SEA URCHIN HYPOTHESES: A STUDY OF PLASTICITY AND HOMOLOGY

TESTING EVOLUTIONARY DEVELOPMENTAL HYPOTHESES WITH SEA URCHINS: A STUDY OF PLASTICITY AND HOMOLOGY

By

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Abstract

Sea urchins traditionally have been considered as model organisms for developmental studies, as they transform from bilaterally symmetric larvae to pentaradially symmetric adults. They are classified universally as members in the phylum Echinodermata, but skeletal homologies between the class in which sea urchins are contained and other echinoderm classes remain contested. And, culturally, the high demand for sea urchin sushi, a delicacy known as uni, has spiked interest in sea urchin farming and how to capitalize on making a commercially more-desirable food product for human consumption.

In this thesis, experiments were conducted to test evolutionary developmental hypotheses about sea urchin life history plasticity, skeleton homologies, and reproductive energetics. I found that sea urchin rudiments can be resorbed, exhibiting extreme plasticity and, thereby, functioning as capacitors for ensuring metamorphose in favourable conditions; sea urchin primary podia may be considered as nonhomologous with sea cucumber ambulacral podia, in accordance with the extra-axial theory; and gravid sea urchins fed a carrot-only diet produced gonads that were more desirable commercially than were gonads produced by sea urchins fed a seaweed and carrot diet.

Acknowledgments

I began this study with a keen interest in expanding on my undergraduate thesis project. Over the course of my Master's candidacy, I have been able further investigate the commercial value of sea urchin roe in addition to gaining a breadth knowledge of larval morphology, plasticity and homology. With this thesis, I am pleased to contribute to the growing body of literature on sea urchins.

I would like to thank Julie Lederer, Priyanka Kapoor and Amir Khoshdel for their assistance and cooperation throughout my research. Your daily support allowed me to stay on track and reach my goal.

To my lab ladies, Maria Abou Chakra and Carmen Cheung, thank you for providing me with resource material and moral support.

I would also like to thank, Erin Leonard, Erin O'Leary, Christina Pierre, Sarah Riekotter and Theresa Wright for believing in me and being a constant source of optimism. And to my family, your endless love, and encouragement has allowed me to successfully complete this chapter in my life.

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Finally, I devote an immense amount of gratitude to my supervisor, Jonathon Stone. I greatly appreciate the opportunity you have provided me with, which has enabled me to expand my research skills on a topic that has become a fond familiarity. Your continuous guidance, patience and generosity of time knows no bounds. Thank you for your continuous support throughout this endeavour.

Table of Contents

List of Figures

Introduction

Chapter 1

Chapter 2

Chapter 3

List of Figures

Chapter 3 - Continued

List of Tables

Chapter 3

Introduction

Sea urchins are remarkable marine organisms for testing evolutionary developmental hypotheses. They manifest as numerous species, live for decades, easily are obtained and manipulated to rear larvae in laboratories and undergo fascinating life histories. A typical sea urchin life history may be divided into two main developmental stages, the larva followed by the adult (the larva first transitions to a juvenile and becomes an adult upon sexual maturation) (Figures 1 & 1.1).

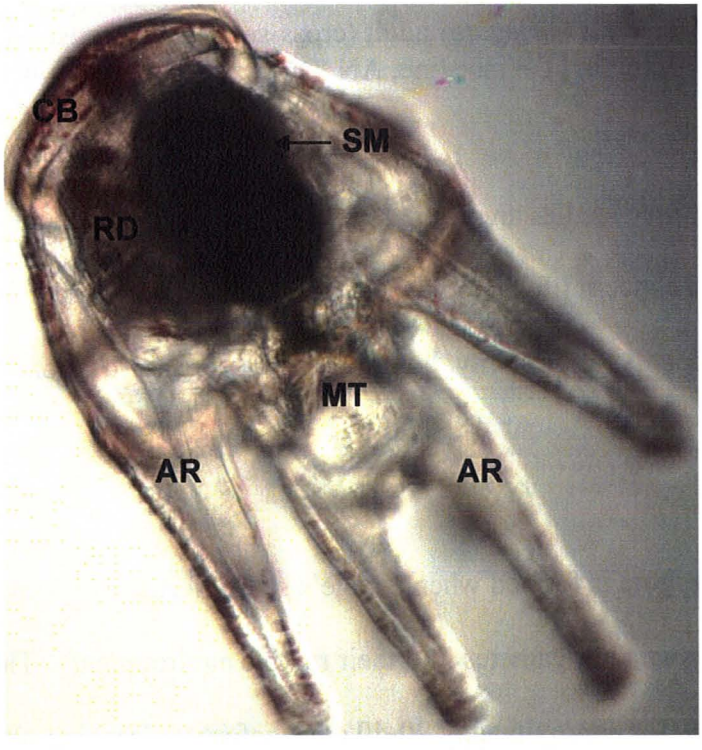

Figure 1 *Strongylocentrotus purpuratus* larva (at the eight-arm stage; CB – cilliary band, AR – arm, SM – stomach, RD – rudiment, MT – mouth).

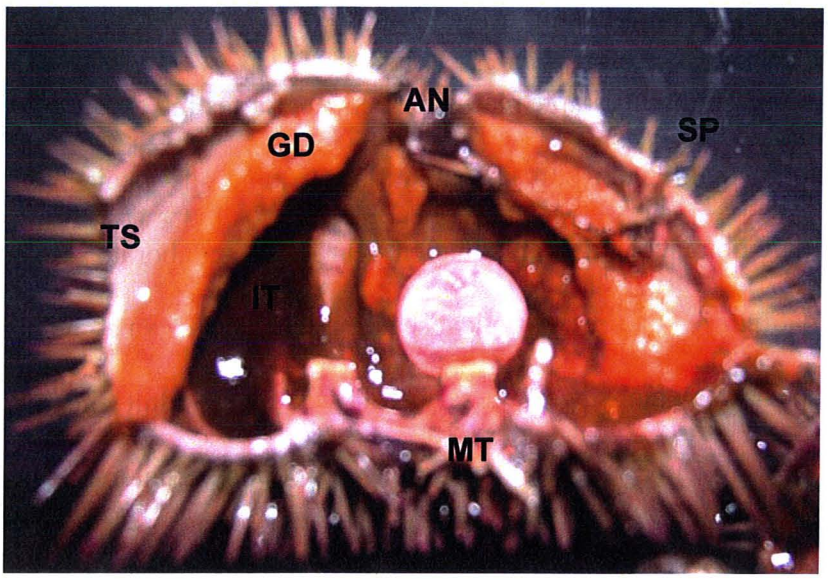

Figure 1.1 *Lytechinus variegatus* adult (cross-section; TS – test, SP – spines, GD – gonad, MT – mouth, IT – intestine, AN – anus; copper penny included for scale).

In this thesis, three studies are presented (two involving larvae and one involving adults), which concern life history, skeletogenesis and reproduction and encompass phenotypic plasticity, homology and energy allocation.

Chapter 1

A sea urchin zygote is formed when a male's sperm fertilizes a female's egg (both sexes spawn their gametes into their marine environment). The embryo and then larva grow for approximately 20-40 days before undergoing a process known as metamorphosis (Sewell et al., 2004). Metamorphosis is a dramatic event in which the larva ceases to exist and the adult starts to live an independent existence (Hart and Strathmann, 1994). The adult first develops as a structure within the larva, known as the rudiment. The rudiment appears as a dark mass on the left

side in the larval body and contains the prospective structures for the newly formed juvenile, the primary podia, test (shell or skeleton), spines and mouth (Hinegardner, 1969).

Authors commonly state that a larva undergoes metamorphosis only if the rudiment is formed completely and conditions are 'appropriate' (Hart and Strathmann, 1994). Appropriate conditions include a substrate for the juvenile to attach to and proper nutrition. I demonstrate that, if all food sources are removed, larvae delay metamorphosis by resorbing their rudiment. This response can be considered as a compensatory mechanism, providing larvae time to find suitable conditions or wait until favourable condition return before progressing to the next developmental stage.

Chapter 2

Sea cucumbers and sea urchins are classified in the Phylum Echinodermata. They are grouped together in a clade as members in sister taxa, on the basis of shared derived traits and, on the basis of more-specific shared derived traits, separately in the classes Holothuroidea and Echinoidea, respectively (Wray et al., 1991).

The extra-axial/axial theory (EAT) was formulated on the basis of the ocular plate rule (OPR) to explain skeleton growth among echinoderm classes. The EAT stipulates that skeletons among echinoderms comprise axial elements and extraxial elements (Mooi and David, 1997). Axial elements are grown

according to the OPR, which stipulates that those elements are added in 5 contiguous growth zones (Mooi and David, 1997).

Sea cucumber bodies are predominantly extraxial in origin, whereas sea urchin primary podia are axial in origin, however both comprise skeletal cells (Mooi and David, 1997). This constitutes the basis for my interest and the homology study between the two classes. Using the antibody 6a3, which is a marker for Meso1, a skeletogenic protein, I consider the EAT at the cellular level and find that hypotheses derived in accordance with it remain unfalsified (contrary to a pilot project conducted by J. Stone).

Chapter 3

Sea urchin gonads are consumed as a sushi delicacy known as uni. Adult sea urchins contain five gonads, which derive from their pentaradial symmetry. Sea urchin gonads play a dual role, as they are used for nutrition storage as well as reproduction (Russell, 1998). Gonads are assessed using five characteristics, which include: size, colour, smell, texture and firmness (Agatsuma et al., 2005; Senaratna et al., 2005). The characteristics for a gonad harvest depend on the reproductive cycle and diet at the time that sea urchins are collected (Garrido et al., 2001).

The species *Strongylocentrotus purpuratus* is known commonly as the purple sea urchin. Purple sea urchin populations around North America achieve maximum gonad indices in February or March (Garrido et al., 2001). These

gravid sea urchins invest predominantly in gamete production, creating large, firm gonads. Non gravid sea urchins, however, invest energy in gonad maintenance and test growth. Previous studies have demonstrated that a β-carotene-rich diet increased the orange pigment in gonads (Robinson, 2002), whereas a microalgal diet increased gonad yields (Pearce, 2002). I test the hypothesis that fed gravid sea urchins produce the most-desirable gonad product (increased gonad yield and darker yellow/orange colour), as energy invested in growth will be directed primarily at gonads rather than tests. It was found that a carrot diet improved the desired orange gonad colour in both gravid and non gravid species where as a combined seaweed + carrot diet had varying results on gonad yield in each species.

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Preface

The following study was completed in collaboration with P. Kapoor and J. Stone. I independently established the experimental setup. P. Kapoor assisted with daily development documentation through image capture. This chapter has been prepared as a manuscript for The Biological Bulletin.

