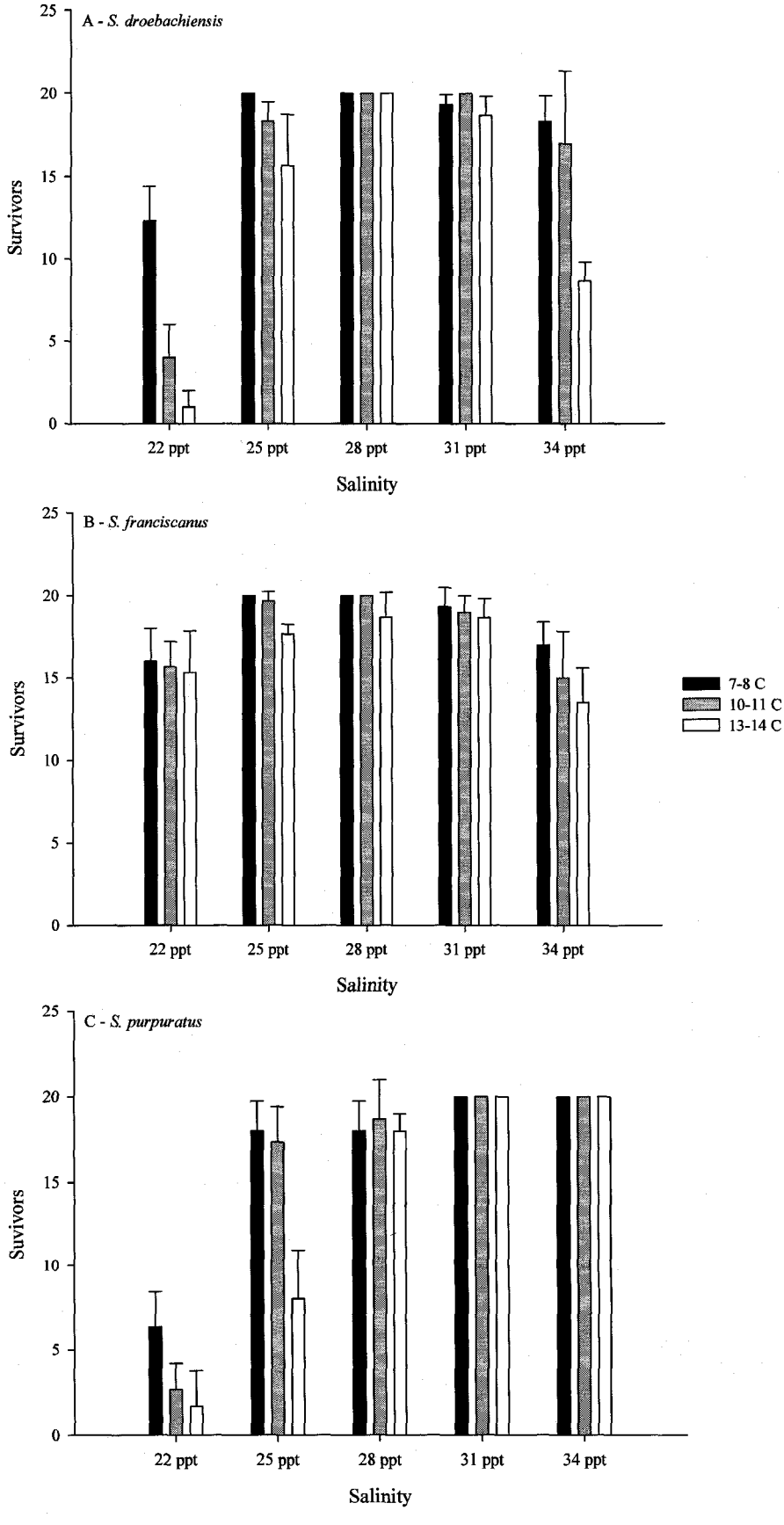
another and normal cleavage patterns were observed although at an asynchronous rate among embryos (Fig. 5-1C).

5.3.2 Hatching

Hatching was characterized as any motile embryo outside a fertilization envelope. There was a significant interaction between salinity and temperature on hatching (G^2= 17.6, df= 8, p= 0.024. Table 5-1B). The incidence of hatching was highest for all temperatures at 28 and 31 ppt (Fig. 5-2 A,B,C). At 22, 25 and 34 ppt, the highest incidence of hatching occurred in the coldest temperature range (7-8°C) and survivorship was reduced in each higher temperature (Fig. 2). There was also an interaction between species and salinity (G^2= 90.74, df=8, p<0.0001). Among species, *S. franciscanus* had the highest overall survivorship of hatched embryos over all salinity treatments (Fig. 5-2B), followed by *S. droebachiensis* (Fig. 5-2A). *S. purpuratus* had the lowest survival in low salinity conditions and the highest survival rates at 34 ppt (Fig. 5-2C).

The morphology of motile embryos, particularly those in hypotonic conditions, was largely abnormal (Fig. 5-1D). Hypotonic blastulae had a convoluted epithelium and were not spherical, many had blastomeres that partly protruded from the epiderm and some embryos had more than one blastocoel. Blastulae in the 28 - 34 ppt range were spherical, and the epithelium was of consistent density (Fig 5-1E, F). The epithelium of *S. franciscanus* and *S. droebachiensis* blastulae in 34 ppt appeared slightly thicker relative to the diameter of the blastocoel compared to embryos in 28 and 31 ppt. After hatching,

Figure 5-2. Effect of temperature on the mean survivorship to hatching in different salinities for A) *Strongylocentrotus droebachiensis*, B) *S. franciscanus*, and C) *S. purpuratus*), N= 60 (3 replicates of 20) for each temperature and salinity combination. Black bars represent 7-8°C, grey bars 10-11°C, and white bars 13-14°C.


