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Comparative studies of regeneration and cloning in the planktotrophic larvae of echinoderms.

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COMPARATIVE STUDIES OF REGENERATION AND CLONING IN THE PLANKTOTROPHIC LARVAE OF ECHINODERMS

by

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Regeneration and cloning have been examined in numerous invertebrates and vertebrates in an effort to understand these phenomena. Among the invertebrates, adult echinoderms are well known for their capacity for regeneration and asexual cloning. However, regeneration and cloning have not been as thoroughly examined in larval echinoderms as in adults. It was suggested that larval regeneration and cloning might occur in the ophioplutei of brittle stars about a century ago and in the bipinnaria larvae of sea stars about 70 years ago. However, cloning and complete regeneration of missing body parts in echinoderm larvae were only confirmed to occur fairly recently. In the last 2 decades several incidences of larval asteroid (sea star) cloning in the natural environment have been reported; very recently, cloning of ophioplutei was documented, with the regeneration of the missing larval parts being described in detail. However, to date, no studies have fully examined the capabilities of sea star larvae to undergo regeneration. Also, cloning in sea star larvae has not been studied and documented in larval cultures under controlled laboratory conditions. The studies presented in this dissertation document and describe in detail for the first time the complete regeneration process after surgical bisection in planktotrophic larval sea stars of *Luidia foliolata* and *Pisaster ochraceus*. Furthermore, potential biotic

(food) and environmental (temperature) factors that might influence regeneration and cloning in planktotrophic larval sea stars were examined under controlled laboratory conditions. In addition, planktotrophic echinoid larval regeneration was examined in the sand dollar *Dendraster excentricus* and the sea urchin *Lytechinus variegatus*, demonstrating for the first time the regenerative capacity of planktotrophic echinoid larvae. These studies of regeneration and cloning in echinoderms not only will contribute to our basic understanding of these phenomena but may ultimately benefit humans, as echinoderms share a deuterostome phylogeny with the chordates.

DEDICATION

This dissertation is dedicated to my wonderful parents, Ken-ichi and Akiko Sugi-yama, for their lifetime of encouragement and warm support and for the sacrifice they made for me to pursue my dream. I cannot thank you enough.

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GENERAL INTRODUCTION

Regeneration of lost body parts is known to occur in a variety of invertebrates and vertebrates, including planarians, echinoderms, crustaceans, and amphibians (Goss, 1969; Mattson, 1976; Emson and Wilkie, 1980; Baguñà *et al.*, 1989; Hopkins, 2001). The regenerative capacity of adult echinoderms is legendary, and regeneration processes have recently been examined in both adult and larval echinoderms at both molecular and cellular levels (Thorndyke *et al.*, 1999; Vickery, 2001; Vickery *et al.*, 2001a, 2001b). Cloning is an alternative method of asexual reproduction which requires *de novo* development of missing tissues; thus regeneration and cloning are closely related biologic phenomena. Among adult echinoderms, asteroids, commonly know as sea stars, are especially known for their considerable regenerative and cloning capacities (Thorndyke *et. al.*, 1999). However, in larval echinoderms, regeneration and cloning have received far less attention than in adults, although early studies had suggested that these phenomena might occur in planktotrophic asteroid and ophiuroid larvae (MacBride, 1921; Mortensen, 1921; Tattersal and Sheppard, 1934; Hörstadius 1973). Interestingly, Thorson (1950) discounted the possibility of the regeneration of larval organs, and the idea that marine invertebrate larvae might be capable of complete regeneration was further discouraged based upon the results of other developmental studies (Hörstadius, 1973; Wilson, 1978). Perhaps because of these studies, larval regeneration and cloning in general were not examined in detail until cloning via budding in planktotrophic sea star larvae was discovered (Bosch, 1988;

Bosch *et al.*, 1989; Rao *et al.*, 1993; Jaeckle, 1994).

Plankton samples collected from the Sargasso Sea and the Gulf Stream waters of the Atlantic revealed that 30% of the bipinnaria larvae of the sea star *Luidia* sp. had highly modified posterolateral arms that were in the process of transformation into secondary larvae (Bosch, 1988; Bosch *et al.*, 1989). Jaeckle (1994) further reported that cloning occurred not only in sea star bipinnaria larvae but also in unidentified sea star brachiolaria larvae collected in plankton samples from the northwest Atlantic. This report suggested that not only luidiids but also other asteroids with planktotrophic larvae may have the ability to produce larval clones. Furthermore, Jaeckle (1994) observed that secondary larvae emerged not only from the posterolateral arms but also by fission of the posterior portion of the larval sea star body. Rao *et al.* (1993) observed larval budding in asteroid larvae from plankton samples collected from coastal waters of Visakhapatnam, India, and presented information on the frequency of this phenomenon. Most recently, cloning and regeneration of larval ophiuroids were reported (Balser, 1998).

Collectively, these studies suggest that not only adult echinoderms but also their larvae may be capable of both regeneration and cloning, exploiting both to enhance their reproductive potential. Even in light of these reports, the occurrence of cloning is still widely considered to be an unusual event in most planktotrophic larvae of echinoderms.

Compared with nonfeeding lecithotrophic larvae, feeding planktotrophic larvae generally spend longer periods in the water column (Young and Chia, 1987); therefore, planktotrophic larvae are associated with high dispersal potential and extensive gene flow across broad geographic ranges in marine invertebrates (Levin and Bridges, 1995). Planktotrophic larvae are also known to have high mortality rates during their developmental

process when compared with nonfeeding lecithotrophic larvae. For instance, it has been estimated that a single female of the asteroid *Pisaster ochraceus* produces over 40 million ova (each a potential embryo) in her lifetime, with a cumulative mortality rate of over 99% (Menge, 1975). The existence of larval cloning in the natural environment suggests a mechanism for the enhancement of individual gamete survival. Hence larval regeneration and cloning in asteroids may be an adaptation to facilitate increased dispersal potential and lower mortality rates. On the other hand, larvae differ in their physiologic and morphologic adaptation to limited food sources (Strathmann, 1978); therefore, cloning may be an adaptation for increased survival under certain optimal environmental and biotic conditions of high food abundance, low population density, and increased seawater temperature.

The study animals used for the experiments presented in this dissertation were the sea stars *Luidia foliolata* and *Pisaster ochraceus* and the sand dollar *Dendraster excentricus* from the Pacific Northwest coast, and the sea urchin *Lytechinus variegatus* from the Gulf of Mexico. Both of the sea stars we examined possess planktotrophic larval development however, *L. foliolata* is considered to be primitive among sea stars, including *P. ochraceus* (Downey, 1973; Clark and Downey, 1992; McEdward and Janies, 1993). Embryos of *L. foliolata* develop into bipinnaria larvae and then complete metamorphose completely while swimming in the water column. In contrast, embryos of *P. ochraceus* first develop into bipinnaria larvae and then later develop into brachiolaria larvae. The fact that *L. foliolata* lack a brachiolaria larval stage is the major argument for its consideration as a more primitive form in comparison with *P. ochraceus* (McEdward and Janies, 1993), although others argue that *P. ochraceus*, with a brachiolaria stage, is more

primitive (Blake, 1988).

In the work presented in this dissertation we report for the first time regenerative capacity after surgical bisection in larvae of two representative sea star species with bipinnaria and brachiolaria developmental types (Dissertation Manuscript 1) and discuss in detail the differences in regenerative capacity in these two larval types (Dissertation Manuscript 2). In addition, we examine factors regulating regenerative and cloning capacities in laboratory larval cultures of these two sea star species, simulating the types of environmental conditions that might be encountered during their planktonic existence and offering the first confirmed report of cloning capacity in larvae of both species (Dissertation Manuscripts 3 and 4). Embryos and larvae of echinoids (sea urchins and sand dollars) have been employed extensively in embryologic research; however, the regenerative capacity of adult echinoids is very limited, and the regenerative capacity of larval echinoids has not been examined previously. Planktotrophic larval echinoids display many similarities with planktotrophic larval brittle stars (ophiuroids). Therefore, since cloning and *de novo* development of missing body parts during asexual reproduction had recently been reported in ophiuroid larvae (Balser, 1998), we examined and subsequently first documented regenerative capacity in larval echinoids in the sea urchin *L. variegatus* and the sand dollar *D. excentricus* (Dissertation Manuscript 5).

These studies were undertaken to further our understanding of regeneration and cloning in echinoderm planktotrophic larvae; it is our intention that they will provide a solid foundation for future studies of these remarkable phenomena. Echinoderms are adjacent to the chordates in most phylogenetic trees (Willmer, 1991; Halanych and Passamaneck, 2001) and, like the chordates, possess deuterostome type development. There-

fore examining the cellular mechanics and genetics of the regeneration and cloning processes in echinoderms not only will contribute to our understanding of these phenomena in invertebrates but may ultimately benefit our understanding of regeneration in chordates, including human beings.

REGENERATION IN ECHINODERM LARVAE

by

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The capacity for regeneration of lost body parts requiring organogenesis has never been documented in metazoan larvae. In contrast, regeneration is known to be relatively common in the juvenile and adult phases of the life history of select groups of metazoans, including the reptilia, amphibia, annelida, arthropoda, and echinodermata (Goss, 1969; Mattson, 1976; Lawrence, 1987). Some amphibian larvae can regenerate lost limbs, but this regeneration requires no organogenesis (Lawrence, 1987). Here we describe for the first time the capacity of metazoan larvae (planktotrophic bipinnaria and brachiolaria sea star larvae) to regenerate rapidly and completely to fully functional individuals after surgical division of the larval body. As planktonic larval mortality is considered to be extremely high (Thorson, 1950; Menge, 1975; Young and Chia, 1987), the capacity to completely regenerate larval tissues and organs lost to either partial predation or abiotic stress is likely to be an important adaptation to enhance recruitment into adult populations.