Rudiment Resorption in *Strongylocentrotus purpuratus*

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Abstract

Environmental factors, including nutrient availability, greatly affect embryological development for many organisms. Environmental fluctuations have been found to affect arm rod length, internal tissue development and ciliated band formation via resource reallocation within developing purple sea urchins (*Strongylocentrotus purpuratus*). Larvae grow for approximately 20-40 days before undergoing a dramatic metamorphosis. Juveniles erupt to live as independent individuals while larval tissues degrade and larvae cease to exist. Each juvenile initiates development as a structure, known as the rudiment, within its larva. The rudiment appears as a dark mass on the left side in the larval body and contains the prospective primary podia, test, spines and mouth. A larvae metamorphoses if the rudiment is fully formed and conditions are appropriate. Nutrient availability constitutes a major factor in promoting and regulating metamorphosis. We show that larvae containing early rudiment tissue that are subjected to starvation can use the rudiment as capacitor for maintenance and buffer for metamorphosis. Sea urchins may utilise this plasticity to adapt to dynamic environments and delay development until environmental conditions are favourable, potentially enhancing fitness.

Introduction

Sea urchin larvae constitute useful models for phenotypic plasticity studies. Phenotypic plasticity may be defined as the ability for an organism to adopt different phenotypes in response to different environments (Miner, 2003). Numerous nutrition-ration experiments have been conducted to show the relationship between altered, atypical environments and resulting non rudiment, morphological larval changes (Fenaux et al., 1988; McEdward, 1999; George S.B. et al., 2004; Sewell. et al., 2004). Echinoid larvae exhibit phenotypic plasticity in response to different food levels in their environments (Hart and Strathmann, 1994). In this study, we document the plastic nature for sea urchin rudiments in different environments.

An embryological sea urchin usually develops as two entities, a feeding larvae and a rudiment (Hart and Strathmann, 1994). These entities are considered essentially as being functionally and developmentally independent until metamorphosis starts (Hart and Strathmann, 1994). The rudiment then becomes independently active (Hart and Strathmann, 1994). Throughout, the sea urchin encounters fluctuating environmental conditions, in which functional requirements may effect ontogenetic changes; a larva responds with compensatory modifications to its morphology (McEdward, 1999).

Researchers have shown that larval lifespan can be increased by underfeeding (Hinegardner, 1969). Underfeeding results in slow development and, consequently, delays metamorphic competence and metamorphosis

(Hinegardner, 1969). These morphological changes may occur with minimal exposure, as larvae respond phenotypically to short-term variations in their environments (Padilla, 2006). Such phenotypic plasticity can alter development, life histories and behaviours in an evolutionary sense, when changes persist over long time periods, especially if the modifications are adaptive (Hart and Strathmann, 1994).

Phenotypic plasticity early in echinoid development may depend on egg size; the capability for plasticity later in development depends primarily on environmental factors, such as energy, which may be assimilated from nutrition sources (Hart and Strathmann, 1994). Rudiment formation relative to larval body formation is an energetically highly demanding process (McEdward, 1999). We investigate herein alterations in allocation from a nutritient source (algae) to larval body structures, especially the rudiment.

Whereas other researchers have shown that food rationing affects sea urchin larvae and their rudiments (Hinegardner, 1969; Vaïtilingon et al., 2001; George et al., 2004; Sewell et al., 2004), no documented data exist on rudiment resorption. In this study, we test if rudiments in *Strongylocentrotus purpuratus* larvae can act as phenotypically plastic traits in unfavourable environments.

Materials And Methods

Fertilization and Larval Rearing

Adult *Strongylocentrotus purpuratus* sea urchins were obtained from WestWind Sealab Supplies (Victoria, British Columbia). Upon arrival, specimens were injected with approximately 5 mL 0.5 M KCl in the soft tissue around the peristome, causing the gonads to contract and release gametes. Injected adults were placed aboral surface downward in 100 mL beakers containing artificial seawater (Instant Ocean, Cincinnati, Ohio). Sperm and eggs were collected separately. Eggs were fertilized with sperm in 500 mL beakers. Fertilized eggs were transferred to Pyrex bowls filled with artificial seawater, forming monolayers, and left overnight. Larval embryos that were floating 12 hours later were collected and placed in 1.8 L FÖRVAR glass jars (IKEA) containing artificial seawater, for rearing. To simulate an ocean current, jars were placed on magnetic stirrers and water was agitated with magnetic stir bars. All jars were placed in a cold room maintained at 10 °C.

Larval Maintenance

Larvae were fed 14 mL from a solution containing *Dunaliella tertiolecta* + 1% Reef Crew algal growth medium that was centrifuged at 4500 rpm for 1 minute then resuspended in artifical seawater, every other day. To prevent algae stocks from crashing, flasks housing *D. tertiolecta* were swirled 50 times daily and transferred to new flasks every other day. Water salinity for each jar was monitored and adjusted by adding distilled water to maintain specific gravity equal to 1.020-1.023. Jars were cleaned using glass pipettes to remove debris,

when necessary. Images depicting developing larvae were captured daily using Openlab Improvison software on a Leica DM5000B digital microscope.

Starvation Experiment

Larvae at the 8 arm stage with early rudimentary tissue and visible tube feet were collected and stored in large Pyrex bowls containing artificial seawater. Larva then were isolated in 12-well tissue culture plates (BD Falcon), one larva per well. On day 1in the experiment, larvae were assigned to fed and unfed groups. Images were captured on a daily basis, to record any changes in rudiment size and tube feet formation. Specimens in the fed group were administered 3 mL of pure *D. tertiolecta* daily, while the specimens in the unfed group were administered 3 mL artificial sea water. Approximately 0.25 mL distilled water were added to each well (fed and unfed), to maintain salinity and compensate for evaporation. Evaporation was minimized by covering wells with a plate lid.

Results

Typical Larval Fate

Fed larvae realized a typical larval fate, reaching metamorphosis by day 25 (Fig. 1).

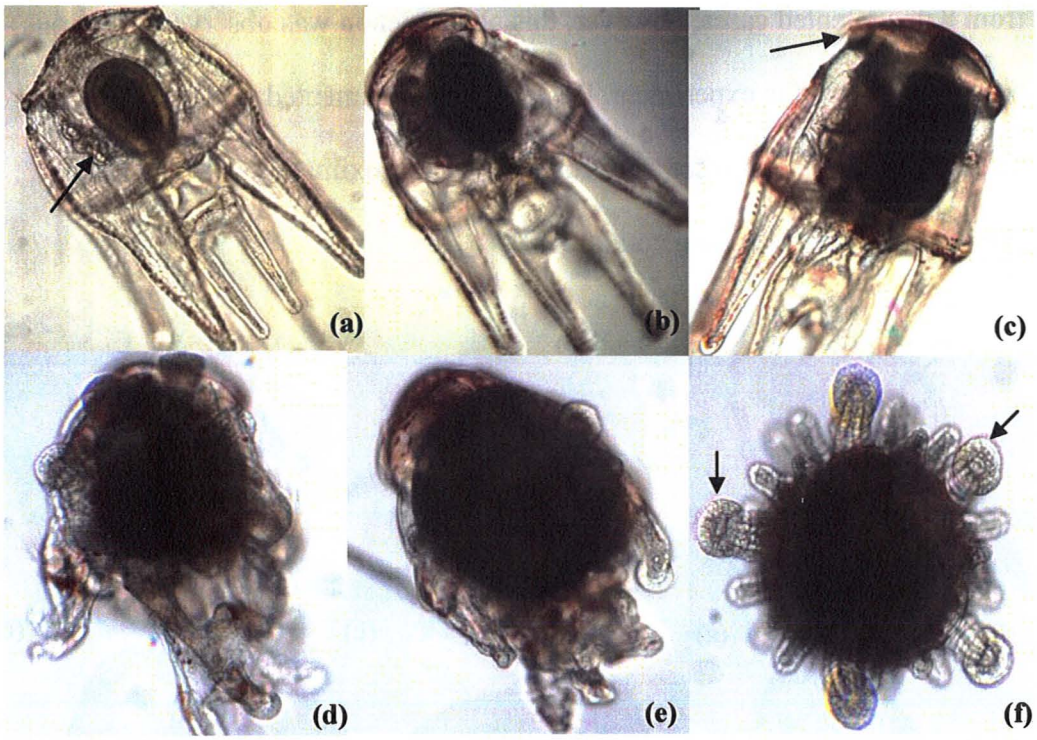

Figure 1. Typical fate for *S. purpuratus* larvae in the fed group. Images (a)-(c) were captured from an anterior view, (d)-(f) from a lateral view and (f) from an anal view. (a) Day 1 – larva with early rudiment tissue and tube feet (indicated with arrow). (b) Day 14 – larva with well developed rudiment; the increased pigment in the gut is due to algae. (c) Day 19 –rudiment increased in size and ciliary band becoming visible (indicated with arrow). (d) Day 23 – competent larva starting metamorphosis. (e) Day 24 – larva progressing through metamorphosis. (f) Day 25 – newly metamorphosed juvenile with visible primary podia (indicated with arrows).

Rudiment Resorption

Unfed larvae realized an alternative fate, involving rudiment resorption; rudiment ingression and disappearance was evident by day 46, and, ultimately, specimens started to revert to a blastula-like, spherical shape. Similar results were obtained from 8 documented cases; however, this phenomenon was observed numerous times throughout the experiment. Based on the documented results, we suggest that adaptive rudiment resorption could occur approximately 25% the time in completely nutrient-lacking conditions.

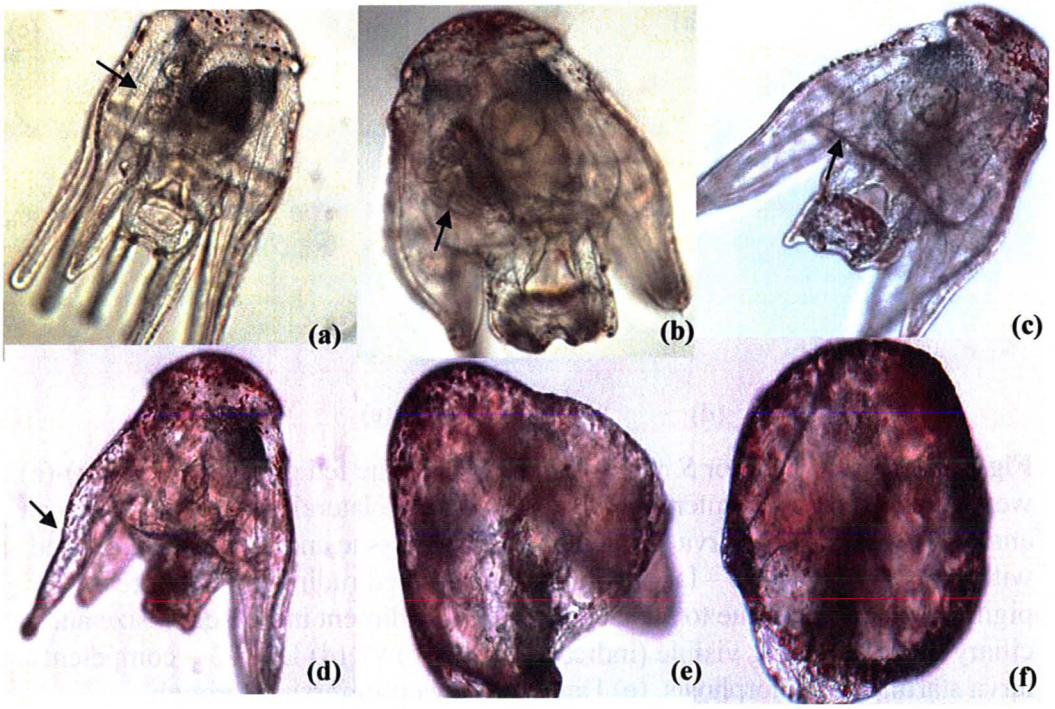

Figure 2. Alternative fate for *S. purpuratus* larvae in the starved group, all images were captured from an anterior view. (a) Day 1 – larva with well developed rudiment and tube feet (indicated with arrow). (b) Day 17 – larva with very little pigment in the gut, due to lacking algae, and rudiment tissue increased in diameter (indicated with arrow). (c) Day 36 – invagination where rudimentary tissue and tube feet once were (indicated with arrow). (d) Day 43 – invagination and rudimentary tissue completely resorbed; skeletal rods and arms still present. (e) Day 56 –skeletal rods diminished and arms regressed. (f) Day 70 – larva reverted to spherical shape, similar to a blastula.