A - S. droebachiensis
B - S. franciscanus
C - S. purpuratus
Survivors
Suvivors
Salinity
22 ppt
25 ppt
28 ppt
31 ppt
34 ppt
0
5
10
15
20
25
7-8 C
10-11 C
13-14 C

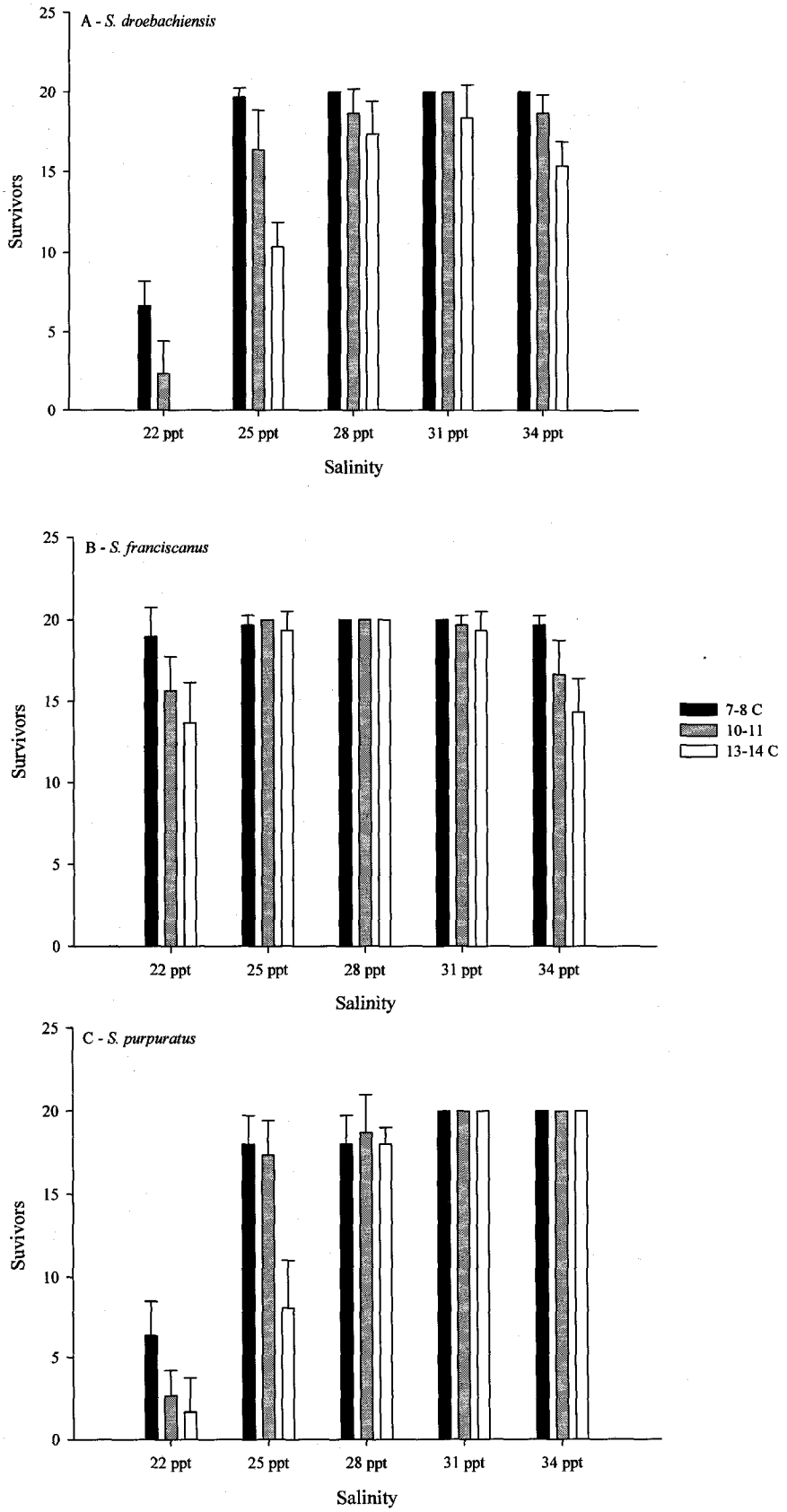
nearly all embryos of all three species swam near the surface in the cultures, only very abnormal embryos swam near the bottom.

5.3.3 Gastrulation

The coldest temperatures yielded the highest incidence of gastrulation among all species in all salinity conditions, particularly at the lower salinity range (Fig. 5-3 A, B, C) (G^2= 17.72, df= 8, p< 0.02 for the interaction between temperature and salinity, see also Table 5-1C). Among species, at 22 ppt *S. fransicanus* (Fig. 5-3B) had a five-fold greater survival rate than *S. purpuratus* and *S. droebachiensis* (Fig. 5-3A, C). These two factors had a significant interaction (G^2= 152.1, df= 8, p< 0.0001). The discrepancy among species was narrower at 25 ppt, and all three species had a high incidence of survival to gastrulation at 28 and 31 ppt. *S. purpuratus* had a slightly higher incidence of survival at 34 ppt. These two factors also had a significant interaction (G^2=152.1, df=8, p<0.0001).

In hypotonic blastulae of *S. purpuratus* and *S. droebachiensis*, mesenchyme cells ingressed throughout the blastocoel (Fig. 5-1G) instead of being localized in the vegetal hemisphere as in 25-34 ppt salinities (Fig. 5-1H). Among the majority of specimens, no animal-vegetal polarity became apparent and these embryos did not develop further. Among the few embryos that did gastrulate at 22 ppt (Fig. 5-1G), the archenteron was short and the future stomodeal end contacted the inner wall of the blastocoel at a site very close to the blastopore instead of extending across the blastocoel to the apical region of the embryo as observed in normal salinity. In hypersaline conditions mesenchyme cells ingressed at the vegetal hemisphere and either the embryo underwent normal gastrulation or, the mesenchyme cells did not migrate properly throughout the blastocoel and

Figure 5-3. Effect of temperature on the mean survivorship to gastrulation in different salinities for A)*Strongylocentrotus droebachiensis*, B) *S. franciscanus*, and C) *S. purpuratus*), N= 60 (3 replicates of 20) for each temperature and salinity combination. Black bars represent 7-8°C, grey bars 10-11°C, and white bars 13-14°C.