Larvae of the sea stars *Pisaster ochraceus* (bipinnaria and brachiolaria) and *Lu-idia foliolata* (bipinnaria) that had been surgically divided across the horizontal larval axis equidistant between the anterior and posterior poles regenerated into fully functional larvae over a 12- to 14-day period. No mortality occurred in any of the experimental or control larval treatments. The ontogeny of larval regeneration was similar in both species. Figures 1-3 show the regenerative process in the surgically divided larvae of *P. och-raceus*. On day 1, immediately following surgical manipulation (Fig. 1B, C), both the anterior and posterior portions of bipinnaria larvae (hereafter referred to as anterior and posterior larvae) displayed swimming behaviors similar to those of control larvae. Feeding was observed in the posterior larvae, indicating that these larvae do not lose the ability to capture and ingest food particles even when the upper portion of the esophagus and

mouth has been surgically removed. By day 3 the anterior larvae had begun to regenerate the surgically separated postoral ciliary bands, as well as elongate along the larval axis (Fig. 1D). These larvae displayed the ability to capture and transport phytoplankton to the region of the former larval mouth. By day 4, the coelom of posterior larvae had elongated and showed signs of fusing above the larval mouth (Fig. 1E). After one week, both the anterior and posterior larvae completed the regeneration of the digestive organs and coelom (Fig. 1F, G). At this point in the regenerative process, anterior larvae became competent to feed. Capture and ingestion of phytoplankton were observed in culture; guts of anterior larvae contained phytoplankton by day 9 (Fig. 1H). The posterior larvae completely regenerated the preoral ciliary bands by day 10 (Fig. 1I). By day 12 most anterior and posterior larvae had regenerated all of their body components (Fig. 1J, K), and surgically divided bipinnaria larvae of both sea star species were essentially indistinguishable from control larvae by day 14.

To determine whether regenerated bipinnaria larvae were capable of sustaining their regenerative capacity, a single bipinnaria from the regenerated posterior larval treatment of *P. ochraceus* was once again surgically divided. By day 4 this larva had attained a regenerative state similar to that seen after a 4-day period after the first surgical treatment (Figs. 2A, B). As regenerated larvae were further along in the developmental sequence, the posterior larva also displayed an elongation of posterior arms. By day 7, the digestive organs in the redivided anterior and posterior larvae had completely regenerated (Fig. 2C, D), and the posterior larva displayed continued elongation of the larval arms as it neared the brachiolaria stage (Fig. 2D).

Brachiolaria larvae of *P. ochraceus* that were surgically divided as described above showed a similar pattern of regeneration (Fig. 3). By day 3 the anterior and posterior larvae exhibited an elongation along the larval axis (Fig. 3B, C). Similarly to surgically manipulated bipinnaria, the posterior brachiolaria larva continued to feed throughout regeneration. By day 10 most anterior larvae had regained the capacity to feed, and both anterior and posterior larvae possessed regenerated coeloms, ciliary bands and digestive organs (Fig. 3D, E). Complete regeneration of lost body parts required a maximum of 14 days in brachiolaria of *P. ochraceus*.

In an additional experiment, bipinnaria larvae *of L. foliolata* that had attained a developmental stage distinguished by the presence of the adult rudiment (Fig. 4A) were surgically divided. By day 3 anterior larvae had begun to regenerate postoral ciliary bands and were capable of capturing but not ingesting phytoplankton (Fig. 4B). In contrast, by day 3 all posterior larvae had undergone complete metamorphosis into juveniles (Fig. 4C). Unmanipulated control larvae possessing juvenile rudiments did not undergo complete metamorphosis for at least another 10 days. Juveniles resulting from surgically divided posterior larvae were morphologically indistinguishable from juveniles resulting from control larvae (Mean experimental and control juvenile diameters were 0.5 ± 0 mm; $n = 3$ in each treatment.)

To the best of our knowledge our study is the first to demonstrate the capacity of metazoan larvae to regenerate lost body components that require organogenesis. Several investigators have manipulated very early echinoderm embryos, separating blastomeres at the 2- to 4-cell stage and documenting subsequent development to larvae (McEdward, 1996). In addition, asexual reproduction has been documented in planktotrophic larvae

of sea stars (Bosch *et al.*, 1989; Rao *et al.*, 1993; Jaeckle, 1994) and brittle stars (Balser, 1998). While the capacity for asexual reproduction in echinoderm larvae reflects the indeterminate developmental nature of deuterostomial embryonic cells, such observations provide no direct measure of the ability of larvae to regenerate lost body components.

Models of the recruitment dynamics of marine and freshwater invertebrates consistently factor in high estimates of larval mortality in species with planktonic larvae (Gaines and Lafferty, 1995). This high mortality is attributed to predation (Morgan, 1990; Shanks, 1995), abiotic factors such as hydrodynamic shear stress (Levitan, 1995), or dispersal to inappropriate habitats (Rummill, 1990). Our observations indicate that at least some larvae are capable of surviving and rapidly regenerating lost body parts after radical surgery that simulates partial predation or abiotically induced damage. Therefore, population models that include rate functions of larval mortality in the plankton may be utilizing mortality estimates that are unrealistically high. It is also significant that damaged sea star larvae possessing a juvenile rudiment rapidly metamorphose to the benthic juvenile phase, reducing time spent exposed in the plankton and refuting current hypotheses that resorption of the larval body is an energetic prerequisite to successful metamorphosis (Chia and Burke, 1978). Regenerative capacity may be particularly important in small, abundant, planktotrophic larvae that require longer periods to develop in the plankton (Emlet *et al.*, 1987) and exploit chemical defenses less often than large, less abundant, yolk-laden lecithotrophic larvae (Lindqust and Hay, 1996; McClintock and Baker, 1997).

MATERIALS AND METHODS

Adult sea stars (*Pisaster ochraceus* and *Luidia foliolata*) were collected using SCUBA in the early summer of 1997 in Puget Sound, Washington, USA. Mature gametes were obtained by intracoelomic injection of 10^{-4} M solution of 1-methyladenine (Kanatani, 1969). Fertilized ova were raised using standard culture protocols (Strathmann, 1987). Once larvae attained a feeding stage (bipinnaria) they were fed an *ad libitum* diet of mixed cultures of the single-celled marine algae *Chaetocerous calcitrans*, *Dunaliella tertiolecta* and *Isochrysis galbana*.

Bipinnaria (Figs. 1A and 2A) and brachiolaria (Fig. 3A) were gently held in a vertical fixed position with microforceps and surgically divided equidistant between anterior and posterior poles and horizontal to the larval axis (Figs. 1B, C; 2B, C). Only brachiolaria from *P. ochraceus* were examined because *Luidia* lack a brachiolaria stage (Chia *et al.*, 1993). Experimental and control (identical culture conditions but no manipulation) larval treatments consisted of sample sizes of 10 for *P. ochraceus* and 12 for *L. foliolata*, respectively. After surgical treatment, manipulated larvae were returned to separate culture vessels and cultured according to methods described above. Larval regenerative processes were documented daily using a Nikon compound microscope equipped with a camera. Daily observations were conducted to determine whether mortality occurred in either experimental or control treatments and to determine rates of regeneration of larvae in manipulated treatments.

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Figure 1. Bipinnaria of *Pisaster ochraceus* at various stages of regeneration. (A) Bipinnaria prior to surgical treatment. (B, C) Day 1. Anterior and posterior larvae after surgical division. Posterior larva with gut sufficiently intact for feeding. (D) Day 3 and (E) Day 4. Elongation along larval axis. (F, G) Day 7. Anterior larvae regenerates functional digestive system. (H) Day 9 through (I) Day 10. Complete regeneration of ciliary bands in both larvae. Note phytoplankton in gut of anterior larva. (J, K) Day 12. Both larvae have regenerated and are similar to control larvae. cb, ciliary band; cp, coelomic pouch; ds, digestive system; p, phytoplankton. Scale bar in Figure is 200 μm and applies to A-E. Scale bar in Figure F is 200 μm and applies to F- K.

Figure 2. Regeneration sequence of a regenerated bipinnaria of *Pisaster ochraceus* subject to a second surgical division. (A, B) Day 4. Elongation along the larval axis. (C) Day 7. Anterior larva regenerates digestive system. (D) Day 7. Posterior larva regenerates ciliary bands and elongates larval arms. Scale bar = 200 μm (A-D).

Figure 3. Brachiolaria of *Pisaster ochraceus* at various stages of regeneration. (A). Brachiolaria prior to surgical treatment. (B, C) Day 3. Elongation along larval axis. Posterior larva with gut sufficiently intact for feeding. D through E (Day 10). Both larvae complete regeneration of ciliary bands. Anterior larva regenerates complete digestive system. cb. ciliary band; ds. digestive system. Scale bar = 200 μm (A-E).

Figure 4. Metamorphosis induction of *Luidia foliolata*. (A). Bipinnaria of *Luidia foliolata* with adult rudiment. Scale bar = 200 μm. (B) Day 3. Anterior larva elongating along larval axis. C (Day 3). Juvenile resulting from metamorphosed posterior larva. Scale bar = 100 μm (B-C).