Discussion

Rudiments in *S. purpuratus* larvae are phenotypically plastic structures. This observation is consistent with those made in previous studies that have revealed phenotypic plasticity in other structures in sea urchin larvae and adults. In *Evechinus chloroticus*, growth in larvae fed small algal rations or no algae was stalled at the 4-arm stage and larvae were much smaller in size when compared to larvae in a large algal ration treatment group (Sewell, 2004). Deficient nutrients after initial larval formation can result in developmental arrest in size and structures such as arms (Herrera et al., 1996; Sewell et al., 2004). In environments with low nutrient levels, sea urchins increase their arm lengths, facilitating food collection, and ultimately delay rudiment formation (Fenaux et al., 1988; Hart et al., 1988; Strathmann et al., 1992; Hart and Strathmann, 1994; Shilling, 1995; Mieidel et al., 1999). Such phenotypic plasticity is adaptive because the morphological changes enhance survival (Strathmann et al., 1992).

The observations that are documented herein also may be considered as adaptive in that rudiment morphology changed to accommodate nutrient-lacking conditions (Fig. 2), a plastic response relative to the typical fate exhibited in control, nutrient-rich conditions (Fig. 1). Plasticity might prolong survival until adequate (nutrition-rich) conditions present for the larvae to metamorphose, enhancing fitness. By isolating individual larvae and averting competition, algal pigment accumulated in stomachs (Fig. 1). Additionally, remarkable structures, such as the ciliary band, provide evidence for near competency. By day 24, fed

larvae almost had completed metamorphosed, and, by day 25, juveniles with hallmark tube feet were produced. Our observations are consistent with previous studies, showing that larvae provided with large food rations achieve competence relatively quickly (at 13-14 days post-rudiment-formation) while larva with small food rations achieve competence relatively slowly (at days 40-41) (Hart and Strathmann 1994).

In our study, however, larvae were starved, and these larvae staved-off rather than delayed metamorphosis. Rudiments continued to grow until day 17, then started shrinking. This finding is consistent with results that were obtained in a previous study, in which unfed competent larvae showed an initial increase in rudiment size and, afterwards, failed to further develop and increase in size (Vaïtilingon et al., 2001). Those larvae retained their metamorphic capabilities and eventually underwent metamorphosis; however, they died shortly after. In another study, researchers showed that sand dollar larvae provided with small food rations eventually reached competence and metamorphosed (Hart and Strathmann 1994).

In the present study, starved larvae showed complete regression. The discrepancy among results might be explained by supposing that starvation imposes a 'window of opportunity' for rudiment resorption (*i.e.*, that regression can take place only within a particular time frame during larval development). As metamorphosis is an energetically demanding process, larvae likely would conserve stored nutrients until conditions became favourable. However,

competent larvae still progress toward metamorphosis, even if they encounter poor nutrition environments; they just progress at a slower rate (Hart and Strathmann 1994). Additionally, larvae in the starved group had formed only early rudiment tissue and lacked particular anatomical features and behaviours (such as protrusion of the tube feet from the larval body), which allow larvae to progress as a juvenile.

Under appropriate conditions, larvae develop via the typical mode that is described herein (Fig. 1). In the fed group, food was abundant, and, therefore, more energy could be allocated toward rudiment development and less toward resource acquisition. Similar observations have been made on the species *Paracentrotus lividus*, where a priority to increase rudiment size over body width was documented (Vaïtilingon et al., 2001).

Under unfavourable conditions, larvae may develop via the alternative mode that is described herein (Figure 2). In the starved group, larvae used their acquired energy stores (nutrition from algae contained in the stomach), and, when those became depleted, rudiment resorption commenced. Consistent observations have been made previously in that, as larval development progresses, most energy is used to make juvenile structures (Strathmann, 1971; Hart and Scheibling, 1988; Boidron-Metairon, 1998). Thus, rudiment tissue, including tube feet, continued to develop after larvae were introduced to a nutrition-lacking environment (Fig. 2b) In this study, once rudiments had been resorbed completely, larval arms started to

shrink, skeletal rods began to degrade and bodies eventually reverted to a spherical shape (Figure 2f.)

In algae-depleted environments, larvae generally have been shown to increase arm length to facilitate food acquisition (Strathmann et al., 1992; George S.B. et al., 2004; Byrne et al., 2008). The shrinking larval arms described herein constitutes a different response. Lengthening arms would require more energy; thus, decreasing arm length might conserve energy and allow for enhanced survival. Under this scenario, as acquired energy becomes depleted, larvae utilise rudiments as calcium sources, to maintain existing larval features, such as the skeletal arms. Once all rudiment tissues have been resorbed, larvae lack the stores for maintenance and, thus, start to change morphologically, perhaps to an energetically less-demanding (for example blastula-like) form.

The mechanics involved in this phenomenon are unknown. Cell death constitutes a potential mechanistic component. Apoptosis is a physiological process involved in sea urchin development (Roccheri et al., 2002). Apoptotic cells decrease over larval life span, as cells that need to be eliminated decrease when individuals approach metamorphosis (Roccheri et al., 2002). In this study, the opposite effect may be realized in unfed larvae: as rudimentmenary tissues start to regress in unfavourable conditions (Fig. 2), apoptotic cells increase in number.

The rudiment is a cellular mass that contains juvenile tissues (such as the primary podia, test, spines and mouth), (Yajima, 2006), and evidence has been

marshalled to show that larval tissue may regenerate pre-competence, which may involve cell death regulation (Runnström 1915, 1917, 1918a,b, 1925). Embryonic echinoids can reorganize and reconstitute body parts from disaggregated cells (Hörstadius, 1973). The inability for tissue to differentiate as well the ability for larval structures to be resorbed is observed in starved echinoid larvae in early development (Runnström 1915, 1917, 1918a,b, 1925). Upon re-feeding, tissues are able to properly differentiate and structures can be regenerated, enabling the larvae to progress toward metamorphosis (Runnstrom 1915, 1917, 1918a,b, 1925). Future studies should investigate the potential for rudiment re-growth after larvae have been reintroduced to favourable conditions.

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Preface

The following study was completed in collaboration with J. Lederer and J. Stone. I independently established the experimental setup and optimized staining for the *Strongylocentrotus purpuratus* images. J. Lederer assisted in daily development documentation through image capture. Unpublished *Eupentacta quinquesemita* images were provided by J. Stone. Patterns obtained from the *Strongylocentrotus purpuratus* images presented in this thesis are inconclusive, as staining produced a diffuse pattern. Additionally, recently recovered data demonstrates strong clear signalling in the primary podia of *Dendraster excentricus*, a sand dollar species. Those data are unpublished and were produced by J. Stone at the Department of Ecology & Evolution, Stony Brook, under mentorship from G. Wray in 1999, with a different secondary antibody and laboratory set up. These more-distinct patterns provide evidence supporting the notion that in the ambulacral podia on sea cucumbers are homologous to the primary podia on sand dollars (echinoids like sea urchins), at the cellular level. This conclusion contradicts the EAT theory at the cellular level and ultimately might be included in a publication. This chapter has been prepared as a manuscript for Journal of Evolutionary Biology.

Testing Holothuroid and Echinoid Skeleton Homology

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Abstract

The Extraxial-Axial Theory (EAT), an elaboration on the Ocular Plate Rule (OPR), has been proposed to explain skeletal homologies in the phylum Echinodermata. The EAT suggests that echinoderm body walls comprise two main components: axial and extra-axial elements. The OPR states that axial elements are added adjacent to ocular plates; all other skeletal elements are extra-axial. The proportions in which axial and extra-axial body walls are expressed characterize organisms within echinoderm classes. But ambiguities in determining homology among echinoderm classes remain.

The class Holothuroidea, which contains sea cucumber species, and the class Echinoidea, which contains sea urchin species, are sister-taxa. According to the EAT, holothuroid body walls are extra-axial and echinoid body walls are axial (predominantly). Consequently, ambulacral podia on sea cucumber larvae are extraxial and primary podia on sea urchin larvae are axial. On the basis of the EAT, therefore, these structures are nonhomologous. We investigated the EAT at the cellular level, by observing skeletogenic protein expression (Meso1) in ambulacral podia on *Eupentacta quinquesemita* larvae and primary podia on *Strongylocentrotus purpuratus* larvae. Immunohistochemical staining revealed

that ambulacral podia on holothuroids express Meso1 whereas primary podia on echinoids lack expression. This observation supports the morphological interpretation that these structures are nonhomologous.

Introduction

The Echinodermata is a diverse yet distinctive phylum in the animal kingdom. The phylum contains approximately 16 extinct classes and 5 extant classes, including Asteroidea, Crinoidea, Echinoidea, Holothuroidea, and Ophiuroidea (Lawrence 1987; David and Mooi, 1998). The phylum is characterized by many traits that are shared among the classes, such as a water vascular system derived from the coelom and an internal skeleton; more-specific traits are used to characterize different groups at other taxonomic levels (Lawrence 1987, Nichols 1966). Among these traits, many are thought to be homologous.

Homologous traits may be defined on the basis of similarities (Owen, 1848). Similarities in this sense derive from descent from a common ancestor (Gilbert, 2003) and may be genetic, developmental (process based), physiological, structural, behavioral, or ontogenic (patterned based) (Gilbert, 2003). Ontogenic homology is similarity in growth patterns among organisms (Wray and McClay, 1989).

The macroevolutionary change in the timing for events during ontogeny is known as heterochrony (Wray and McClay, 1989). Heterochrony, itself, is characterized by two observational components: the change in the timing (e.g., faster or slower, earlier or latter) and an ensuing change in phenotype (Wray and McClay,

1989). For example, a change in the time required to reach sexual maturation can produce a change in body size (Wray and McClay, 1989). Relational events during development like these provide clues for gathering together different taxa into phyla.