A - S. droebachiensis
B - S. franciscanus
C - S. purpuratus
Survivors
Salinity
22 ppt
25 ppt
28 ppt
31 ppt
34 ppt
0
5
10
15
20
25
7-8 C
10-11
13-14 C

inundated the blastocoel with dark mesenchyme cells (Fig 5-1I). Ultimately, gastrulae appeared the most normal in 28-34 ppt for *S. purpuratus* and *S. droebachiensis*. *S. franciscanus* tolerated all salinities.

5.4 Discussion

Pelagic larvae of benthic marine invertebrates endure far more variable abiotic factors than adult animals (Saranchova et al. 2006). In addition, early developmental stages of most invertebrates have much lower tolerances than later developmental stages or adults (Verween et al. 2007). We found salinity to have the most significant effect on survival through embryogenesis, when developing organisms are most vulnerable to salinity extremes (Roller and Stickle 1994). However, this sensitivity was evident only after ontogenesis had begun, as fertilization rates were indistinguishable among different temperature and salinity combinations for all species. Estimates of fertilization likely overestimate rates of survival under conditions of osmotic stress and are therefore poor predictors of developmental success (Hintz and Lawrence 1994; Roller and Stickle 1994).

Salinity also had the greatest effect on morphogenesis in all species. The morphological abnormalities induced were consistent among all species in both hypotonic and hypertonic conditions. Most notably, in hypotonic conditions the loose association of blastomeres during ontogeny resulted in irregular cleavage patterns and abnormal embryos. In contrast, hypertonic conditions supported normal cleavage patterns and tightly associated blastomeres. However, ingression of primary mesenchyme cells

was sometimes abnormal, yielding a blastocoel filled with darkly pigmented mesenchyme cells and disrupting gastrulation.

Interestingly, at lower temperatures all species could tolerate a wider range of salinities. Temperature is regarded as being the most influential abiotic factor on the rate of development; as temperature increases, development accelerates (Thorson 1950; Scheltema 1967; Hoegh-Guldberg and Pearse 1995). However, development at 'optimal' temperatures can accommodate broader environmental parameters. For example, mud crab larvae (*Scylla serrata*) withstand broader salinity conditions at optimal growth temperature (28°C) (Nurdiani and Zeng 2007). The higher survivorship observed at the lower temperatures likely occurred because that cold temperature better reflected oceanic conditions to which local species are exposed subtidally. These conditions also coincide with the temperature and salinity parameters required for the reproduction of kelp species of *Laminaria* (Druehl 1967), a food source of Strongylocentrotids. Distribution of adult *S. droebachiensis* coincides with the distribution of laminarian kelps (Scheibling and Hatcher 2007). Based on the low temperatures and high salinities required for sporophyte production by gametophytes of *Laminaria groenlandica* (Druehl 1967), we predict the distribution of the *S. droebachiensis* and *S. purpuratus* examined in the present study to coincide with the distribution of the kelp *L. groenlandica*, whereas the distribution of the lower-salinity tolerating *S. franciscanus* to correspond with more broadly distributed *L. saccharina* (Druehl 1967).

5.4.1 Adult habitat determines embryonic survival

The key determinant of environmental stress tolerance is the habitat of the adult (Stickle and Diehl 1987). For example, sea urchin (*Psammechinus miliaris*) embryos derived from subtidal adults develop to gastrulation at salinities of 30 and 32 ppt, but not at 28 ppt (Lindahl and Runnström 1929; reviewed by Stickle and Diehl 1987). In contrast, embryos derived from the same species collected from littoral and more euryhaline adults could gastrulate in salinities as low as 28.3 ppt (Lindahl and Runnström 1929; reviewed by Stickle and Diehl 1987). Similarly, larvae of several sublittoral species of White Sea invertebrates are less euryhaline than littoral-sublittoral species, and show similar patterns of thermotolerance (Saranchova et al. 2006). A similar adaptive pattern of thermotolerance is observed for embryos derived from adult sand dollars (*Dendraster excentricus*). Embryos have a much greater thermotolerance range when gametes are obtained from intertidal adults (Bingham et al. 1997). In contrast, embryos derived from subtidal adults not subjected to a range of temperatures have a greatly reduced thermotolerance range. The difference in thermotolerance between intertidal and subtidal *D. excentricus* is capacitated by the egg cytoplasm (Fujisawa 1993).

5.4.2 Acclimation or speciation?

Despite pre-conditioning of sympatric adults prior to spawning, the three species exhibited different salinity tolerance ranges. Differences in environmental tolerance among species generally correlate with the conditions under which adults are found (Stickle and Diehl 1987). However, short-term (2-4 weeks) acclimation of adult *S. droebachiensis* and *S. pallidus* to lower salinities does not increase larval survival in

hypotonic conditions (Roller and Stickle 1993, 1994). In fact, embryo mortality was higher for hypotonic pre-conditioned adults, likely due to gamete deterioration in the gonads from osmotic stress (Roller and Stickle 1994).

With overlapping reproductive seasons (Strathmann 1987), habitat and spawning events (Levitan 2002), and the ability to cross-fertilize (Strathmann 1987), did among-species variation in environmental tolerance evolve as a result of speciation, or preclude it? Likely among-species variation arose during a period of sea level fluctuation that has been correlated to the timing of strongylocentrotid cladogenesis (Biermann et al. 2003; Lee 2003). These same phylogenies place *S. droebachiensis* and *S. purpuratus* as closely related sister groups, further supporting the observation of their similar temperature and salinity tolerance ranges compared to the more distantly related *S. franciscanus*. However, acclimation to variable environmental conditions also occurs within species, as observed among sand dollars (Bingham et al. 1997).