MORPHOGENESIS AND ORGANOGENESIS IN REGENERATING PLANKTOTROPHIC LARVAE OF THE SEA STARS *LUIDIA FOLIOLATA* AND *PISASTER OCHRACEUS*

by

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Format adapted for dissertation

Abstract

In a previous study, we described complete body regeneration (with organogenesis) after surgical bisection in the planktotrophic larvae of the sea stars *Luidia foliolata* and *Pisaster ochraceus* (Vickery and McClintock, 1998, Dissertation Manuscript 1). In the present paper we describe further detailed observations of these unique regenerative processes not presented in the previous paper. Larvae of both species displayed a capacity for rapid regeneration regardless of their developmental stage. Within 48 hours after bisection, aggregations of mesenchyme cells with pseudopodia were observed at the site of surgical bisection. These cellular aggregations were similar in appearance to the mesenchymal blastema that form in adult echinoderms prior to their arm regeneration and have been described in other deuterostomes that undergo regeneration. When larvae were surgically bisected in the early-stages of larval development, clusters of mesenchyme cells developed into completely new pairs of coelomic pouches located anterior to the newly regenerated digestive tract, indicating that cell fate in regenerating sea star larvae remains indeterminate during early development. In the larvae of *P. ochraceus*, regardless of the developmental stage at the time of bisection, both the anterior and posterior portions regenerate all their missing organs and tissues. However, the larvae of *L. foliolata* displayed differential regenerative capacity in bisected larval halves at the late bipinnaria stage. The differences observed may be caused by the differences in larval development (*L. foliolata* has no brachiolaria stage) and may have evolutionary implications. Our observations confirm that sea star larvae provide an excellent model for studies of regeneration in deuterostomes.

Introduction

Regeneration has been described at both the cellular and the tissue levels in many animals, including planarians, crustaceans, reptiles, and amphibians (Goss, 1969; Mattson, 1976; Baguñà *et al.*, 1989; Martin, 1997; Hopkins, 2001). Many adult echinoderms, including sea stars, brittle stars, and sea cucumbers, are widely known to possess considerable regenerative capacities, as well as the ability to reproduce by clonal division (Emson and Willkie, 1980; Mladenov and Burke, 1994). The occurrence of cloning in planktotrophic larvae of sea stars and brittle stars has also been reported (Bosch, 1988; Bosch *et al.*, 1989; Rao *et al.*, 1993; Jaeckle, 1994; Balser, 1998). However, complete regeneration in echinoderm larvae after surgical bisection has only recently been documented (Vickery and McClintock, 1998, Dissertation Manuscript 1). Bipinnaria and brachiolaria larvae of the sea stars *Luidia foliolata* and *Pisaster ochraceus* were surgically bisected into anterior and posterior portions, and the regeneration process was followed over a 2-week period. Both portions of the larvae in both species were observed to regenerate all missing organs and tissues completely. The discovery of regeneration in planktotrophic sea star larvae provided a new (and previously unknown) model system for the molecular study of regeneration in deuterostomes (M. C. L. Vickery, 2001; Vickery *et al.*, 2001a, b, submitted).

Sea star larvae provide a unique model for the study of regeneration that offers a number of advantages. For example, echinoderms share many developmental traits with other deuterostomes, including vertebrates (Willmer, 1991; Halanych and Passamaneck, 2001). Also, sea star larvae possess simple body structures compared with adults, and their transparent bodies make it possible to observe the regeneration process not only externally but also internally.

In the present study we performed experiments using larvae of *L. foliolata* and *P. ochraceus* to provide detailed information regarding the regeneration process at the cellular/organ level. We examined the regenerative capacity and organogenesis of larvae of both sea star species throughout all developmental stages. Since no other experiments conducted to date have examined the ability of discrete stages of larvae to regenerate, this study provides the first assessment of this capacity. We also report on the regenerative capacity of surgically removed portions of the larval body in *P. ochraceus*.

Materials and Methods

Sexually mature *Luidia foliolata* and *Pisaster ochraceus* were collected near San Juan Island, Washington, in the late spring to early summer of 1998. Artificial fertilizations were conducted by intracoelomic injection of 1-methyladenine (Kanatani, 1969), and ambient seawater temperature (12-15°C) was maintained during the experiments. After the embryos established functional digestive systems, larvae were fed a quantitatively equal mixture of the single-celled algae *Chaetocerous calcitrans*, *Dunaliella tertiolecta*, and *Isochrysis galbana*. Standard protocols for larval culturing were followed (Strathmann, 1987).

First we investigated larval regenerative capacities throughout three major stages of larval development. The three representative developmental stages chosen were based upon larval anatomy. Early-stage larvae were defined for both species as embryos (Fig. 1A) that had developed into early bipinnaria larvae with a functional digestive system and a pair of nonconnected coelomic pouches (Fig. 1B). Mid-stage larvae were defined as fully grown bipinnaria larvae in *L. foliolata* (Fig. 1C) and as either fully developed bipin-

naria (Fig. 1D) or brachiolaria (Fig. 1E) larvae in *P. ochraceus*. All mid-stage larvae possessed long larval arms and, in *P. ochraceus* brachiolaria larvae, possessed a brachiolar apparatus in the anterior region. Internally, the left and right coelomic pouches were connected at the anterior ends of the larvae; in brachiolaria larvae, the brachiolar apparatus possessed an underlying coelom. Late-stage larvae were defined as either bipinnaria or brachiolaria larvae with an adult rudiment on the posterior portion. At this late stage, the coelom had formed hydrolobes that would eventually form the water vascular system in the juvenile. Moreover, spicules were present that would eventually form the juvenile skeletal system.

At each discrete larval stage, subsamples of larvae ($n = 20$) of both species were surgically bisected with a scalpel across the horizontal larval axis at a point equidistant between the anterior and posterior poles after first measuring their longitudinal length. Bisected anterior and posterior portions and nonbisected control larvae ($n = 20$) for each stage were maintained separately over a 2-week period in fingerbowls placed in a water table with a circulating seawater system and were examined daily using bifocal dissecting and compound light microscopy. Their growth, as determined by their increase in length, was measured daily. The seawater in each fingerbowl was changed every 3 days (Strathmann, 1987). A Nikon Optiphoto-2 compound microscope was used to take photographs of larvae. All the measurements made during the experiments were analyzed statistically, and $P < 0.05$ was employed to assess statistical significance.

To further assess larval regenerative capacity further, we examined *P. ochraceus* because this species possesses both bipinnaria and brachiolaria larval stages. The far anterior tips of the larval body were surgically removed in both larval stages (Fig. 1C, D). The

anterior tip containing the axocoel was removed from both mid-stage bipinnaria (n = 20) and brachiolaria (n = 20) larvae (including the brachiolar apparatus) (Fig. 1C, D). Moreover, sets of posterior larval arms were removed from brachiolaria larvae (n = 20) (Fig. 1C, D). The dissected fragments and the larvae from which they were removed were maintained separately and observed over a 2-week period as described above.

Results

Regeneration in early-stage larvae

Surgical bisection was performed 21 days after fertilization in early-stage larvae of *Luidia foliolata*. Prior to bisection the bipinnaria larvae measured 710 ± 80 μm in length (n = 20, Fig. 2A). After surgical bisection, both the anterior and the posterior portions of the larvae continued utilizing their ciliary bands to swim and collected phytoplankton to feed. Although the anterior portions had lost the gut due to surgical bisection, phytoplankton was still accumulating in the oral cavity. The posterior portions of the larvae collected phytoplankton directly through the esophagus. Within 24 hours, rough edges of tissues originally seen at the site of bisection in both the anterior and posterior portions of the larvae had become smooth. Within 48 hours of bisection, mesenchyme cells with pseudopodia appeared at the site of bisection (Fig. 2B, E) in both the anterior and posterior larval portions. In the anterior portions, a thickening of the former upper esophagus/lower mouth region became evident (Fig. 2B); aggregations of mesenchyme cells around this thickened area became more prominent. Eventually this thickened wall invaginated into the body cavity, forming a tube structure (Fig. 2C). Similar mesenchyme cell aggregations

were observed in the bisected posterior portions at various locations throughout the regenerating larval body, most notably at the site of bisection.

The anterior portions of bisected larvae gradually decreased in size after bisection (immediately after bisection = 306 ± 47 μm in length; 11 days later = 233 ± 29 μm in length; n = 20 ± 1 SD). However, the posterior portions of the bisected larvae, which retained a functional digestive system and continued feeding throughout the entire regeneration process, continued to grow (immediately after bisection = 378 ± 31 μm in length; 11 days later = 495 ± 136 μm in length; n = 20 ± 1 SD). Seven days after bisection (28 days after fertilization), the aggregation of mesenchyme cells (described above) seen in the anterior portions of the larvae appeared to be involved in development of a new pair of coelomic pouches that exvaginated from the anterior portion of the newly formed "digestive tube" (Fig. 2C). The digestive tube then elongated toward the posterior, eventually reconnecting to the posterior portion of the larval wall, forming an opening that eventually became a new anus (Fig. 2D). By 7 days after bisection the posterior portions of the bisected larvae had almost completely regenerated the mouth (Fig. 2F, G), and an elongation of the coelomic pouches was prominent. Once the anterior portions of the bisected larvae had reformed a complete digestive tube, actual feeding and digestion of phytoplankton began; differentiation of the digestive tube into a new esophagus, stomach, and intestine was observed by day 10 (Fig. 2D). After approximately 12-14 days, both the anterior and posterior portions of the bisected larvae had completely regenerated all missing body structures, including all internal organs.

Based upon our observations, we constructed a schematic diagram of regeneration and organogenesis of the anterior portion of bisected sea star larvae (Fig. 3). Immediately

after bisection, the anterior portion is left with no coelom components (Fig. 3Aa) but retains one complete preoral ciliary band and one incomplete postoral ciliary band. Invagination of the body wall begins in order to regenerate the missing digestive system (Fig. 3Bb, Cc), eventually forming a new anus (Fig. 3Dd). During this time, the postoral ciliary band extends toward the posterior. Meanwhile, from the anterior portion of the new gut, an entirely new set of coeloms is formed by lateral exvagination of the newly formed digestive system (Fig. 3Ee), which then separate from the gut and elongate anteriorly and posteriorly alongside the newly formed digestive system (Fig. 3Ff).