Macroevolutionay changes in timing often manifest as changes in morphology (Wray and Mclay, 1989; Raff, 1989). These changes can lead to differences among classes. Evidence reinforcing this idea can be obtained by manipulation experiments, in which timing in gene expression is altered and causes development and physiology to change (De Beer, 1954). Perturbing 'normal' gene expression temporally constitutes the basis for heterochrony as a developmental mechanism producing evolutionary change (Gould, 1977; Raff, 1987).

Together, homology and heterochrony may be used to develop an evolutionary developmental model, in which closely related organisms share common stages in development during early embryogenesis and diverge then after (von Baer, 1928; Gilbert, 2003). This notion can be applied to holothuroids and echinoids, classes in the phylum Echinodermata. Holothuroids, which contain sea cucumbers, and echinoids, which contain sea urchins, are characterized by distinct features. For example, sea cucumbers contain one gonad that branches and sea urchins have five gonads arranged in a radial pattern (Byrne, 2001; Ziegler et al., 2008). Also, in the transition from the larval stage to the adult stage in development, only sea urchins undergo metamorphosis; sea cucumbers arrest at the developmental stage before metamorphosis and, therefore, may be considered as giant larvae (Nichols, 1966; Mooi and David, 1998). Although these two classes are

characterized by distinct morphologies, they are hypothesized to have evolved from a common ancestor (Mooi and David, 1997).

Skeleton homology has been used to substantiate the phylogenetic kinship between these classes, as it integrates similarities in ontogeny and morphology (David and Mooi, 2000). To explain skeletal homologies specifically and reduce discrepancies among extant classes in Echinodermata generally, David and Mooi (1997) proposed the Extraxial-Axial Theory (EAT) as an elaboration on the Ocular Plate Rule (OPR). The OPR states that "all the plates in a given growth zone are produced adjacent to the ocular plate that is at the head of that growth zone" (David and Mooi, 1998). The EAT, therefore, is formulated on the basis of the way in which echinoderm skeletons grow. The axial skeleton embodies all the elements that are formed next to the ocular or terminal, plates, in accordance with the OPR (David, Mooi, 1998). All other elements are extraxial (David and Mooi, 1998). The extraxial skeleton is divided further into perforate and non-perforate components (David and Mooi, 1998). Cladistic analyses conducted on the basis of information derived from the EAT yield holothuroids and echinoids as sister taxa, the main difference between them being that holothuroid skeletons predominately are extraxial in origin whereas echinoid skeletons mainly are axial (David and Mooi, 1998).

Whereas this new approach to echinoid classification has been tested at morphological and developmental levels, scant research has been conducted at the cellular level. We used immunohistochemical staining techniques to examine skeletogenic protein expression during development in sea cucumbers and sea

urchins. The protein that we chose, Meso1 is known to be a marker for skeletal tissue (Wessel and McClady, 1985, Wray, 1987). Meso1 is a 380 kd protein, the first germ-layer-specific protein that arises in sea urchin larvae, especially in the rudiment, which contains the developing adult tissues (Wessel and McClady, 1985). We examined Meso1 expression in ambulacral podia on the sea cucumber species *Eupentacta quinquesemita* and primary podia on the sea urchin species *Strongylocentrotus purpuratus* to test skeletal homology at the cellular level.

Materials and Methods

Fertilization and Larval Rearing

Adult *Strongylocentrotus purpuratus* sea urchins were obtained from WestWind Sealab Supplies (Victoria, British Columbia). Upon arrival, specimens were injected with approximately 5 mL 0.5 M KCl in the soft tissue around the peristome, causing the gonads to contract and release gametes. Injected adults were placed aboral side down in 100 mL beakers containing artificial seawater (Instant Ocean, Cincinnati, Ohio). Sperm and eggs were collected separately. Eggs were fertilized with sperm in 500 mL beakers. Fertilized eggs were transferred to Pyrex bowls filled with artificial seawater, forming monolayers, and left overnight. Larval embryos that were floating 12 hours later were collected and placed in 1.8 L FÖRVAR glass jars (IKEA) containing artificial seawater, for rearing. To simulate an ocean current, jars were placed on magnetic stirrers and water was agitated with magnetic stir bars. All jars were placed in a cold room maintained at 10 °C.

Larval Maintenance

Larvae were fed 14 mL centrifuged at 4500 rpm for 1 minute. *Dunaliella tertiolecta* + 1% Reef Crew algal growth medium solution, re-suspended in artificial seawater every other day. To prevent algae stocks from crashing, flasks housing *D. tertiolecta* were swirled 50 times daily and transferred to new flasks every other day. Water salinity for each jar was monitored and adjusted by adding distilled water to maintain specific gravity equal to 1.020-1.023. Jars were cleaned using glass pipettes to remove debris, when necessary. Images depicting developing larvae were captured daily using Openlab Improvison software on a Leica DM5000B digital microscope.

Larval Collection and Fixing

Larvae at the 8-arm stage with well-developed rudiment tissue and visible tube feet primordia were collected. Specimens were rinsed twice with artificial seawater, to remove any ciliates or other organisms that may have been included in the sample collected with the specimen. Specimens were then immersed in a test tube containing a solution comprising 5 mL 7% $MgCl_2$ and 5 mL seawater for 15 minutes, to relax them. Specimens were fixed in a solution comprising 5 mL 4% formaldehyde in artificial seawater, for 30 minutes. Specimens then were dehydrated in 25%, 50%, and 75% EtOH solutions for approximately 5 minutes each.

Larval Staining

To knock out endogenous peroxidases, specimens were dehydrated in 95% EtOH, then MeOH, for 1 minute each. Specimens then were placed in 3% H_2O_2 in MeOH for 15 minutes. Next, specimens were rinsed in MeOH and rehydrated in 95%, 75%, 50%, 25% EtOH and 95%, 75%, 50%, 25% PBS solutions for approximately 1 minute each. To prevent nonspecific binding with antibody, specimens were blocked using ~5% Goat Serum (Sigma-Alrdich) in PBT and maintained in solution at 4 °C overnight. To induce primary antibody incubation, specimens were covered directly with 5 µL 6a3 antibody at room temperature for 1 hour. Next, specimens were rinsed in PBT for 5 minutes, twice, then once for 30 minutes, and one final rinse immediately before storing overnight at 4 °C.

To induce secondary antibody incubation, specimens were immersed in a solution containing 3 µL secondary antibody (Rodamine-x: Anti-mouse; Sigma-Aldrich) and 250 µL ~5% Goat Serum in PBT, which bound to 6a3. Specimens were incubated in this solution at room temperature for 1 hour. Next, specimens were rinsed in PBT for 5 minutes, twice, then once for 30 minutes, and one final rinse, immediately before storing overnight at 4 °C. To visualize Meso1 protein fluorescence, a triton-X-filter on a Leica DM5000 B microscope was used.

Results

Staining in *E. quinquesemita* larvae was conducted at the two-ambulacral-podia stage. A distinct staining pattern was observed at the tips on each podium (Fig. 1) Distinct and localized staining also was observed at the 4-ambulacral-podia stage (Fig. 2).

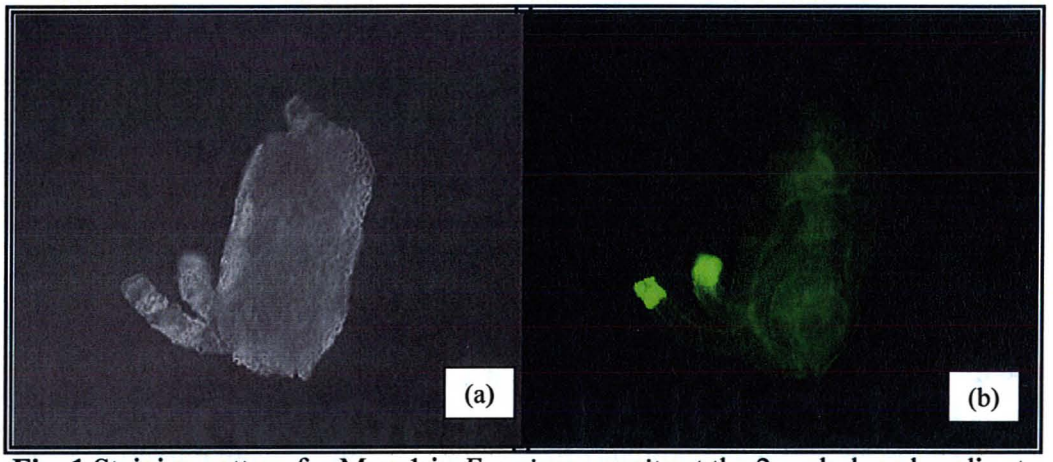

Fig. 1 Staining pattern for Meso1 in *E. quinquesemita* at the 2-ambulacral-podia stage, treated with antibody 6a3. (a) *E. quinquesemita* viewed under brightfield illumination. (b) *E. quinquesemita* viewed with FITC-filter.

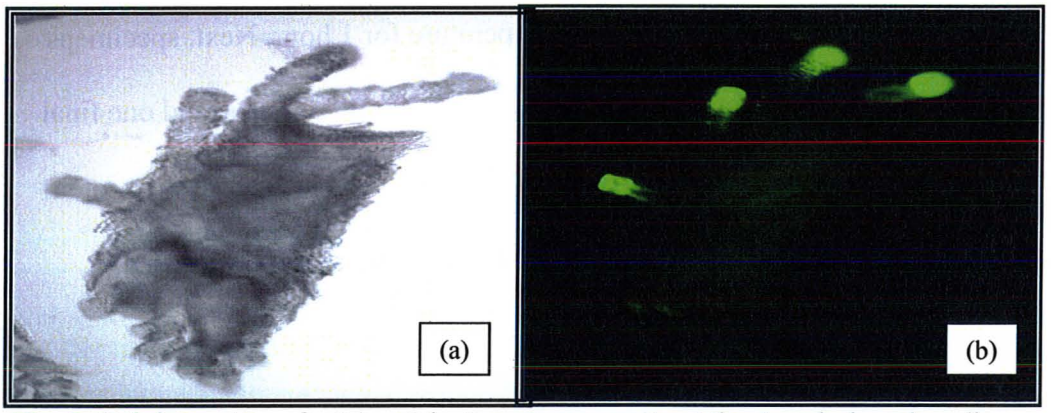

Fig. 2 Staining pattern for Meso1 in *E. quinquesemita* at the 4-ambulacral-podia stage, treated with antibody 6a3. (a) *E. quinquesemita* viewed under brightfield illumination. (b) *E. quinquesemita* viewed with FITC-filter.

Staining in *S. purpuratus* larvae was conducted at the 6-arm stage (Fig. 3). At this stage, the rudiment was developed incompletely. Faint staining was observed at the site where the rudiment ultimately would form, later in development. Staining in *S. purpuratus* specimens also was conducted at the 8-arm stage (Fig. 4-6). The primary podia appeared to be unstained (Fig. 4a). Intense staining was unobserved in the rudiment (Figs. 4-5), although a faint signal was perceived in primary podia on some speciemens (Fig. 6b).