5.4.3 Ecological implications

Survival of any organism is determined by its capacity to tolerate various abiotic factors (Verween et al. 2007). Differences in the tolerances of congeneric species could result in changes in species dominance in benthic habitats. For example, outbreaks of crown-of-thorns starfish (*Acanthaster planci*) correlate with abnormally high larval recruitment in years following major terrestrial runoff events such as typhoons (reviewed by Olson and Olson 1989). In 2002 an unusually high mass transport of warm Atlantic water introduced spat of blue mussels (*Mytilus edulis*) into the high Arctic, where they

had not existed for the previous millennium (Berge et al. 2005). Similarly, an outbreak of *S. droebachiensis* and consequent destruction of ecologically invaluable kelp beds along the North-Western Atlantic in the 1960's and 1970's may have resulted from an isolated temperature peak in 1960 producing a larger-than-average juvenile settlement cohort in 1960 (Hart 1988). Unfortunately as global climate change accelerates (Walther et al 2002), examples such as these will become more frequent and severe, thus characterization of among-species variation in environmental tolerance can help predict where catastrophic ecological imbalances might arise.

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Chapter VI

General Conclusions

6.1 Novel findings

Despite the longstanding status of sea urchin larvae as model organisms, studies of their development continue to yield surprises. For example, the discovery of larval cloning in echinoids (Eaves & Palmer 2003, Chapter II, Vaughn & Strathmann 2008) has radically altered our understanding of the sea urchin life cycle. The discovery that the gut may be massively remodeled depending on food supply (Chapter III) raises the intriguing possibility that the gut wall may also function as a storage organ. Both of these phenomena potentially confound estimates of larval dispersal ranges and developmental times because larvae may either a) reproduce asexually in supra-optimal food conditions, or b) sustain themselves through barren oceanic conditions previously regarded as barriers to dispersal (chapter III). Finally, the ability to withstand significant physical trauma apparently without consequence to the duration of development (chapter IV) is another unexpected finding. The developmental plasticity of these organisms provides them with substantially greater resilience in the face of heterogenous marine environments than was previously understood and has potentially significant implications for our understanding of larval development in other marine organisms.

6.2 Implications of larval cloning

Only recently has larval cloning in echinoderms received proper attention; it has now been documented in all extant classes with a protracted larval period (Eaves & Palmer 2003, Chapter II, Appendices 1, 2). Overall, larval clones can form from nearly

any part of the echinoderm larval body at nearly any time during development and most commonly detach as a small ectodermal bud containing several mesenchyme cells (Chapter II), whereas in some asteroid larvae an entirely new second larva can form prior to detachment (Vickery & McClintock 2000). Aside from possibly inhibiting swimming and feeding efficiency in the later example, no other costs to larval cloning are apparent to the primary larva other than a transient size reduction. However, cloning into smaller larvae (and eventually developing into smaller juveniles than non-cloning counterparts) in response to predator cues for the sand dollar *Dendraster excentricus* may be an advantage (Vaughn & Strathmann 2008). The same authors concluded that smaller size could be an adaptive feature rendering the larvae less vulnerable to predation by large predators. Cloning yields increased fecundity, increased dispersal potential, and increased chances of representatives from a genet finding suitable post-metamorphic habitat (Jaeckle 1994). Surprisingly, disadvantages to cloning are not obvious.

So why hasn't larval cloning been observed in other phyla whose larvae have an extended feeding developmental period? I strongly suspect this phenomenon is not limited to the echinoderms. Any larva with an elaborate, largely ectodermal feeding structure may also be capable of cloning, including: Müller's larvae of flatworm turbellarians, pilidium larvae of nemertean rhynchocoels, the trochophore larvae of some polycheates, the veliger larvae of some gastropods, the pelagosphera larvae of some sipunculids, the actinotroch larvae of phoronids, and the tornaria larvae of hemichordates. Examples should be sought in carefully collected plankton tows and robust larval cultures. Many more examples may yet be discovered.

6.3 Implications of gut reduction

Although phenotypic plasticity of larval feeding structures occurs in a variety of organisms including gastropods (Klinzing & Pechenik 2000), bivalves (Strathmann et al. 1993), and brittlestars (Podolsky & McAlister 2005), the majority of studies have been on echinoids (Boidron-Metairon 1988; Fenaux et al. 1988, 1994; Hart & Scheibling 1988; Strathmann et al. 1992; Hart and Strathmann 1994; Sewell et al. 2004; Miner 2005, 2007; Reitzel & Heyland 2007). However, few studies have examined the effects of extreme food limitation on the development of larvae that depend entirely on food sources and none of these studies considered the gut as a phenotypically plastic structure as well. Based on the observations in Chapter III, the larval gut is capable of undergoing a size reduction under food-limited stress, and this process is possibly mediated by the ingression of pigmented mesenchyme cells from the endoderm. This provides yet another dramatic example of the tremendous cellular re-specification capabilities of echinoid larvae and demonstrates how the developmental trajectory of morphogenesis is reversible: organs can shrink during development, and then re-grow.

Ecologically, gut reduction demonstrates how larvae might be capable of defying sub-optimal food conditions thought to be prevalent in the ocean environment (Thorson 1950) and capable of superceding estimates of planktonic dispersal potential based on developmental times determined in the laboratory (Paulay & Meyer 2006). Recently, the potential for veliger larvae of the gastropod *Fusitriton oregonesis* to remain as larvae for an inconceivable length of time (4.5 years) was reported (Strathmann &Strathmann 2007). Although in the wild such a protracted larval period seems unnatural, a 'growth-

stasis' has been previously reported for gastropod larvae (*Cymatium parthenopeum*) capable of trans-Atlantic dispersal (Pechenik et al. 1984).