Afterward, complete differentiation of the newly formed digestive system, completion of regeneration of the lost components of the postoral ciliary band, and further development of both coeloms are observed (Fig. 3Gg). Our observations of the regeneration process in early-stage bipinnaria larva of *P. ochraceus* were virtually identical to those described above for *L. foliolata*.

Regeneration in mid-stage larvae

Regenerative processes in bisected mid-stage larvae of both *L. foliolata* and *P. ochraceus* were observed to be virtually identical to those described above for early bipinnaria larva of *L. foliolata* (as previously reported by Vickery and McClintock, 1998, Dissertation Manuscript 1). The only difference was that the anterior portions of mid-stage bisected larvae contained an axocoel and therefore did not have to regenerate the coelomic pouches as they were already present. Bisected anterior portions continued to swim and collect phytoplankton in the oral cavity (Fig. 4A). An invagination of the body wall occurred after the appearance of mesenchyme cells, and the coelomic pouches elongated to-

ward the posterior (Fig. 4B). A new digestive system completed regeneration approximately one week after bisection (Fig. 4C). The posterior portions, which retained a functional gut, continued to digest phytoplankton (Fig. 4D). During the process of regeneration of the lost anterior portions, mesenchyme cells with pseudopodia were evident (Fig. 4E).

Regeneration in late-stage larvae

In *L. foliolata*, the anterior portions of bisected larvae underwent regeneration as described above for mid-stage bipinnaria larva (n = 30, Fig. 5A). However, the posterior portions of bisected larvae, each possessing a juvenile rudiment, invariably metamorphosed into juveniles within 3 days (n = 30, Fig. 5B) (Vickery and McClintock, 1998). The resulting juveniles measured approximately 500 μm in diameter, which was not statistically different ($P > 0.05$) from the size of control juveniles (n = 20) from nonbisected larvae.

In contrast, both the anterior and posterior portions of late-stage larvae of *P. ochraceus* underwent complete regeneration without exception and with no mortality (n = 350, Fig. 5C, D) in a process similar to that described above for mid-stage larvae. However, formation of a new juvenile (adult rudiment, including the spicules and hydrolobes) in association with the anterior portions of bisected larvae was observed to occur at an accelerated rate (within only 7 days). In comparison, the control larvae required an additional 7-10 days (14-21 days total) to form an equivalent adult rudiment on the posterior portions of the larvae. Within 7 days of late-stage bisection, the anterior portions of bisected larvae were morphologically and functionally indistinguishable from control larvae

and later metamorphosed into juveniles that were indistinguishable both morphologically and functionally from juveniles obtained from nonbisected control larvae.

During the regeneration process of the posterior portions of bisected larvae, the formation of a new larval body was observed in each posterior portion (Fig. 5E). The newly formed secondary larval body first developed into a bipinnaria (Fig. 5F) and then later formed a brachiolar apparatus, without exception (Fig. 5G). After completing regeneration of the brachiolar apparatus, the posterior portions metamorphosed into juveniles that were morphologically and functionally indistinguishable from juveniles resulting from nonbisected control larvae. The juveniles resulting from metamorphosis of regenerated larvae (both anterior and posterior portions) measured approximately 500 μm in diameter ($n > 20$), a size not found to be statistically different ($P > 0.05$) from that seen in control juveniles ($n = 20$) from non-bisected larvae.

Regeneration of partial larval body parts in Pisaster ochraceus

The far ends of the anterior tips were removed from both bipinnaria and brachiolaria larvae of *P. ochraceus* ($n = 10$ each). Immediately after removal, the anterior tips, which contained both the axocoel and portions of the preoral and postoral ciliary bands, continued to swim (Fig. 6A). After 2 weeks the anterior tips showed no signs of regeneration but were observed both to retain their ability to swim and to display muscle contractions (Fig. 6B). In contrast, the posterior portions of the larvae partially regenerated (Fig. 6C) and proceeded to become brachiolaria larvae. When the tips were removed from brachiolaria larvae, the tips, which retained the brachiolar apparatus, continued to swim using cilia (Fig. 7A); however, after 2 weeks no signs of regeneration of the larval body

were evident (Fig. 7B). The posterior portions did regenerate the lost brachiolar apparatus within 7 days, as shown in Fig. 7C.

Regeneration of short, stubby arm buds was observed in many brachiolaria larvae when the larval arms were surgically removed (Fig. 8A). In most cases, larval arms regenerated but never reached the same length as before. Sometimes no regeneration of the larval arms occurred at all. The severed larval arms continued to swim in a spiral fashion (Fig. 8B); after one week, muscular contractions could still be observed in the still-swimming severed arms (Fig. 8C). After 2 weeks, no regeneration was observed in the severed arms; however, it is noteworthy that the arms themselves continued to swim actively with no mortality (Fig. 8D).

Discussion

The present study demonstrates that planktotrophic larvae of the sea stars *Luidia foliolata* and *Pisaster ochraceus* possess extensive regenerative capacities regardless of their developmental stage. In early- and mid-stage surgically bisected larvae, both species regenerated missing body components within 2 weeks; absolutely no mortality occurred due to bisection, a result similar to our previous observations (Vickery and McClintock, 1998, Dissertation Manuscript 1). Mesenchyme cells possessing pseudopodia were observed to play a regenerative role in the formation of an entirely new digestive system. In early-stage larvae, a new pair of coelomic pouches was formed by exvagination of the anterior portion of the newly formed digestive system in association with aggregations of mesenchyme cells (possessing pseudopodia). A similar type of coelom formation was documented by Runnström in sea urchin larvae after surgical manipulation (reviewed in

Vickery *et al.*, 2001b). The resultant coelomic structures are essential body components that eventually form the body cavity in adult organisms, ultimately surrounding the digestive and reproductive organs. Coelomocytes (located within the coelom) have been reported to play a role in regenerative processes in adult echinoderms (Thorndyke *et al.*, 1999). Therefore it is noteworthy that our observations indicate that bisected larvae, lacking coelomic pouches in their anterior regions, completed regeneration. Thus coelomocytes do not appear to play a critical role in larval regeneration.

In adult sea stars, regeneration has been reported to be initiated by a proliferation of epidermal cells forming a mesenchymatous blastema, which later gives rise to the missing body structures (Candia Carnevali and Bonasoro, 1994; Thorndyke *et al.*, 1999). Our observations demonstrate that regenerative processes occur in a similar fashion in planktotrophic sea star larvae. Our microscopic examinations revealed that a proliferation of cells was initiated at the larval epithelium near the surgical plane. We observed that during the process of regeneration these cells appeared to dedifferentiate into mesenchymal-like cells and then to redifferentiate into new types of cells that later gave rise to the regenerated structures (Thorndyke *et al.*, 1999). However, further confirmation is necessary.

Cell fate in echinoderm larvae has previously been considered to be determinate and irreversible once larvae attain discrete developmental stages (Cameron *et al.*, 1987; Thorndyke *et al.*, 1999). Nonetheless, the flexibility of developmental pathways and morphogenesis has been questioned lately (Balser, 1998). Our results demonstrate that fully differentiated larval body tissues are capable of additional proliferation and apparent dedifferentiation and redifferentiation in order to reconstruct missing body parts, including the larval epidermis and coelomic pouches, suggesting organogenesis from previously dif-

ferentiated cells. Moreover, our observations are supported by those of Balser (1998) in asexually cloning ophiuroid larvae, in which cloning apparently required both development and growth of new tissues, as well as a reorganization of some existing tissues. Thus many cells apparently remain omnipotent in the larval stages, at least in the planktotrophic echinoderm species examined to date.

Peterson *et al.* (1997), discussing larval forms with what they termed maximal indirect development (including planktotrophic echinoderm larvae), suggested that larva-specific embryonic cell lineages with a fixed fate and a limited capacity for division give rise to the larval structures, while pluripotent "set-aside" cells, capable of relatively unlimited cell division, give rise to the adult form. The results of the present study and others (Jaeckle, 1994; Balser, 1998; Vickery and McClintock, 1998, Dissertation Manuscripts 1 and 5) suggest that in planktotrophic echinoderm larvae the fate of larval-specific cells is not fixed and that their division capacity does not appear to be limited to only a few division cycles as was suggested by Peterson *et al.* (1997). Since it has been demonstrated that omnipotent/pluripotent cells in the larval body are capable of replacing missing larval structures, it is questionable whether pluripotent set-aside cells are required to give rise to the adult structures as was suggested by Peterson *et al.* (1997). Further studies will shed more light on this subject.

In both species we examined, the anterior portions of bisected larvae lost their functional gut as a result of surgical bisection, retaining only the upper part of the mouth and oral cavity. Therefore these larval anterior portions could not feed on phytoplankton until they had regenerated a functional digestive system and were observed to decrease in size until they had regenerated a functional digestive system (a period of 7 days) and re-

stored their capacity to feed. It is possible that they obtained some nutrients directly through the larval epidermis in the form of dissolved organic matter (DOM) from the surrounding seawater (Jaeckle and Manahan, 1989; Manahan, 1990). Also, the bisected, regenerating anterior portions apparently reabsorbed some larval body parts as a nutrient source during regeneration of the digestive system (thus explaining their decrease in body size). Absorption and utilization of the larval body during regeneration were suggested by Chia and Burke (1978). In contrast, the posterior portions of bisected larvae, which retained functional digestive systems from the esophagus to the anus (and therefore continued to actively feed) increased in size over a 2-week period. Both anterior and posterior portions of bisected larvae of both species somehow obtained the required energy/nutrition to support regeneration of their missing structures through active feeding, absorption of DOM, or absorption of larval body tissues. Future investigations are necessary to evaluate the energetics of the regeneration process in planktotrophic sea star larvae.