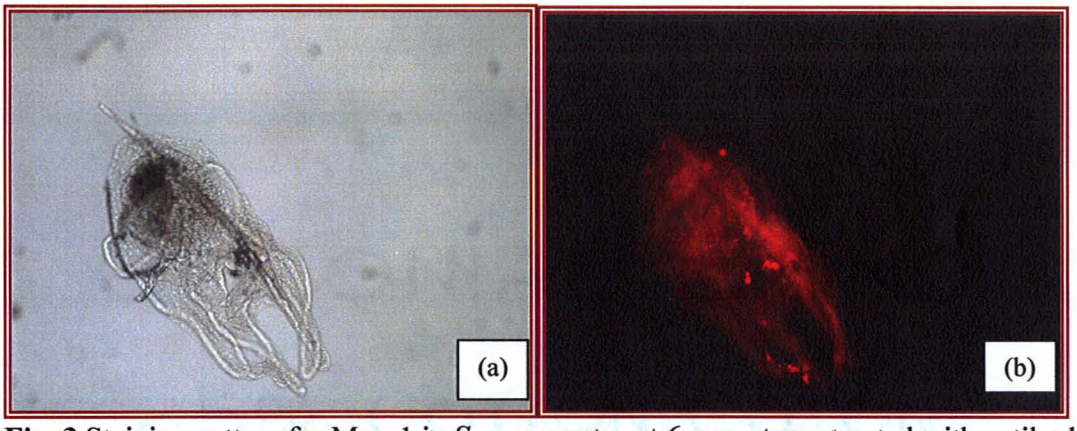

Fig. 3 Staining pattern for Meso1 in *S. purpuratus* at 6-arm stage, treated with antibody 6a3. (a) *S. purpuratus* viewed under brightfield illumination. (b) *S. purpuratus* viewed with N21, TRITC-filter.

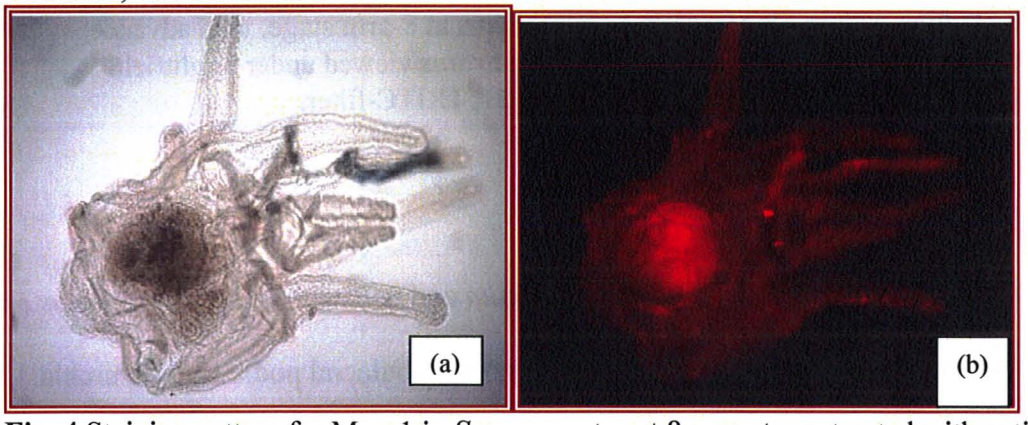

Fig. 4 Staining pattern for Meso1 in *S. purpuratus* at 8-arm stage, treated with antibody 6a3. (a) *S. purpuratus* viewed under brightfield illumination. (b) *S. purpuratus* viewed with N21, TRITC-filter.

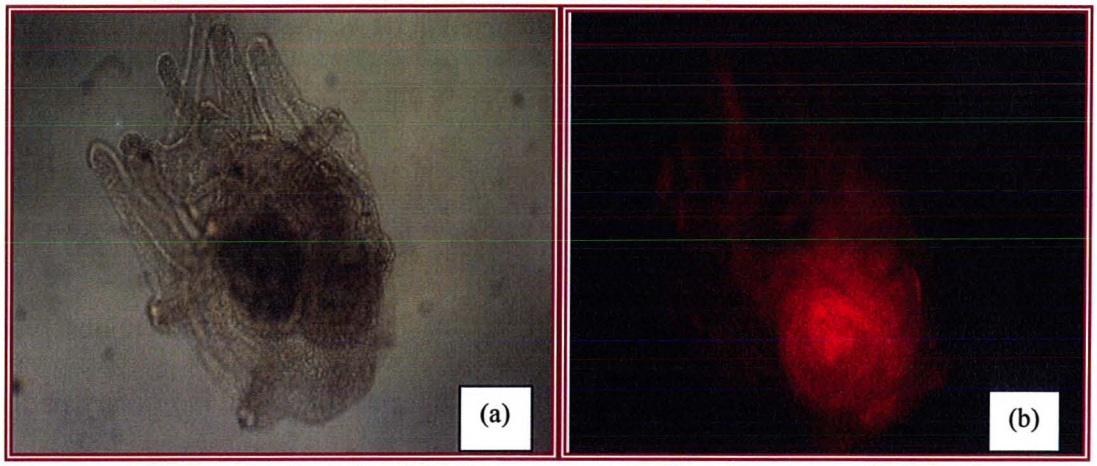

Fig. 5 Staining pattern for Meso1 in *S. purpuratus* at 8-arm stage, treated with antibody 6a3. (a) *S. purpuratus* viewed under brightfield illumination. (b) *S. purpuratus* viewed with N21, TRITC-filter.

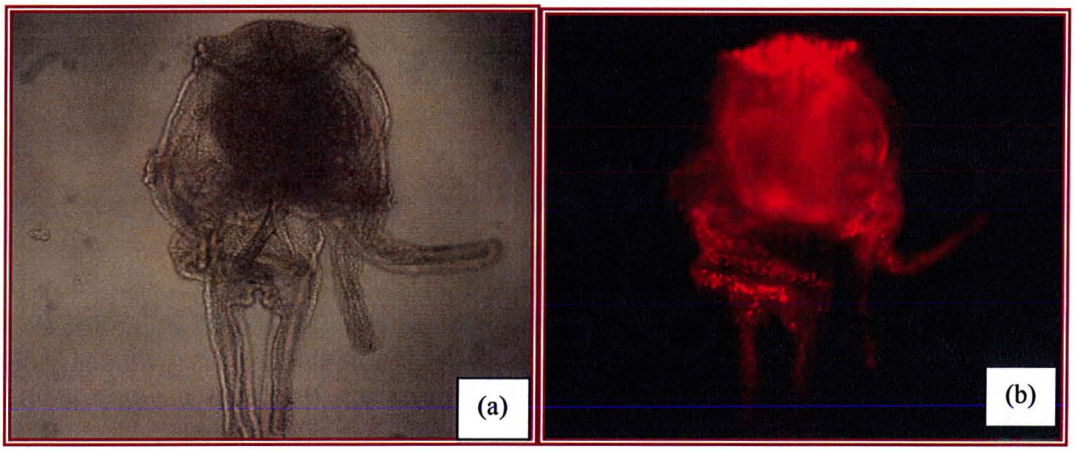

Fig. 6 Staining pattern for Meso1 in *S. purpuratus* at 8-arm stage, with advance rudiment treated with antibody 6a3. (a) *S. purpuratus* viewed under brightfield illumination. (b) *S. purpuratus* viewed with N21, TRITC-filter.

Discussion

The distinct staining pattern in *E. quinquesemita* and nonstaining in *S. purpuratus* are consistent with homology between sea cucumber ambulacral podia and sea urchin primary podia, supporting the EAT at the cellular level. Meso1, a marker for skeletogenic protein, has been found in all holothuroid and echinoid species (Wessel

and McClady, 1985). In the sea cucumber species *E. quinquesemita*, ambulacral podia stained distinctly for Meso1 at the 2-ambulacral-podia stage (Fig. 1b). Increased intensity in staining was observed later in development, at the 4-ambulacral-podia stage (Fig. 2b). This increase in staining intensity indicates an increase in skeletogenic protein expression. As holothuroids develop, skeletogenic proteins become more prevalent in the ambulacral podia, probably as a means to increase strength during ontogeny, allowing enhanced locomotion (Gilbert & Raunio, 1997).

In *S. purpuratus* mesenchymal tissue expression, Meso1 can be detected as early as when cells delaminate from the blastula wall (Spencer and Wright, 1966; Lawrence 1987). Primary mesenchyme cells are known to add skeletal material throughout larval life; therefore, a variety of developmental stages could be examined (Spencer and Wright, 1966; Lawrence, 1987; Gilbert & Raunio, 1997). The staining pattern observed at the 6-arm stage shows Meso1 expression in skeletal rods and on the left side, where the rudiment ultimately will form (Fig. 3b). At the late 6-arm stage in larval development, amniotic invagination and hydrocoel development begin to form the adult rudiment (David and Mooi, 1998). Skeletal elements also start to form (Smith et al., 2008). By the 8–arm stage, rudiments were well developed and contained structures such as the primordial test and spines for future juveniles (Smith et al., 2008). Also within rudiments, primary podia become visibly distinct and proteins such as Meso1, specific to these structures, may be detected. In *S. purpuratus*, staining was conducted at the 8-arm stage. No strong signal was observed

in rudiments (Figs. 4b, 5b, 6b). Staining was expressed most strongly in the gut, ciliary bands and skeletal rods. Faint staining was detected in three among five visible primary tube feet. However, since the signal was weak, we cannot determine with certainty whether primary podia contained in the rudiment express Meso1. Homology has been suggested between *E. quinquesemita and S. purpuratus*, as ambulacral podia and primary podia arise from the five primary lobes in the hydrocoel in holothuroids and echinoids, respectively (Lowe et al., 2002). On the basis of our results, ambulacral podia in sea cucumbers stain differently with respect to primary podia in sea urchins, consistent with the EAT at the cellular level.

Other proteins, such as DIx, also have been used to support homology among echinoderm structures. DIx encodes transcription factors that play important roles in patterning among many metazoan groups (Duboul 1994; Finkelstein and Boncinelli, 1994; Holland et al., 1996; Panganiban et al., 1997; Lee and Jacobs 1999; Shoguchi et al., 2000). DIx expression is localized to ciliated ectodermal cells throughout development and in the five developing primary tentacles in holothuroid larvae (Lowe et al., 2002). DIx expression in echinoids is less pronounced and more scattered. Expression was observed in tube feet in larvae approaching metamophosis and in distal ends in postmetamorphic juveniles. Ectodermal and coleomic components in adult rudiments express DIx throughout development. These similar gene expression patterns were interpreted as evidence for homology (Lowe et al., 2002). That finding is consistent with the results that are reported in the current study, as different structures were considered. In the study by Lowe (2002), sea

cucumber feeding tentacles were compared to primary podia in sea urchin rudiments. According to the EAT, these structures are axial; the morphological homology described herein, in conjunction with the cellular results from Lowe et al. (2002), thus provide consistent support for the EAT.