Due to their transparent anatomy, echinoids have been an ideal candidate for examining phenotypic plasticity of larval feeding structures. However, a mechanism similar to that described here for echinoids (Chapter III) may also occur in larva with feeding structures capable of a phenotypically plastic response to available food conditions, such as gastropods, bivalves, brittlestars and likely others.

6.4 Implications of wound repair

Until recently investigations of larval predation consistently assume that all predator encounters are lethal. However, phenotypically plastic responses to predator cues have been reported in both a gastropod veliger (Vaughn 2007) and an echinoid pluteus (Vaughn & Strathmann 2008). For an adaptive response to have evolved, larvae must have a means of surviving predator attack. Gastropod veliger larvae both collected from plankton samples and reared in the laboratory are capable of repairing damaged shells following non-lethal attacks by predatory crustacean larvae (Hickman 2001). Although echinoid larvae are vulnerable to lethal predator attacks when they are small (Rumrill et al. 1985; Allen 2008) larger larvae may be less vulnerable to the same predators, resulting in sub-lethal attacks. By improvising a non-lethal attack on the vulnerable feeding arms, echinoid larvae revealed not only are they capable of repairing damaged structures using an array of mechanisms in a matter of several hours, but also that the timing of development to metamorphic competence did not appear to be affected

by injury (Chapter IV). The seemingly limitless regenerative potential of echinoid larvae likely enhances larval survival rates in the plankton (Vickery & McClintock 1998; Vickery et al. 2002). Due to the inherent necessity for planktotrophic larvae to feed I predict that all planktotrophic larvae are capable of regenerating damaged or lost feeding structures, including groups with an exoskeleton. Moreover, the capacity to regenerate missing larval structures is required for asexual reproduction to occur. Therefore I predict that any soft-bodied larva capable of extensive regeneration should also be capable of asexual reproduction.

6.5 Implications of heterospecific temperature and salinity tolerance

Abiotic factors such as temperature and salinity are the two most influential physical factors affecting early development of benthic marine invertebrates (Kinne 1964, Scheltema 1965). Furthermore, marine organisms are most sensitive to the physical conditions of their environment during embryogenesis (Stickle & Diehl 1987). The range of environmental conditions that are tolerated by developing organisms is determined by the conditions to which the adults are acclimated (Saranchova et al. 2006), particularly for echinoderms, which are stenohaline (Stickle & Diehl 1987). Therefore, by comparing the early environmental parameters of three congeneric species of echinoid from a sympatric population I was able to test whether differences in environmental tolerance exist among species. Although neither temperature nor salinity had a significant effect on fertilization rate, both factors had a significant effect on the rate of hatching and gastrulation in all three species examined. In general, colder temperatures accommodated development in a broader range of salinities, and the highest survival rates occurred in

salinities between 28 and 31 ppt for *S. droebachiensis* and *S. franciscanus*, and 28-34 ppt for *S. purpuratus*. Sub-optimal salinity induced consistent morphological abnormalities in each species: embryogenesis was disrupted in low salinities and gastrulation was disrupted in high salinity. Overall, *S. franciscanus* tolerated the broadest range of salinities at all temperatures. Therefore, *S. franciscanus* appears to have the potential to develop in a broader range of habitats than either *S. droebachiensis* or *S. purpuratus*.

Does among-species variation in environmental tolerance evolve as a result of speciation, or precede it? The rapid diversification of this group occurred only recently, approximately 3 million years ago (Biermann et al. 2003), and has been correlated to a period of intense sea level fluctuation (Biermann et al. 2003, Lee 2003). Changes in the physical environment can affect entire populations simultaneously, whereas mutation initially affects only a single individual (West-Eberhard 2005). The capacity of adults to adapt to new environmental conditions is essential for the survival of a species when conditions change (Visser 2008). However, among-species variation in environmental tolerance is more likely to produce an ecological shift in species diversity and abundance in response to climate change.

6.6 Consequences of underestimating development potential

At the outset of this study, a popular – but controversial – theory persisted in the field of developmental biology: the set-aside-cell-theory (Petersen et al. 1997; Davidson et al. 1998). Among other things, this theory postulated that the largely ectodermally derived larval body was only a vector for cultivation of a distinct population of pluripotent 'set-aside' cells uniquely capable of giving rise to the juvenile body, a process

dubbed 'maximally indirect development' (Davidson et al. 1995). The set-aside cell theory further described cells of the larval body as being "essentially eutelic", capable of a limited number of cellular divisions and incapable of differentiating into mesoderm or other juvenile structures (Davidson et al. 1998). The foremost example of a maximally indirect developing set aside cell organism was the echinoid larva, specifically that of *Strongylocentrotus purpuratus* (Petersen et al. 1997; Davidson et al. 1998). Eventually this theory was irrefutably disproved by observations of larval clone formation from ectodermal buds that could differentiate a juvenile rudiment (Eaves & Palmer 2003).

However, the set-aside-cell theory highlighted an unsettling observation about science. Prior to the discovery of larval cloning in echinoids, it had previously been observed in ophiuroids (Mortensen 1921; Balser 1998) and asteroids (Bosch et al. 1989; Bosch 1992; Rao et al. 1993; Jaeckle 1994; Vickery & McClintock 2000). Yet despite these well-documented examples, the set-aside-cell theory was staunchly promoted and widely accepted in the field of evolution and development. Soon after this theory was proposed, the dramatic regenerative potential of echinoderm larvae following surgical bisection into anterior and posterior halves was reported (Vickery & McClintock 1998; Vickery et al. 2002), further demonstrating a) the vast regenerative potential of larval ectoderm, and b) that cells of the larval body that Davidson and others (1995) had described as "essentially eutelic", instead retain almost unlimited developmental potential after specification into larval ectoderm.