In late-stage larvae, the anterior portions of both species regenerated in a process identical to that described for the early- and mid-stage larvae. The posterior portions (with adult rudiment) of *L. foliolata*, however, instead of regenerating their missing anterior larval components, rapidly completed metamorphosis into juvenile sea stars within 3 days of bisection (Vickery and McClintock, 1998, Dissertation Manuscript 1). Apparently surgical bisection either initiated or dramatically accelerated the process of metamorphosis suggesting that incorporation of the entire larval body mass into the juvenile is not required during the metamorphic process, and therefore contradicting the report of Chia and Burke (1978) for larvae of the sand dollar *Dendraster excentricus*. Future studies examining the

energetics of larval regeneration and metamorphosis and comparing the internal anatomy, survival, fate of juveniles resulting from bisected larvae with the internal anatomy, survival, fate of juveniles resulting from nonbisected controls will be of great interest.

In contrast, the posterior portions of *P. ochraceus* larvae (which lacked the brachiolar apparatus) did not readily complete metamorphosis after surgical bisection. Instead they regenerated their missing larval anterior components, including the brachiolar apparatus, prior to metamorphosis. The brachiolar apparatus is considered to be important for metamorphosis (Barker, 1978). These observations are likely caused by developmental differences (described below) that characterize *L. foliolata* and *P. ochraceus*. *L. foliolata* is a member of the Luidiidae family, which lacks a brachiolaria larval stage in its ontogeny (Chia *et al.*, 1993). These larvae complete metamorphosis while swimming in the water column (Chia *et al.*, 1993). In contrast, *P. ochraceus* is a member of Asteridae family and possesses a brachiolaria larval stage. *P. ochraceus* larvae require attachment of the brachiolar apparatus to a favorable substrata to complete metamorphosis (Barker, 1978; Chia *et al.*, 1993). Therefore, it is possible that metamorphically competent posterior portions of bisected larvae of *L. foliolata*, which do not require a brachiolar apparatus for substrata attachment to trigger metamorphosis, would rapidly complete metamorphosis after surgical bisection, thus avoiding unnecessary feeding and growth to reform anterior tissues that are not required for metamorphosis. The remaining portion of the larval body is absorbed into the adult rudiment during metamorphosis. In contrast, *P. ochraceus* requires the brachiolar apparatus for attachment and metamorphosis; thus it is reasonable that this structure would have to be regenerated prior to metamorphosis.

A number of theories about the evolution of developmental modes in asteroids and marine invertebrate larvae in general have been proposed (reviewed by McEdward and Janies, 1993; Wray and Raff, 1991). Within planktotrophic modes of development in Asteroidea, the Luidiidae are often considered to possess more primitive development than the Asteridae (Downey, 1973; Clark and Downey, 1992; McEdward and Janies, 1993). Others argue exactly the opposite (Blake, 1988). Our observations do not clearly support either proponent, however, they do demonstrate the requisite of possessing the brachiolar apparatus for development in the Asteridae and the clearly regenerative differences between Luidiidae and Asteridae.

In instances where small portions of the larval body were removed from *P. ochraceus*, regeneration of the missing portion of the larval body after removal of these small portions usually occurred. Some of these surgical procedures produced anterior larval body fragments almost identical to those observed by Jaeckle (1994) in larva from plankton tow samples, which were produced by autotomization of the far anterior portion of the larval preoral lobe. Jaeckle (1994) reported that development of this detached anterior fragment of the larval body into a bipinnaria larva occurred in 1-2 days. We did not consistently observe regeneration of the entire larval body from these surgically removed far anterior portions of the preoral lobe over the 2 weeks they were followed. However, In Jaeckle's (1994) study the fragments were kept at tropical seawater temperature; thus the developmental processes might have been accelerated as compared with the larvae from the present study that were maintained at the cold temperature of the seawaters of the Pacific Northwest.

Exceptions were limited to brachiolaria larvae that had lost their larval arms. In mid-stage larvae, missing portions were regenerated even as development progressed. (The adult rudiment was forming) Interestingly, no mortality of small larval fragments was observed over the 2-week course of these studies. Subsequent observations indicated that these small larval pieces continued to swim in the water column for up to 4 weeks, providing evidence that larvae or larval parts may obtain energy/nutrients through the uptake of DOM from the surrounding seawater (Manahan, 1990; Jaeckle and Manahan, 1992).

The area of regeneration of echinoderm larvae has only begun to be explored (Balser, 1998; M. C. L. Vickery, 2001; Vickery *et al.*, 2001a, b; Vickery and McClintock, 1998, Dissertation Manuscripts 1, 2, and 5). Echinoderms share many developmental traits with other deuterostomes. Therefore understanding the processes of regeneration in echinoderm larvae may lead to a better understanding of regeneration in many higher animals, including vertebrates.

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Figure 1. Schematic diagram of two different types of planktotrophic larval sea star development. The embryo (A) develops into a planktotrophic bipinnaria larva (B). Bipinnaria larvae of *Luidia foliolata* develop into a late bipinnaria (C) and complete metamorphosis to become juvenile sea stars (F) while swimming in the water column. Bipinnaria of *Pisaster ochraceus* (D) develop into a brachiolaria (E), after which they complete metamorphosis to the juvenile stage (F). Arrows with * in (C) and (E) show area of larva removed to examine regeneration of partial body parts. a,anus; bra, brachiolar apparatus; cp, coelomic pouches; ds, digestive system; la, larval arms; m, mouth; s:,sucker.

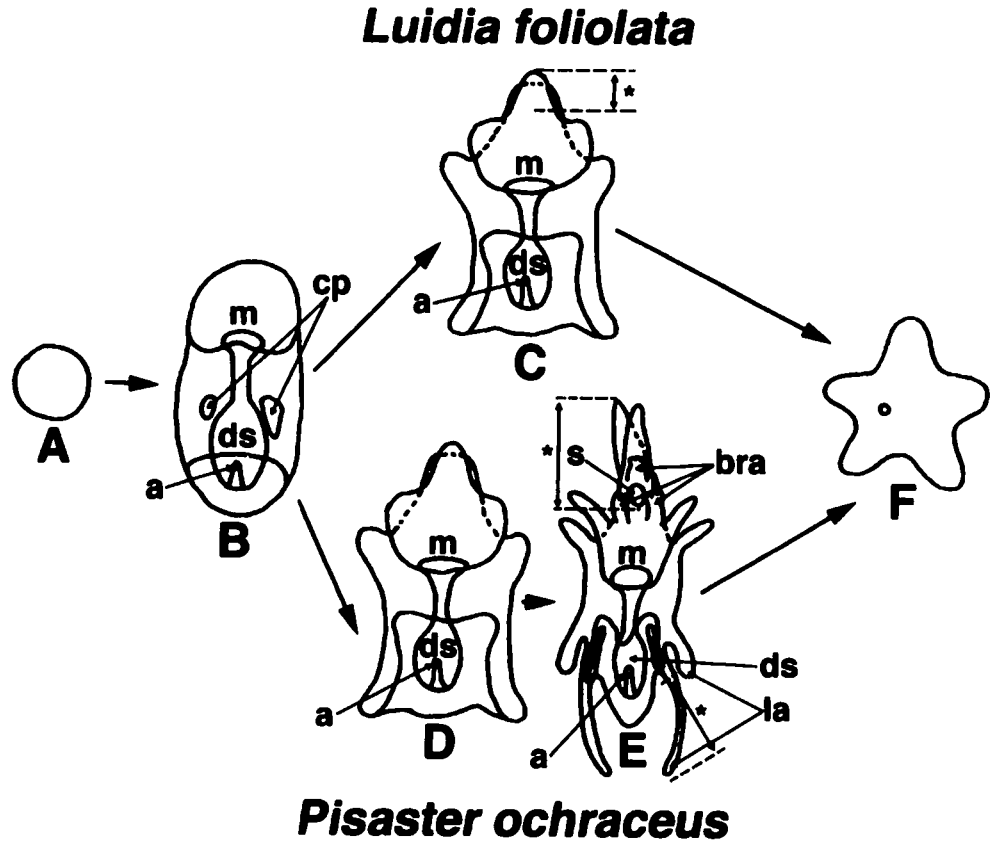


Figure 2. Light photomicrographs of regenerating early-stage bipinnaria larvae of *Luidia foliolata*. (A) 21 day old bipinnaria larvae with a pair of coelomic pouches located on the sides of the larval esophagus. Two days after bisection, mesenchyme cells are apparent at the surgical plane in the anterior (B) and posterior (E) portions. Note that the anterior portion (B) does not possess any coelomic components after surgical bisection and that a thickening of the epidermis at the rear part of the former mouth is evident. Three days after bisection, the larval epidermis invaginated into the body cavity, forming a new pair of coeloms originating from the upper portion of the newly formed rudimental gut. The posterior portion (F) increases in size, and regeneration of the missing mouth and elongation of the right and left coeloms are evident (F). Seven days after bisection, the newly formed coeloms are clearly separated from newly-formed gut in the anterior portion (D); further extension of the right and left coelomic pouches is evident, and the lost mouth has completely regenerated (G). Scale bars = 200 μm. a, anus; cb, ciliary band; cp, coelomic pouches; e, esophagus; g, gut; m, mouth; mc, mesencyme cell; rg, rudimental gut; s, stomach.

Figure 3. Schematic diagrams of organogenesis in regenerating early-stage anterior portions of surgically bisected regenerating bipinnaria larvae. (A-G) represent a frontal view, (a-g) represent a lateral view. (A) and (a) represent the anterior portion immediately after surgical bisection. (B) and (b) through (D) and (d) represent the formation of a new gut described in Fig. 2B, C. (E) and (e) through (G) and (g) represent the formation of a new set of coeloms and differentiation of the newly-formed gut into esophagus, stomach, and intestine described in Fig. 2D. a, anus; cb, ciliary band; cp, coelomic pouch; g, gut, rcp, rudiment of coelomic pouch; rg, regenerating gut.