In this study, we determined cellular homology on the basis of gene expression, using Meso1. Meso1 is one skeletogenic protein found in echinoids, but antibodies to other skeletongenic proteins, like P4 and IG9, also can be used to detect skeletogenic cells (Yajima and Kiyomoto, 2006). Like Meso1, P4 and IG9 are detected in primary mesenchyme cells in growing echinoids (Wessel and McClady, 1985; Yajima and Kiyomoto, 2006). However, P4 and IG9 detect skeletogenic cells at the tips in developing tube feet (Yajima and Kiyomoto, 2006). Similar patterns were developed in late formed skeletogenic cells in primary podia. A possible rationale for this discrepancy is the idea that expression patterns are variable, producing proteins in different amounts at different points in development (Mooi et al., 2005).

Gene expression constitutes an acceptable criterion for testing homology at the cellular level; however, other criteria for testing cellular homology should be examined, and, consequently, our results provide only partial evidence for establishing homology at the cellular level. To strengthen a homology statement, other criteria for cellular homology should be studied. Features for further study include investigating morphology and movement, time and position for origin and cell fate (Raff, 1987). In addition, we can examine other skeletogenic protein

markers, such as antibodies P4 and IG9, in holothuroids and echinoids to test if the EAT holds at the cellular level. Such studies would help solidify using the EAT as a basis for establishing phylogenetic relationships among classes in the phylum Echinodermata, as it encompasses homologies at different levels.

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Preface

The following study began as an independent undergraduate thesis project (BIOLOGY 4C09 course). All results presented were collected solely by myself during my M.Sc. candidacy. J. Stone provided suggestions on experimental design as well as manuscript emendation. S. Dudley contributed to statistical analysis and formatting. This chapter has been prepared as a manuscript for Aquaculture Research.

Seasonal & Dietary Effects on Gonads and Tests in *Strongylocentrotus purpuratus* and *Lytechinus variegatus* Sea Urchins

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Abstract

Adult sea urchins are harvested primarily for their gonads ('uni' or 'roe'), which are considered a delicacy among sushi eaters. Quantity and colour are the two most important factors considered in selling, purchasing and consuming sea urchin gonads. We assessed gonad plasticity in gravid *Strongylocentrotus purpuratus* and non gravid *Lytechinus variegatus* sea urchins in response to a carrot or a seaweed + carrot diet. We also assessed which diet yields a commercially more-appealing product than what is available currently, specifically by enhancing weight and colour. Additionally, we examined how the diets affected test size. Results showed that a combined seaweed + carrot diet has variable effects on gonad yield while a carrot diet alone produced the best gonad colour. Neither diet significantly effected test growth. Understanding gonad plasticity could enhance the growing sea urchin gonad harvesting industry.

Introduction

The demand for producing and extracting healthy sea urchin gonads for human consumption is rising, but natural sea urchin stocks are declining (Yokota

et al., 2002). Thus, commercial harvesting and laboratory culturing are becoming prominent activities. The ability to produce commercially desirable gonads (in size and colour terms) can be achieved through environmental influences. Phenotypic plasticity may be defined as the ability for an organism to adopt different phenotypes in response to different environments (Miner, 2003). A new diet may constitute a changed condition, and previous studies have shown that diet constitutes an important factor in assessing sea urchin roe quality (Daggett et al., 2004; Senaratna et al., 2005; Shpigel et al., 2005 Hammer et al., 2006). Gonad size is determined by the food (quantity and quality) that sea urchins can obtain. Sea urchins along the Gulf of Mexico with limited food resources are characterized by small overall weight and gonad size (Hill and Lawrence 2003). Similar observations have been recorded under laboratory conditions (Pearce 2002; Robinson et al., 2004). Food-ration experiments have revealed that underfed sea urchins are characterised by lower gonad indices compared to sea urchins provided minimal and abundant food, suggesting a positive correlation between food administered and gonad size (Garrido et al., 2001).

Algae diets have been shown to increase gonad growth (Russel, 1998). Micro-algal diets are rich in protein and are known to be the quickest and most-efficient means for enhancing gonad indices (Jaquin et al., 2006). But algal diets also have been observed to yield nonsignificant increases in gonad size (Senaratna et al., 2005; Shipigel et al., 2005). These contradictory results may have derived from differences among algae (amounts and types) used.

Gonad colour is another important factor in marketing sea urchin gonads. Desired colour ranges from yellow to dark orange. Different diets affect gonad colour (Pearce, 2002; Shpigel, 2005): algae diets produce dark orange eggs and prepared pellet diets produced pale yellow, brown and orange eggs. Gonad colours obtained from prepared diets are indistinguishable from gonad colours obtained from wild control specimens (Pearce, 2002). Individuals in the sea urchin species *Strongylocentrotus droebachiensis* produce a significantly more-appealing gonad colour when fed prepared diets than when fed kelp exclusively; and gonad colour is more appealing in smaller than in larger sea urchins (Pearce 2004).

Diet in conjunction with season affects gonad colour. Gonads from individual *S. franciscanus* that were fed a low carotenoid pigment diet or a high carotenoid pigment diet or kelp maintain red colour in both seasons, whereas yellow colour decreased significantly in fall (McBride 2004). Also, gonads from laboratory treatments contain less red and yellow pigment, resulting in an overall lighter colour than commercially processed roe (McBride 2004). Additionally, gravid sea urchins produce larger, commercially more-appealing gonads than do nongravid sea urchins (Brady and Scheibling, 2006). Individual *S. droebachiensis* off the Nova Scotia coast provide evidence for two spawnings: a primary period in spring and shorter, secondary period in fall. Both periods yielded higher gonad indicies compared to nongravid periods (Brady and Scheibling, 2006).

Several factors influencing sea urchin gonad quantity and quality are known to impact sea urchin tests (skeletons), too. The main factor affecting growth is diet (Dix, 1972). Studies indicate that a fed sea urchin is a growing sea urchin (Ellers, 1998). Fed sea urchins have looser sutures (indicating growth) compared to unfed sea urchins (Ellers, 1998). In wild *S. purpuratus* populations, food shortage result in lower overall growth relative to growth during nonfamine periods (Dix, 1972). Varying feeding rate induces test growth (Lawrence and Lane, 1982).

A growth equation for sea urchins has been described: $g(w) = A\text{-}M\text{-}R$,where g represents growth rate, w represents total urchin weight, A stands for assimilation rate, M stands for maintenance costs and R represents allocation to gamete reproduction (Middleton, 1998). Echinoderm species that grow quickly direct more energy toward reproductive resources (R) and less on maintenance (M) (Ebert,1975). Therefore, quickly growing sea urchins may experience increased mortality rates due to a minimal investment in maintenance (Ebert, 1975).

Diet constitutes an influential factor in sea urchin reproduction and growth (Lawrence and Sammarco, 1982). Whereas previous studies have involved combination diets, no research has been conducted on a seaweed + carrot diet. In this study, we report the effects imparted by a carrot diet or a combined seaweed + carrot diet on sea urchin gonads and tests. This research specifically involves the important commercial factors gonad weight and colour. We compared

individuals from two sea urchin species at a time in which they were in different reproductive phases, gravid *S. purpuratus* and nongravid *Lytechinus variegatus*. We anticipated that both species would exhibit plastic responses. We predicted that *S. purpuratus* would yield enlarged and more-orange-coloured gonads compared to nongravid *L. variegatus*. Additionally, we predicted that *L. variegatus* would produce enlarged tests compared *S. purpuratus*, as the energy obtained from diets would be directed toward test rather than gonad growth (as *L. variegates* sea urchins were 'out-of-season').

Materials and Methods

Specimen Collection

Twelve gravid adult *Strongylocentrotus purpuratus* sea urchins were shipped from WestWind Sealab Supplies (Victoria, British Columbia). Twelve adult nongravid *Lytechinus variegatus* were shipped from Gulf Specimen Marine Laboratories Inc. (Panacea, Florida). Specimens were housed in aquaria (L x W x H: 50 cm x 25m x 30cm) containing approximately 30 L water. Aquaria were acclimated for two days before sea urchins were placed into them.

Experimental Aquarium Maintenance

Four aquaria were established, with a pump and filter to agitate and clean seawater: 1 = *L. variegatus*, carrot only; 2 = *L. variegatus*, seaweed + carrot; 3 = *S. purpuratus*, carrot only; 4 = *S. purpuratus*, seaweed + carrot. Filters were changed every three weeks. Aquaria were cleaned prior to each feeding, using 1

cm and 2 cm bore tubing as a siphon. Aquaria 1 and 2 were maintained at 22 °C-24 °C and housed 6 specimens each. Aquaria 3 and 4 were maintained at 8-10 °C and housed 6 specimens each. Seawater temperature and salinity were measured every other day. Salinity was maintained at specific gravity 1.020-1.023. Treatments continued for exactly 8 weeks. Afterward, *S. purpuratus* and *L. variegatus* were sacrificed and sea urchin wet weight, gonad dry weight and test dry weight were recorded for subsequent analysis.

Diets

Exactly 14 g sliced baby carrots were administered to aquaria 1-4. Nutritional information is presented in Table 1. Exactly 15 g dried brown seaweed (*Phaeophyta*) were administered to aquaria 2 and 4 every third day. Nutritional information is presented in Table 2.

Table 1 Nutritional value for carrot diet

Component	**Amount (mg)**
Beta- Carotene	46
Potassium	7.75
Protein	0.03
Vitamin A	7.5

Table 2 Nutritional value for seaweed diet

Component	**Amount (mg)**
Potassium	7.75
Protein	3.53
Sodium	0.03

Gonad Yield

Net dry gonad yield was calculated as:

Net Dry Gonad Yield= Dry Gonad Mass/Dry Sea Urchin Mass x (100%).

RGB Colour Analysis

A RGB (Red, Green Blue) colour index analysis was conducted, using the software Meazure 2.0 on gonad images, which were captured using a Sony Cyber-shot DSC-W55 digital camera.

Statistical Analysis

All statistical analyses were performed using the software Statistica 7.0 and Microsoft Excel (Windows 2003). An analysis of variance (ANOVA) was conducted on gonad dry mass and test dry mass, with species and diet as treatments (*i.e.*, F-values and chi-square distributions were used to determine P-values). *S. purpuratus* and *L. variegatus* whole urchin wet mass, test circumference and test height were measured pre- and post-experiment and also subjected to ANOVA. Data exploration with the covariate dry body mass

included yielded no significant relationship between this covariate and gonad mass, so performing ANOVAs was justified.

Results

During treatment, one *S. purpuratus* in aquarium 4 (seaweed + carrot treatment) died (4.5 weeks into the experiment); data from that urchin were excluded.