In hindsight it seems obvious: echinoid larvae are clearly capable of spectacular feats of regeneration and cellular re-specification. So, why weren't larval cloning, gut reduction, and wound repair recognized as significant before this study? A classic example of the scientific community consciously dismissing unexpected observations began early in the twentieth century when Mortensen (1921) reported asexual reproduction in ophiuroid larvae. His remarkable observations were dismissed by prominent larval biologists as being "in urgent need of confirmation...totally opposed to what we know of the normal development of ophiuroids" (MacBride 1921 p. 147-149) and simply "audacious" (Bather 1921). Three-quarters of a century later Mortensen's observations of larval cloning in ophiuroids were confirmed by Balser (1998), long after earlier skeptics had passed on. These examples highlight how crucial it is for scientists to be more receptive to what they are looking *at*, instead of what they are looking *for* in their organisms.

6.7 The 'true' potential of larval secondary mesenchyme cells

A common theme among cloning, gut reduction and arm repair is the seemingly limitless potential of secondary mesenchyme derived cells of the larval body to de-differentiate, or regain the ability to differentiate into new cell types and proliferate (such as stem cells). A lineage of stem cells has never been characterized in sea urchin larvae. However, the association of secondary mesenchyme derived cells during major morphological remodeling events such as gastrulation, innate immunity, wound repair, gut reduction and cloning (reviewed in Chapters II, III and IV) suggests these cells retain a pluripotent-like developmental potential throughout larval development and could in

fact be conditionally specified stem cells. Molecular evidence suggests the germ line of *S. purpuratus* is conditionally specified (Juliano et al. 2006), so the hypothesis of conditionally specified stem cells is certainly feasible.

6.8 Larvae as modules for evolutionary change

The ability of echinoid larvae to alter development in a variety of ways in response to different environmental conditions suggests ways that large-scale evolutionary changes might have occurred so readily in this group. For example, acclimation to changing environmental conditions during early development possibly catalyzed the rapid speciation of the genus *Strongylocentrotus* following periods of transient allopatry during glacial events (Biermann et al. 2003; Lee 2003; chapter V). In addition, the ability to re-absorb internal larval structures possibly pre-disposed planktotrophic echinoids towards lecithotrophic development (Strathmann 1992; chapter III). Moreover the remarkable regenerative abilities and conserved developmental potential of echinoid larvae (Eaves & Palmer 2003, chapters II, III, IV) could reflect a conserved genetic program similar to asexual budding in colonial tunicates, and to regeneration and stem cell renewal across the deuterostomes (Swalla 2006). The capacity of the larval body to consistently alter phenotype in response to environmental pressure is not only an adaptive plasticity, but may reflect an example of how much more prevalent genetic assimilation — phenotypic change preceding genotypic change— may actually be (Palmer 2004).

Survival depends on an organism's ability to adapt to whatever conditions it encounters. Given that planktotrophic development has persisted for over half a billion years, echinoid larvae can most assuredly contend with environmental hazards in the pelagic marine environment. This study has demonstrated some of the remarkable mechanisms that facilitate larval survival under varying environmental conditions. Furthermore, this study has re-emphasized the importance of investigating the whole organism, otherwise the underlying molecular mechanisms may not be understood in the proper context. Especially for echinoids, where the complete genome has been sequenced, a prime opportunity now exists to examine molecular mechanisms underlying environmental influence on the expression of phenotype. The resilience of larvae to environmental forces is truly remarkable and we must not underestimate this capacity.

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Appendix A-1.1

Widespread cloning in echinoderm larvae[A-1]

Asexual reproduction by free-living invertebrate larvae is a rare and enigmatic phenomenon and, although it is known to occur in sea stars (Bosh et al. 1989; Jaeckle 1994; Vickery & McClintock 2000; Knott et al. 2003) and brittle stars (Balser 1998; Mortensen 1921), it has not been detected in other echinoderms despite more than a century of intensive study (Mortensen 1921, p. 147-149; MacBride 1921). Here we describe spontaneous larval cloning in three species from two more echinoderm classes: a sea cucumber (Holothuroidea), a sand dollar and a sea urchin (Echinoidea). Larval cloning may therefore be an ancient ability of echinoderms and possibly deuterostomes — the group that includes echinoderms, acorn worms, sea squirts and vertebrates.

To confirm that genuine cloning was occurring, we reared cloning larvae and separated clones individually (Fig. A-1, legend). Although most sea cucumber larvae metamorphosed normally after a month, 5 out of 41 (12.2%) free-swimming doliolaria larvae were half the length of their siblings and had a constriction around the penultimate ciliary band. These buds retained a ciliary band and were still attached by a thin tether into the benthic pentacula stage 12 h later (Fig. A-1A). Eventually, after separation, buds developed into normal auricularia larvae with a juvenile rudiment (Fig. A-1B).

[A-1] A version of this section has been published. Eaves AA & Palmer AR 2003. Nature. 425: 146.