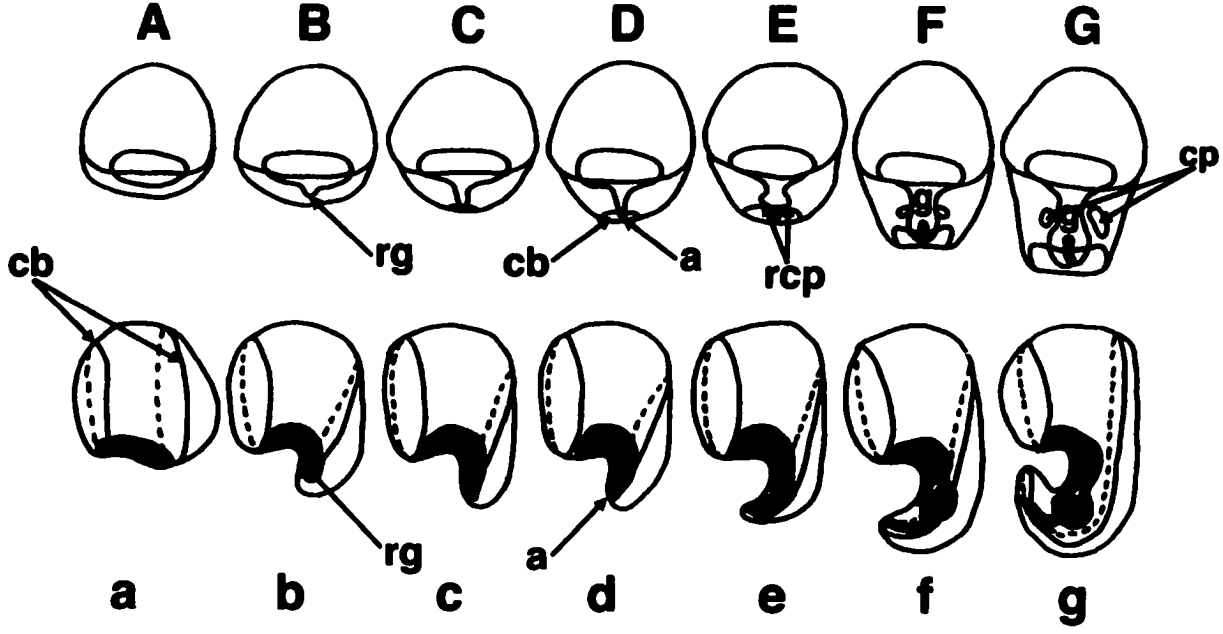


Figure 4. Light photomicrographs of regenerating mid-stage larva of *Pisaster ochraceus*. (A-C) represent an anterior larval portion and (D-E) represent a posterior larval portion. The anterior portion continues to swim in a fashion similar to the non-bisected control larvae and collects phytoplankton into the oral cavity using the ciliary bands (A), although it lost its digestive system during the surgical bisection. (B) shows invagination of the thickened rear part of the former mouth into the body cavity to form a rudimental digestive tube, which later becomes a new digestive system. A lateral view of the anterior portion (C) shows how the rudimental gut forms an external opening, forming a new anus. Note that many mesenchyme cells with pseudopodia are present. The posterior portion (D) continues to swim and feed and increases in size even during regeneration. (E) shows the newly regenerated anterior portion containing mesenchyme cells with pseudopodia. Scale bars = 200 μm. a, anus; cp, coelomic pouches; m, mouth; mc, mesenchyme cell; p, phytoplankton; rg, rudimental gut.

Figure 5. Three days after bisection of a late-stage larva of *Luidia foliolata*, the anterior portion is shown in the process of regeneration (A). In contrast, the posterior portion completes metamorphosis and becomes a juvenile (adult) (B), as we previously reported to occur (Vickery and McClintock, 1998). After three days, in late-stage larvae of *Pisaster ochraceus*, the anterior portion has partially regenerated the lost primary juvenile (adult) rudiment, forming a secondary juvenile (adult) rudiment on the posterior portion. Approximately 7 days after bisection, a lateral view of the anterior larva (D) shows complete regeneration of a secondary juvenile (adult) rudiment, during which the larval arms are absorbed, suggesting a progression of the process of metamorphosis. Three days after bisection, a secondary larval body is in the process of regenerating from the bisected primary juvenile (adult) rudiment (E). The secondary larval body then becomes a seemingly normal late-stage bipinnaria larva (with juvenile rudiment) after 7 days (F). By approximately 9 days after bisection, the secondary larval body (with juvenile rudiment) has regenerated the lost brachiolar apparatus (G). Scale bars = 200 μm. bra, brachiolar apparatus; pl, primary larval body; pj, primary juvenile (adult) rudiment; rsj, rudiment of secondary juvenile (adult); sl, secondary larval body; sj, secondary juvenile (adult).

Figure 6. Two weeks after surgical removal of the far anterior tips of the bipinnariae of *Pisaster ochraceus*, the anterior tips showed no signs of regeneration of the larval body but continued to swim actively, while the posterior portions had partially regenerated the missing tips. (A) Anterior tip (including axocoel) immediately after bisection. (B) Anterior tip 2 weeks after bisection. (C) Posterior portion approximately one week after bisection. Scale bars = 50 μm in (A-B), 200 μm in (C).

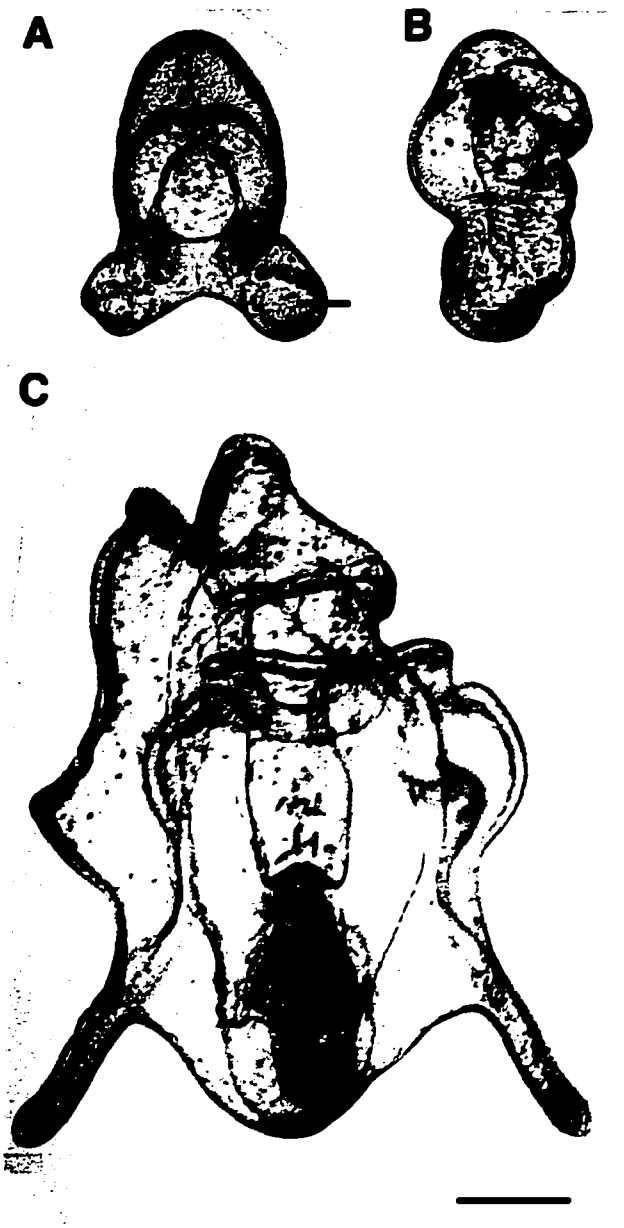


Figure 7. No regeneration of the larval body from the surgically removed brachiolar apparatus was observed in *Pisaster ochraceus*, but the larval posterior portion regenerated the brachiolar apparatus within 7 days; within 2 weeks the larva was morphologically and functionally indistinguishable from nonbisected control larvae. (A) A swimming brachiolar apparatus with axocoel immediately after surgical removal. (B) A swimming brachiolar apparatus (lateral view) 2 weeks after surgical removal. Morphologic changes are evident. (C) Larval posterior portion with regenerating brachiolar apparatus (arrow) approximately 5 days after surgical bisection. Scale bars = 50 μm in (A,B), 200 μm in (C).