ANOVA

Table 3 One – way ANOVAs for dependent variables

Aquaria	P (Whole Urchin Wet Weight)	P (Test circumference)	P (Test height)
1	0.12	0.07	0.03
2	0.83	0.87	0.97
3	0.69	0.76	0.78
4	0.75	0.70	0.29

No statistically significant differences were found between before and after values for whole sea urchin wet mass and circumference around the test in any treatment. A significant difference was observed only for test height in aquarium 1.

Diet affected species with respect to gonad growth in a statistically significant manner ($P = 0.02$). Means for gonad dry mass were, in order: 1.03 g, 0.30 g, 1.46 g and 2.06 g. However, diet imparted no statistically significant effect on species with respect to test growth ($P = 0.59$).

RGB Colour Analysis

Table 4 Mean RGB code numbers generated from individual gonads

Aquarium	RGB Code Number
1	R 190, G 125, B 54
2	R 190, G 125, B 54
3	R 143, G 93, B 31
4	R 127, G 91, B 50

Gonad Yield

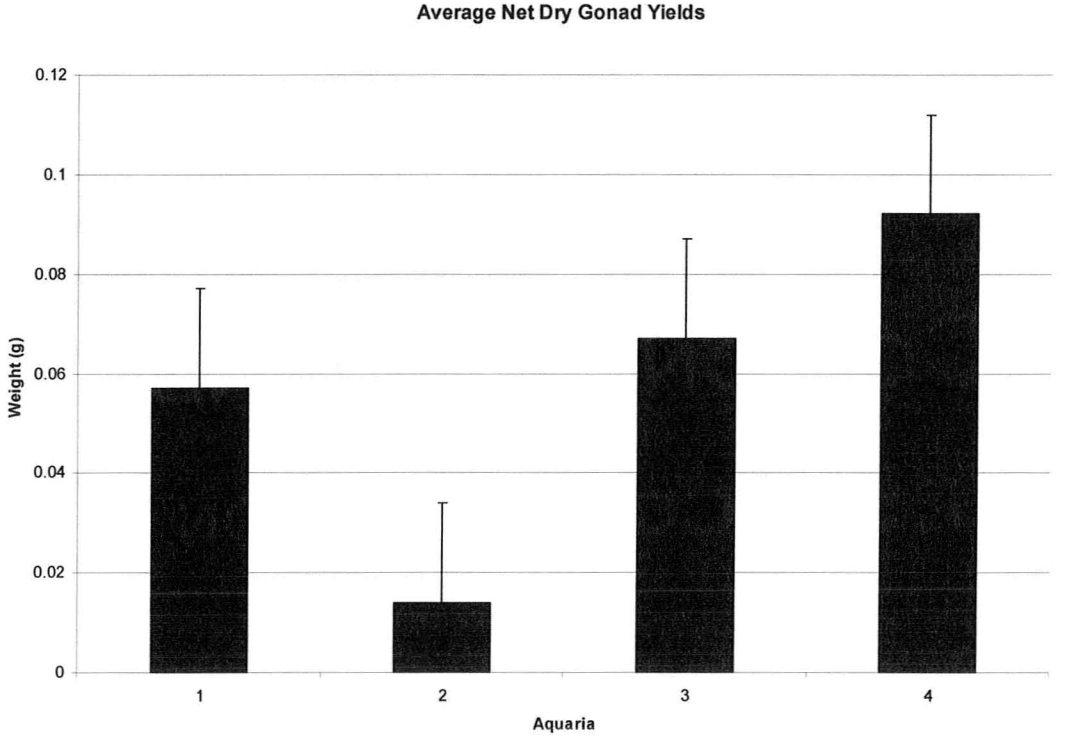

Figure 1 Average net dry gonad yields for sea urchins among experimental treatment aquaria. Aquarium 1 – *L. variegatus* sea urchins fed a carrot diet; 2 – *L. variegatus* sea urchins fed a seaweed + carrot diet; 3 – *S. purpuratus* sea urchins fed a carrot diet; 4 – *S. purpuratus* sea urchins fed a seaweed + carrot diet. Bars indicate the maximum gonad yield in each aquarium.

Visual Analysis

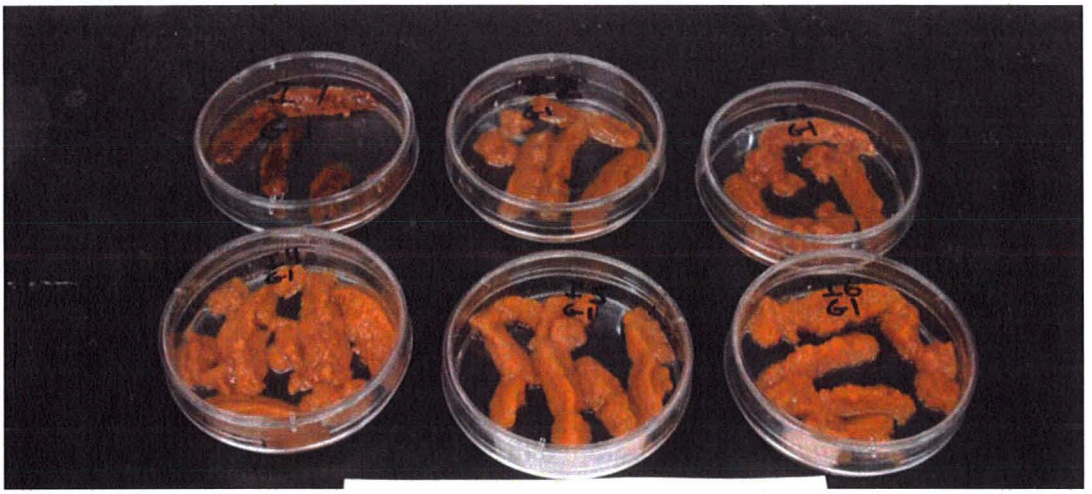

Figure 2 *L. variegatus* gonads from 6 individuals fed a carrot diet.

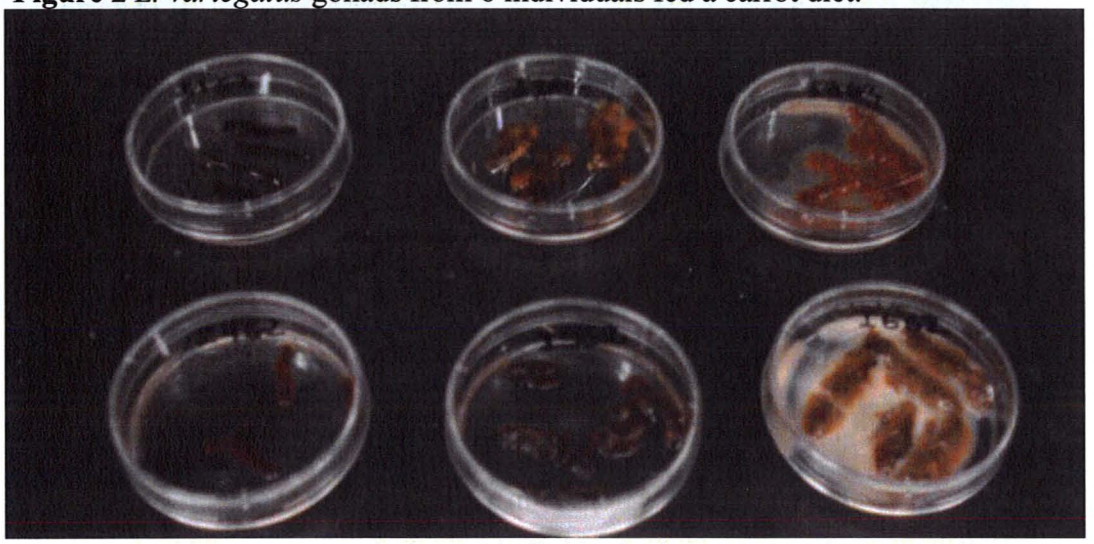

Figure 3 *L. variegatus* gonads from 6 individuals, fed a seaweed + carrot diet.

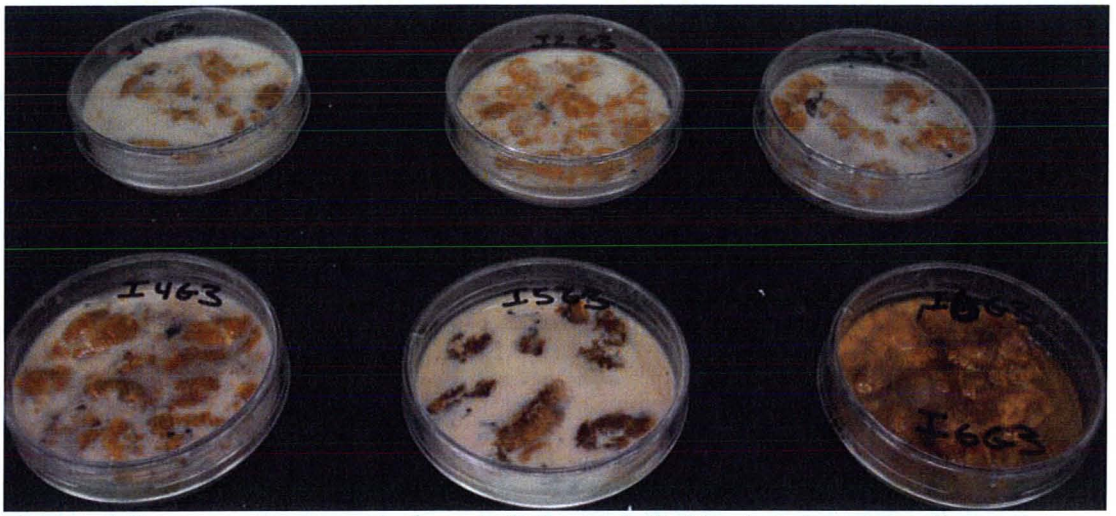

Figure 4 *S. purpuratus* gonads from 6 different individuals fed a carrot diet.

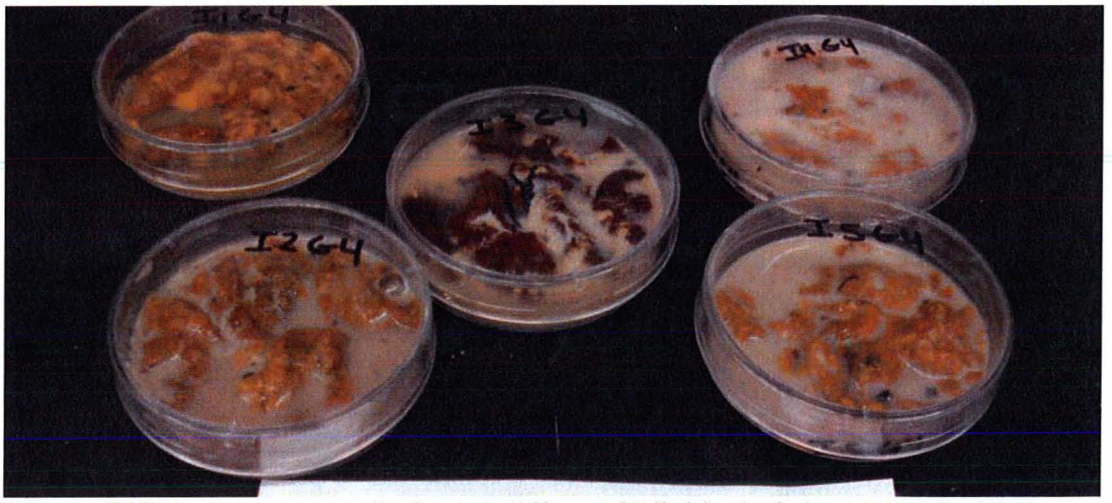

Figure 5 *S. purpuratus* gonads from 6 different individuals fed a seaweed + carrot diet.