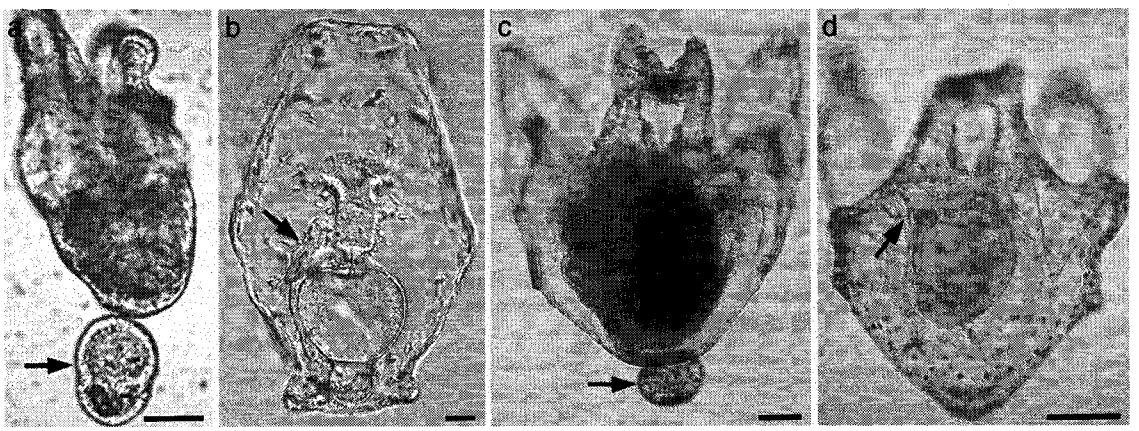

Figure A-1. Asexual budding by echinoderm larvae. A. Sea cucumber (*Parastichopus californicus*; Holothuroidea; Aspidochirotida) early pentacula with clone attached at posterior end (arrowhead). B. Sea cucumber 18 days after separation as a complete auricularia larva, with juvenile rudiment (arrow). C. Sea urchin (*Strongylocentrotus purpuratus*; Echinoidea Echinacea) larva with a clone (arrow) constricting from the posterior end around the robust ciliary band. D. Sea urchin clone three weeks after separation, with early juvenile rudiment (arrow). Scale bars, 50 μm. Adult *P. californicus* were collected sub-tidally from San Juan Island, Washington, and gametes were collected by dissection. Larvae were maintained at 11°C under natural lighting in filtered sea water and were fed a red alga *Rhodomonas salina, ad libitum.* Adult *S. purpuratus* from Prasiola Point (Vancouver Island, Canada) were spawned by intracoelomic injection of 0.55 M potassium chloride. Larvae were maintained in a 19-h light cycle at 14 °C in pasteurized seawater and were fed > 10^6 cells per ml of a golden brown alga *Isochrysis galbana* and *R. salina*; food and water were replaced every 72 h. To monitor their development, cloning larvae and separated clones were isolated in 7-ml glass vials and cultured; most developed to metamorphosis. Cultures were deemed to be healthy as no larval tissue necrosis was seen. Further details are available from the authors.

Most sand dollar metamorphosed within 7 weeks, but 6 of 170 (3.5%) formed a hollow bud near the future mouth (results not shown). Once separated, the evenly ciliated buds developed into an almost solid gastrula with bilateral spicules. Within two days, a tripartite gut and further skeletal spicules formed, and the clones began to feed.

After 4 weeks, nearly 5% of sea urchin larvae (n>500) showed constrictions at their posterior end (Fig. A-1C). These eventually yielded uniformly ciliated buds that began feeding within four days, developed paired skeletal rods within a week, and acquired a normal larval form within two weeks. Juvenile rudiments began to form within three weeks (Fig. A-1D), accompanied by further larval arms.

Larval cloning is therefore known to occur in all classes except crinoids (feather stars and sea lilies), supporting an earlier conjecture that it might be an ancestral ability of echinoderms (Lacalli 2000). The mechanisms by which this cloning occurs, however, are unexpectedly diverse. First, clones may arise from various larval body regions, including arms ((sea stars (Bosh et al. 1989; Jaeckle 1994; Vickery & McClintock 2000; Knott et al. 2003)1-4, brittle stars (Balser 1998)), the oral hood ((sea stars (Jaeckle 1994; Vickery & McClintock 2000; Knott et al. 2003)), the posterior end ((sea stars (Vickery & McClintock 2000), sea cucumbers (Fig. A-1A), sea urchins (Fig. A-1C)) and the lateral body wall ((sea stars (Jaeckle 1994), sand dollars (our results, not shown)). Second, the developmental stage of clones at separation ranges from blastulae 2 to fully formed larvae 3. Third, some clones may not separate until after the primary larva has begun to metamorphose 5 (Fig. A-1A). This indicates either that larval cloning evolved

independently on several occasions or that its mechanisms have diverged widely from an ancestral mode.

Cloning can be surprisingly frequent: up to 12% in laboratory-reared sea cucumber larvae and 10-90% in samples of field-collected sea star larvae (Bosh et al. 1989; Knott et al. 2003). The fact that such a common phenomenon should have been overlooked in heavily studied organisms seems remarkable. Were preconceptions about 'normal' development so strong that cloning was simply dismissed as aberrant? From a theoretical perspective, larval cloning — particularly in sea urchins— challenges a central tenet of the 'set-aside' cell theory (Davidson et al.1998) because, contrary to prediction, larval body cells are not "essentially eutelic" (Davidson et al.1998), but can differentiate into juvenile structures (Fig. A-1B, D). Nevertheless, such cloning offers an exciting opportunity to study the well-characterized developmental regulatory networks of sea urchins (Davidson et al. 2002) in a new ontogenetic context.

Larval cloning represents an intriguing new dimension to invertebrate life histories. The process confers three potential ecological advantages: increased fecundity under optimal growth conditions (Jaeckle 1994; Vickery & McClintock 2000), increased chances of settlement after a protracted larval life (Bosch et al. 1989; Jaeckle 1994; Balser 1998), and recycling of otherwise discarded or reabsorbed larval tissue (Balser 1998). Larval cloning may also be evolutionarily significant, for two reasons. First, clones may subsequently clone (Balser 1998), potentially leading to a new, entirely pelagic bauplan. Second, although it may be merely another idiosyncrasy of echinoderm

development, larval cloning might also be more taxonomically widespread. As nearest relatives to the echinoderms (Cameron et al. 2000), acorn worms offer a critical test. If their tornaria larvae clone, then ancient deuterostomes may have had this ability.