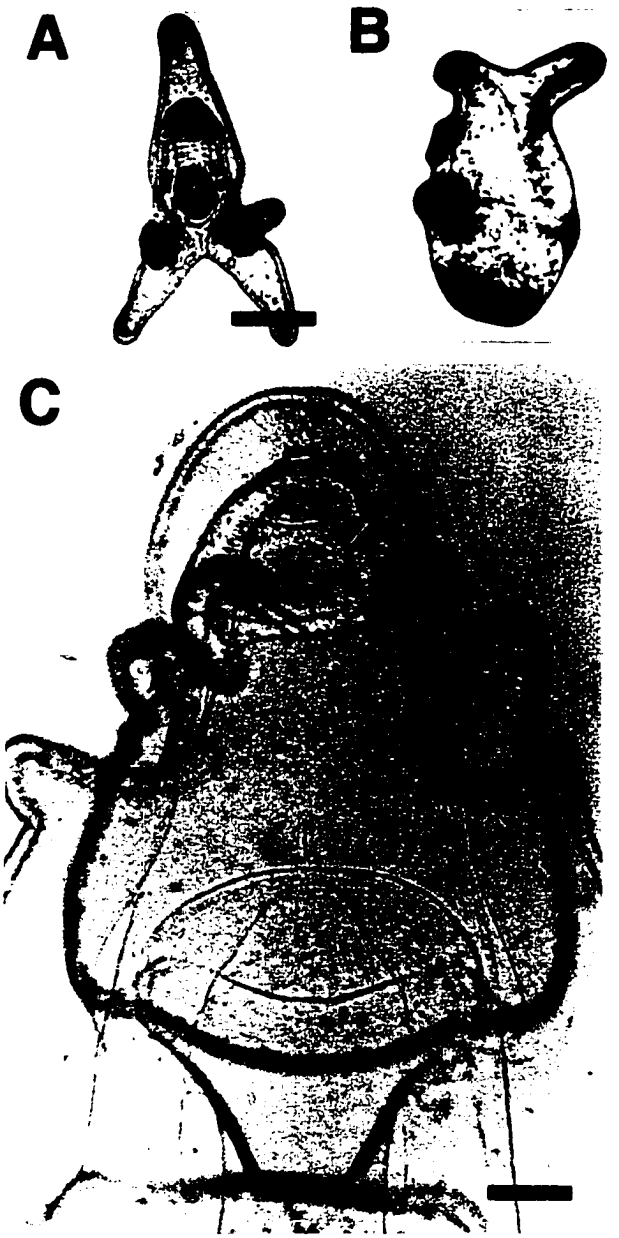
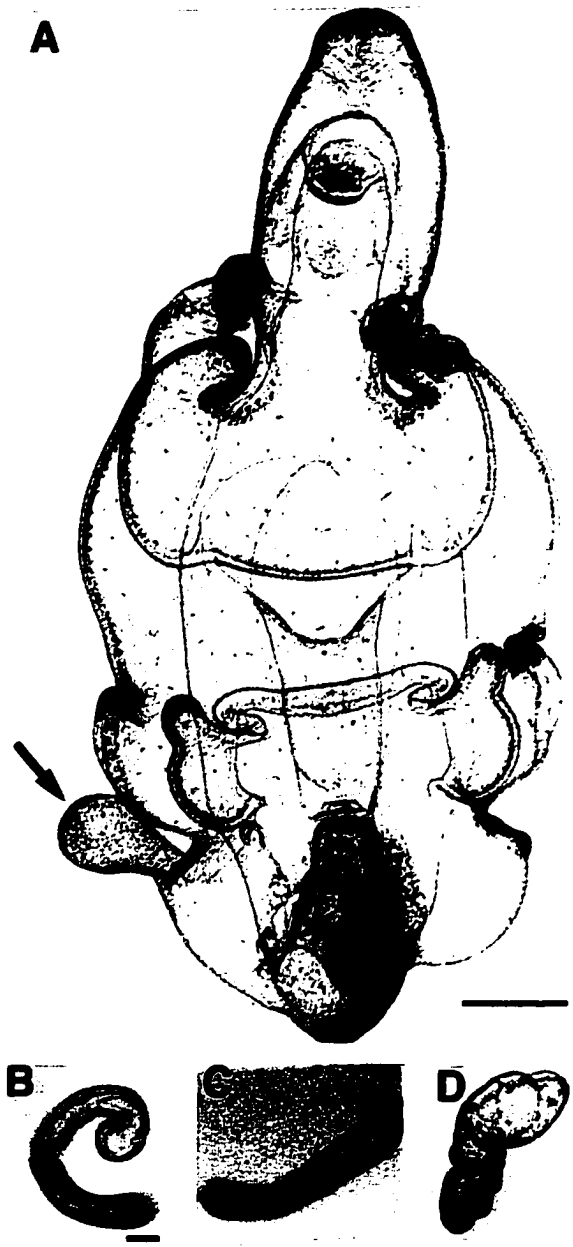


Figure 8. When posterolateral arms were removed from *Pisaster ochraceus* brachiolaria larvae, the formation of (arm) buds was often observed at the site of amputation, while the severed arms continued to swim (with no sign of regeneration) for over 2 weeks. In most cases, regenerated posterolateral arms never reached the same length as before. (A) Regenerating (arm) bud (arrow). (B) Severed posterolateral arm immediately after bisection. After 2 weeks the severed arm still continued to swim actively (C), and muscle contractions were evident (D). Scale bars = 200 μm in (A), 50 μm in (B-D).



AN EXAMINATION OF THE EFFECTS OF QUANTITATIVE AND QUALITATIVE DIFFERENCES IN FOOD AVAILABILITY ON REGENERATION IN PLANKTOTROPHIC LARVAE OF THE SEA STAR *PISASTER OCHRACEUS*

by

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Format adapted for dissertation

Abstract

We examined the role of food quantity and quality on regeneration in planktotrophic larvae of the sea star *Pisaster ochraceus*. Late bipinnaria/early brachiolaria larval stages were surgically bisected and anterior and posterior portions of bisected larvae were either starved; fed three concentrations of monospecific diets of the phytoplankton *Chaetocerous calcitrans, Dunaliella tertiolecta*, or *Isochrysis galbana*; or fed three concentrations of mixed diets comprised of equal numbers of cells of the three phytoplankton species. Control larval cultures consisted of nonbisected larvae fed the highest concentration of mixed phytoplankton over the 20-day experimental period. Regeneration occurred in the bisected anterior and posterior portions of larvae regardless of a complete lack of food or differences in food concentrations or food mixtures. Anterior portions of larvae starved or maintained on low or medium concentrations of single or mixed diets generally showed no growth or decreased in length over the experimental period. In contrast, while posterior portions of larvae starved or fed the lowest concentration of phytoplankton did not grow, those fed medium or high levels of single or mixed diets attained sizes equivalent to nonbisected fed control larvae and also grew larger than posterior portions starved or fed low concentrations of food. These patterns of shrinkage or growth are likely related to the retention of a digestive system in the posterior portion of larvae post-bisection, whereas anterior portions required at least one week for regeneration of a functional gut. Food availability, while not essential to regeneration *per se*, may influence ultimate larval quality (size). Energetic and nutritional requirements associated with regenerative processes, particularly in starved regenerative individuals or in larvae that ini-

tially lack a functional gut, are likely to depend on absorption of dissolved organic material and/or resorption of larval body parts.

Introduction

A variety of environmental and biotic factors have been considered important when modeling patterns of survival and recruitment of marine invertebrate larvae (Connell, 1961; Dayton, 1971; Menge, 1975; Roughgarden *et al.*, 1985; Olson and Olson, 1989). Planktotrophic marine larvae may spend weeks to months in the water column and may be subject to starvation (Thorson, 1950; Vance, 1973), predation (Pennington *et al.*, 1986; Morgan, 1990; Morgan and Christy, 1997), or damage caused by physical factors such as water shear stress (Vickery and McClintock, 1998, Dissertation Manuscript 1). Recently discovered mechanisms that may help offset high rates of mortality in planktotrophic larvae and that complement the high fecundity associated with planktotrophy (Strathmann *et al.*, 1981) include larval fission (Bosch, 1988; Bosch *et al.*, 1989; Rao *et al.*, 1993; Jaeckle, 1994; Vickery and McClintock, 2000, Dissertation Manuscript 4) and larval regeneration (Vickery and McClintock, 1998, in press, Dissertation Manuscripts 1, 2, and 5).

While predators that capture larvae using filtration are likely to ingest intact larvae (Pennington *et al.*, 1986; Lindquist *et al.*, 1992; Lindquist, 1996), other small biting predators may inflict partial predation (Vickery and McClintock, 1998, Dissertation Manuscript 1), leaving behind a sufficient portion of the larva to allow successful regeneration. Moreover, the delicate planktotrophic larvae of some species may be vulnerable to damage via physical shear stresses caused by water motion (Denny, 1995). M. S.

Vickery (unpublished) conducted water shear simulations under controlled laboratory conditions and observed that planktotrophic larvae of the sea star *Pisaster ochraceus* were often torn into smaller portions by moderate stresses. Additional yet unknown factors may cause larval damage. Moreover, the documentation of regenerative capacity in a growing number of planktotrophic marine invertebrate larvae indicates that regeneration is under strong evolutionary selection (Vickery *et al.*, in press).

Vickery and McClintock (1998, in press, Dissertation Manuscripts 1, 2, and 5) demonstrated that complete regeneration occurs in the planktotrophic bipinnaria larvae of the sea star *Luidia foliolata* and in the bipinnaria and brachiolaria larvae of the sea star *P. ochraceus* after surgical bisection. To date no information is available on how the availability of different quantities or qualities of food may influence regenerative capacity or the quality of the resultant larvae. Planktotrophic larvae of echinoderms are generally considered to be obligate feeders of phytoplankton (Fenaux *et al.*, 1985, 1988; George, 1994; Boidron-Métairon, 1995). Nonetheless, it is possible that planktotrophic larvae may not necessarily require phytoplankton for normal development (Olson, 1987; Olson and Olson, 1989) and may absorb dissolved organic material (DOM) to assist with energetic requirements associated with development (Manahan, 1990; Jaeckle and Manahan, 1992). Similarly, Vickery and McClintock (in press, Dissertation Manuscript 2 and 5) suggest that if normal feeding processes are temporarily constrained in regenerating planktotrophic larvae, then the uptake of DOM and/or resorption of larval body tissues may be essential to meet energetic and nutritive demands.

The objectives of the present study included 1) examining the effect of varying the quantity of phytoplankton food on the regenerative capacity of larvae, 2) determining

whether regenerative capacity is influenced by food quality by comparing regeneration of body components in larvae fed single (low-quality) and mixed (high-quality) phytoplankton diets, and 3) assessing a measure of larval quality (larval size) in regenerative larvae either starved or presented phytoplankton diets differing both quantitatively and qualitatively.