Discussion

In this study, we investigated the effects that a carrot diet and a seaweed + carrot diet imparted on gonads and tests in the sea urchin species *S. purpuratus* and *L. variegatus.* We found that diet affected gonad mass in *S. purpuratus* and *L. variegatus* (dry weight, $P = 0.02$).

By inspecting mean values, an interesting interaction can be identified. *S. purpuratus* and *L. variegatus* sea urchins fed the carrot diet yielded similar positive gonad mass increases, whereas *S. purpuratus* and *L. variegatus* sea urchins fed the seaweed + carrot diet yielded species-specific results: gonad mass decreased in *L. variegatus* and increased (almost two-fold when compared to the carrot diet alone) in *S. purpuratus*. The *S. purpuratus* data are consistent with the finding that a seaweed diet increases gonad yield in *Strongylocentrotus droebachiensis* (Russell, 1998). Additionally, the seaweed + carrot diet is higher in protein than is the carrot diet alone. The primary cause for increases in gonad size and quality is attributable to increased protein content in diets (Fleurence, 2001; Martinez and Rico, 2002; Hammer, 2006).

Concerning colour, sea urchins fed a carrot diet yielded gonads closest to the desired golden yellow/orange gonad colour, consistent with RGB code number 240, 165, 68. Sea urchins in aquarium 1 yielded the closest RBG code numbers, followed by sea urchins in aquarium 3. This finding is consistent with the observation that beta-carotene, found in abundance in carrots, produces orange gonads (Griiffiths and Perrot, 1976; Robinson et al., 2002). Sea urchins in aquaria 2 and 4, which included a seaweed component in their diet, likely had altered gonad pigments, producing more-brown colours. Seaweed diets can degrade gonad colour (Senaratna et al., 2005; Shipigel et al., 2005) or improve gonad size and colour (Russell, 1998; Jaquin et al., 2006). The discrepancy in available data most likely resides in the different seaweeds used, which contain different

pigments, in experiments. Most studies have been conducted using green algae and red seaweed, whereas the current experiment used brown seaweed.

Diet imparted no significant affect on test circumference or height, except on sea urchins in aquarium 1. This difference among sea urchins in different environments may be attributed to changes in spine shape morphology or number. Spines loss and re-growth is common, especially when living in changing environments (Bookbinder and Shick, 1986). Losing spines is typical for sea urchins in dire conditions (Bookbinder and Shick, 1986).

Previous studies have revealed a positive correlation among food consumption, gonad size and reproductive state (Kunetzov, 1946; Fugi, 1962; Klinger et al., 1997). In this study, *L. variegatus* sea urchins left scant grazings between feedings compared to *S. purpuratus* sea urchins, which left more grazings. This observation is consistent with studies that showed high food consumption in species that were growing but nonspawning (Agatsuma et al., 1993; Agatsuma and Sugawara 1998). Additionally, large mature gonads are correlated with spawning, low food-consuming sea urchins (Agatsuma et al., 1993, 1996; Agatsuma and Sugawara 1998). These findings are consistent with the results reported herein, as gravid sea urchins possessed the largest gonad dry yields. As well, gametogenesis and spawning are indicated clearly by the milky fluid seen in Figures 4 and 5, which are sperm from *S. purpuratus* sea urchins.

As laboratory conditions poorly simulated natural habitats, the sea urchins used in this study might have invested in maintenance rather than growth.

Utilizing the equation $g(w) = A\text{-}M\text{-}R$, (Middleton, 1998), we hypothesize that the sea urchins used in this study faced high maintenance costs due to the need to acclimatize to their respective environments.

Possible aberrations in gonad quantity and colour as well as test growth can be explained by age. In *Eisenia* and *Crustose* beds, brown-coloured gonads are found in sea urchins with a test diameter greater than 7 cm, which corresponds to an age greater than 7 years, and is associated with a decrease in gonad indices (Agatsuma et al., 2005). These findings suggest that brown colourization is correlated with aging and/or gonad size, as determined by food availability (Agatsuma et al., 2005). Age also is a contributing factor to growth in sea urchins. Tetracycline labeling reveals that although individuals grow throughout their lifetimes, growth decreases as sea urchins age (Ebert, 2003). As the ages for the sea urchins that were used in the study were unknown, age is a plausible factor in explaining inconsistencies with previous studies.

Whereas great variety in traits among individual sea urchins has been observed in their natural habitats (Lawrence, 2007), this study affirms findings that predictable plastic responses can relate diet to factors in gonad growth. Additionally, this study visually demonstrates the importance in obtaining a delicate balance between food, species and spawning period in regulating gonad quality, which is important to commercial aquaculture for optimizing the correct harvesting periods for sea urchins.

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Conclusion

The research in this thesis focused on echinoderms, specifically sea urchins. Research was conducted at the main developmental stages (larval and adult), involved sea urchin life history, skeletogenesis and reproduction and yielded insight into phenotypic plasticity, homology and energy allocation.

Chapter 1

In chapter 1, *Strongylocentrotus purpuratus* sea urchins were examined throughout larval stages to investigate the capability for rudiments to function as plastic traits. Larvae were reared and gathered for experimentation when visible rudimentary tissue appeared. In comparing fed and starved groups, I found that starving larvae (an unfavourable condition) causes rudiments to shrink, preventing larvae from metamorphosing, possibly until favourable conditions present. This study documented complete rudiment resorption, thereby demonstrating an extremely adaptive, plastic nature.

Chapter 2

In chapter 2, *Eupentacta quinquesemita* sea cucumber and *Strongylocentrotus purpuratus* sea urchins were examined at larval stages to investigate homology at the cellular level and test a hypothesis that was formulated on the basis of the extra-axial axial theory (EAT). According the EAT, *E. quinquesemita* ambulacral podia are extra-axial in origin and *S. purpuratus* primary podia are axial. Using immunohistochemical staining techniques, I found that *E. quinquesemita*

ambulacral podia stained distinctly and *S. purpuratus* primary podia stain faintly. These findings support the EAT at the cellular level and provide support of the current phylogenetic classifications among echinoderms.

Chapter 3

In chapter 3, *Strongylocentrotus purpuratus* and *Lytechinus variegatus* adult sea urchins were used to examine the effects imparted by diet and season on gonads and tests. Results show that, in both species, a carrot diet produces gonads closest to the desired yellow/orange colour of commercially processed roe compared to those subjected to a seaweed + carrot diet. A carrot diet imparted a similar increased gonad yield effect on both species, whereas a seaweed + carrot diet caused a decrease in *L. variegatus* and an increase in *S. purpuratus.* Neither diet effected test growth in either species. These findings are important for optimization of diet and timing of gonad harvest for the sea urchin farming industry. This study conducted on these echinoid species could be used as a guide for gonad enhancement for other harvested sea urchin species as well as provide a framework for administering diets to maximize aquaculture industry profits.

Appendix

Protocols

Algal Growth Medium Feeding

- Obtain 1000 ml of artificial seawater in a 1000 mL glass flask.
- Heat seawater in the microwave for 4 minutes. Remove seawater from microwave and swirl gently 20 times. Reheat in microwave for another 4 minutes.
- Let cool for 2 hours.
- Add 10 drops Reef Crew Algae Grow to the cooled 1000 mL artificial seawater.
- Store solution in container with a closed lid.

Dunaliella tertiolecta + Algal Growth Medium Feeding

- Obtain 10 mL of *Dunaliella tertiolecta* + 1% Reef Crew algal growth medium solution.
- Transfer 10 ml to a centrifuge tube.
- Spin the solution in centrifuge for 1 minute at 4500 rpm.
- Pour off excess liquid, leaving the pellet at the bottom in the tube.
- Re-suspend the pellet with 10 mL artificial seawater.
- Shake tube to mix the solution.
- Use to feed one larval jar.

Making PBT

- Obtain 100 mL 10X PBS
- Add 90 ml of H_20 to 10 mL 10X PBS
- Add 100 µL Triton-X

Additional Data

The following is un-reported data for Chapter 3. The tables below display the average score attributed to the gonads in each treatment based on a questionnaire (attached below) relating to colour, texture and general appeal. The scores were obtained with a 1-10 scale, 10 being the best, and the scores were generated by 20 human participants. Certificate of ethics clearance to involve human participants in research was approved by McMaster's Research Ethics Board, April 14, 2009.

SEA URCHIN GONAD QUESTIONNAIRE

Please circle one response for each question:

1. Do you eat sushi – Y N

2. Rank on a scale of 1 – 10 the odour of the gonads (1 being the worst and 10 being the best)

1 2 3 4 5 6 7 8 9 10

3. Rank on a scale of 1 – 10 the texture of the gonads (1 being very rough and 10 being very smooth)

1 2 3 4 5 6 7 8 9 10

4. Rank on a scale of 1 – 10 the firmness of the gonads (1 being squishy/soft and 10 being firm)

1 2 3 4 5 6 7 8 9 10

5. Match the numbered coloured swatches with that which most closely resembles the colour of the gonad.

1 2 3 4 5 6 7 8 9 10

Sushi Eaters				
	Question 2	Question 3	Question 4	Question 5
Aquaria 1	6.85	6.15	6.23	6.83
Aquaria 2	5.15	7.69	5.54	2.42
Aquaria 3	5.77	6.92	4	7
Aquaria 4	5.85	6.62	5.08	6.08

Non Sushi Eaters				
	Question 2	Question 3	Question 4	Question 5
Aquaria 1	7	5.33	7.33	6.33
Aquaria 2	6	7.67	6	3
Aquaria 3	7	5.33	3.33	7.33
Aquaria 4	8.33	4.33	5	7.67

Additional Methods

CT Scanning

Sea urchins were transported in a styrofoam box containing 20 °C, 1.022 specific gravity artificial sea water to CT scanning facility located in the Institute for Applied Health Science Building, McMaster University. During the prescanning and scanning process, sea urchins were out-of-water for approximately an hour and ten minutes. Images were captured during the prescanning process, using a digital camera. Computerised tomography was used to view gonads inside sea urchin tests without animal sacrifice. No images were produced from CT scanning due to the similar density of the internal sea urchin structures. Future attempts might try a higher magnification, using an MRI (Ziegler et al., 2008).

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