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Appendix A-2

Strongylocentrotus purpuratus clone vs. embryo morphology

At the time of separation a clone is approximately 115 μm in diameter and is composed of a transparent, single-cell thick ectoderm with an average of 150 pigment cells. Inside the blastocoel are several transparent mesenchyme cells with no consistent pattern or arrangement among different clones (Fig. A-2A, B). In contrast, a newly hatched, mesenchyme-cell staged blastula also has a transparent, single cell thick ectoderm as well as a slight flattening at the location of vegetal plate formation, and several transparent mesenchyme cells in the vegetal half of the blastocoel. Most notably however, is a complete lack of pigment cells in the early embryo.

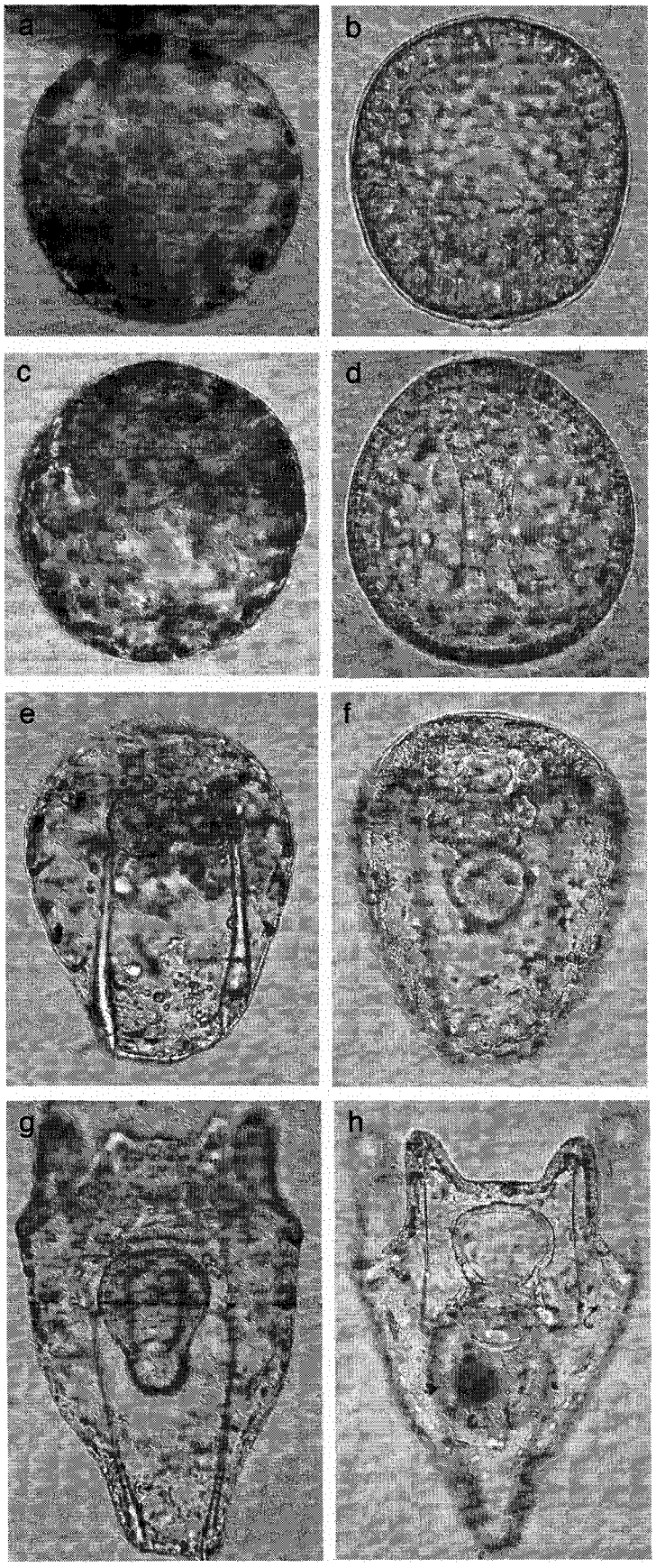
Within a few days of separating, clones have formed a digestive tract by some unknown morphogenetic process (see chapter II). The gut is disproportionately larger than the esophagus and numerous green coloured pigment cells are aggregated in the blastocoel (Fig. A-2C, D). The origin of these green cells is unknown, however they are absolutely not present in clones when they first separate. In contrast, in a normal gastrula an archenteron forms connecting the vegetal half of the embryo to the animal one. The rudimentary gut is tubular, not associated with green mesenchyme cells of any sort within the blastocoel, and pigment cells are only starting to differentiate around the ectoderm.

Approximately one week after separation clones have distinct dorsal-ventral and bilateral axes (Fig. A-2E, F). The gut no longer appears disproportionately large inside the blastocoel, and the apical ciliary band has differentiated from the former attachment

site. There are still several green mesenchyme cells within the blastocoel, as well as transparent ones. Also, a bilateral set of skeletal rods have formed. In contrast, in a normal late prism/ early larva the apical ciliary band and oral field are much larger. Pigment cells are more numerous and the bilateral skeletal rods are less robust than those of clones. Additionally, the gut is not yet stained with lipid from consuming algae, as are the guts clones.

Two weeks after separating clones are largely indistinguishable from regular larvae (Fig. A-2G, H). Except for the slightly-reduced anterior feeding structures (the dorsal hood and mouth are proportionately smaller), the larval clones are almost entirely symmetric and are otherwise identical to normal embryogenesis-derived larvae. However, the larval clones retain two clusters of green mesenchyme cells, one on either side of the gut. The coelomic sacs in the corresponding location in regular larvae are transparent and are derived from outpockets from the archenteron.

Figure A-2. *Strongylocentrotus purpuratus* clone vs. embryo morphology. A) Clone minutes pre-detachement. B) Blastula 1 d post fertilization. C) Clone 4 d post-separation, D) Gastrula 2 d post-fertilization. E) Clone approximately 1 week post-detachment. F) Early larva 4 d post fertilization. G) Clone approximately 2 weeks after detachment, H) Larva 10 d post-fertilization. Approximate sizes indicated in text, otherwise see figure 2-2 for clone sizes.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