Materials and Methods

Adult individuals of the sea star *Pisaster ochraceus* were collected from rocky substrates along East Sound, Orcas Island, WA, during the late spring of 1998. Ovaries and testes were dissected from sexually mature individuals. Mature ova were obtained by treating excised ovaries with 1-methyladenine (10^{-4} M) (Kanatani, 1969). Ripe sperm were diluted in filtered seawater prior to artificial fertilization. Embryos and larvae were cultured in 2.5-liter glass jars maintained at ambient seawater temperatures of 12-15°C after standard larval culture methodology (M. F. Strathmann, 1987). Observations and measurements of larval length were performed using a Wild N-5 dissecting microscope and a Nikon Optishot-2 compound microscope equipped with an ocular micrometer.

Once early bipinnaria larvae in the stock culture obtained functional digestive systems, they were placed on a diet consisting of an equal mixture of three single-celled algae that included *Chaetocerous calcitrans*, *Dunaliella tertiolecta*, and *Isochrysis galbana* (5×10^4 cells/ml seawater). These phytoplankton species are known to be consumed and support growth in larvae of *P. ochraceus* (M. F. Strathmann, 1987). Thirty-nine days after fertilization, a subsample of larvae removed from the stock culture measured 1575 ± 290 μm length (n = 20; measured along the larval axis). These larvae were determined to

be at the late-bipinnaria or early-brachiolaria stages of development. As larval cloning in *P. ochraceus* occurs during early periods of development when growth is rapid (Vickery and McClintock, 2000, Dissertation Manuscript 4), feeding experiments with regenerating larvae were initiated only after larvae were beyond these early developmental stages, thus avoiding any confounding influence of larval cloning.

A total of 14 experimental larval feeding treatments were prepared using different concentrations and combinations of phytoplankton. Three different concentrations (low = 5×10^2; medium = 5×10^3; high = 5×10^4 cells/ml seawater) of each phytoplankton species (single diets) or three similar concentrations comprised of equal proportions of each phytoplankton species (mixed diets) were supplied to cultures of either anterior or posterior portions of bisected larvae. Another two treatments consisted of bisected anterior or posterior portions of larvae that were starved throughout the experimental period. A final control treatment consisted of nonbisected larvae fed the highest concentration of mixed phytoplankton.

Surgical bisections of individual larvae were conducted according to the methodology of Vickery and McClintock (1998, Dissertation Manuscript 1). Phytoplankton cell counts were performed using a hemocytometer (modified after Fenaux *et al.*, 1994; Basch, 1996). Larvae in all experimental treatments and the control treatment were reared for a period of 20 days. Each treatment was replicated three times and consisted of 20 anterior or posterior portions of larvae (experimental treatments) raised in 200ml glass finger bowls. Fresh seawater and food were exchanged in each bowl every 4 days. Larval growth was determined by the increase in larval size. Larval sizes were measured (length along the larval axis) at regular intervals over the course of the 20-day experi-

ment. At the end of the experiment, the larval size was subtracted from the initial larval size and growth was compared among the different food levels within each of the food types utilizing nested analysis of variance with levels of significance set at $P < 0.05$.

Results

After bisection, all anterior and posterior portions of larvae completed regeneration over the 20-day experimental period regardless of the experimental food levels or food types. Moreover, starved anterior and posterior portions of larvae completed regeneration. No larval mortality was documented in any of the experimental treatments or the control treatment. Other than larval size differences, larvae were generally morphologically and functionally similar to nonbisected control larvae by the conclusion of the experiment.

Anterior portions of larvae fed low, medium, and high concentrations of *Chaetocerous calcitrans* grew negatively in length during the 20 days; however, no statistically significant differences ($P > 0.05$) between the 3 food levels were detected (Fig. 1). On the other hand, posterior larval portions fed high concentrations of *C. calcitrans* exhibited a significant ($P < 0.05$) increase in length when compared with posterior larval portions fed medium and low concentrations of *C. calcitrans* (Fig. 2). The amount of growth observed in the posterior portions corresponded to the food level (i.e., more food = more growth, less food = less growth) and the differences in growth between all 3 food levels were statistically significant ($P < 0.05$) (Fig. 2).

Anterior portions of larvae fed medium and low concentrations of *Dunaliella tertiolecta* showed significantly ($P < 0.05$) less negative growth than those fed the high con-

centration of *D. tertiolecta* (Fig. 1). On the other hand, posterior larval portions fed high concentrations of *D. tertiolecta* grew positively in length significantly ($P < 0.05$) when compared with posterior larval portions fed medium and low concentrations of *D. tertiolecta* (Fig. 2). The growth observed in the posterior portions corresponded to the food level (i.e., more food = more growth, less food = less growth) and the differences in growth between all 3 food levels were statistically significant ($P < 0.05$) (Fig. 2).

Anterior portions of larvae fed high and medium concentrations of *Isocrysis galbana* grew negatively in length; however, the anterior portions fed a low food concentration showed significantly ($P < 0.05$) less negative growth when compared to the anterior portions fed the high and medium food levels of *I. galbana* (Fig. 1). Posterior portions of larvae fed low, medium, and high concentrations of *I. galbana* increased in length over the study period, however no statistically significant differences in size were observed between the 3 food levels (Fig. 2).

While anterior and posterior portions of larvae fed all three concentrations of the mixed phytoplankton diet did exhibit changes in length over the study period (negative growth in anterior portions and positive growth in posterior portions), no statistically significant differences were detected between the 3 food levels (Figs. 1, 2).

Both anterior and posterior portions of bisected larvae that were starved over the 20-day study period completely regenerated. Anterior portions of starved larvae were observed to grow significantly ($P > 0.05$) smaller in length over the 20-day experimental period (Fig. 4), while posterior portions of starved larvae grew significantly ($P > 0.05$) larger (Fig. 4).

While starved anterior portions of larvae were similar in length at the end of the experiment to those fed single or mixed phytoplankton diets, starved posterior portions of larvae were significantly ($P < 0.05$) smaller than posterior portions of larvae fed any of the three concentrations of the single or mixed phytoplankton diets. Moreover, most of the posterior portions of larvae fed phytoplankton had formed an adult rudiment by the completion of the 20-day experimental period, whereas no rudiment was evident in starved posterior portions of larvae or in starved or fed anterior portions of larvae. Non-bisected fed control larvae showed no evidence of shrinkage or increased size over the experimental period (mean day 0 = 1600μm versus day 20 = 1400μm). These larvae actively fed throughout the experiment as evidenced by the appearance of phytoplankton in the stomach. Most of the control larvae had formed adult rudiments by the end of the experimental period, indicative of their metamorphic competence.

Discussion

Many intriguing questions remain to be answered about the regenerative processes of planktotrophic marine invertebrate larvae, including aspects of molecular genetics, cell fate, larval energetics, and the ecologic and evolutional implications of these processes. The results of the present study indicate that bisected larvae (simulating potential damage caused by partial predation or shear stress) of the common sea star *Pisaster ochraceus* are capable of regenerating missing body components in the absence of phytoplankton food as a source of energy and nutrients. That larval regeneration is remarkably food independent suggests regenerating larvae may be utilizing DOM (Manahan, 1990; Jaeckle and Manahan, 1992) and/or resorption of body components (Chia and

Burke, 1978; Vickery and McClintock, 1998, Dissertation Manuscript 1) as energy and nutrient resources to support regenerative processes. Larval size (as a proxy of quality) was nonetheless influenced by the amount but generally not the quality (single versus mixed diets) of phytoplankton food present (George, 1994; Vickery and McClintock, 2000, Dissertation Manuscript 4). Bisected larvae of *P. ochraceus* grew and regenerated equally well on single (low-quality) or mixed (high-quality) phytoplankton diets.

After bisection, the anterior portions of larvae of *P. ochraceus* retain the oral cavity, the preoral lobe, an anterior portion of the postoral ciliary bands, and the axocoel (Vickery and McClintock, in press, Dissertation Manuscripts 2 and 5). The esophagus, stomach, intestine, anus, most of the postoral ciliary band, and a significant portion of the coelom (including the hydrocanal and hydropore) are lost. The immediate consequence of these losses is an inability to consume and digest phytoplankton foods. Nonetheless, anterior portions of bisected larvae retain their ability to collect food particles and place them in their oral cavity by employing the limited ciliary bands that remain intact. Moreover, they remain adept at swimming in the water column (Vickery and McClintock, 1998; in press; Dissertation Manuscripts 1, 2, and 5), indicative of the retention of sufficient ciliary bands for coordinated locomotion. The regeneration of a functional digestive system, as evidenced by the presence of phytoplankton in the larval stomach, required a minimum of one week and up to 2 weeks postbisection. This temporary but significant inability to process phytoplankton foods is likely to explain the negative growth (shrinkage) observed in anterior portions of larvae fed phytoplankton over the regenerative period. These larvae were 50% smaller than control nonbisected fed larvae at the end of the 20-day experiment. While not examined in the present study, it is likely that

these have a prolonged period of growth before attaining metamorphic competence, with some presumed negative consequences on larval survival caused by an extended period of vulnerability to planktonic predators (Pennington *et al.*, 1986; Morgan, 1990; Morgan and Christy, 1997). At the end of the 20-day experiment, no evidence of an adult rudiment was evident in any of our treatments that followed regeneration in anterior portions of larvae.

After bisection, posterior larval portions retain the digestive system, but lack the larval mouth region (Vickery and McClintock, 1998, in press, Dissertation Manuscripts 1, 2, and 5). This might be expected to decrease feeding efficiency by impeding necessary ciliary-generated flow associated with capture and ingestion of food particles in bipinnaria and brachiolaria sea star larvae (R. R. Strathmann, 1975, 1987; Hart, 1991). Nonetheless, bisected posterior larval portions initiated feeding almost immediately post-bisection as evidenced by the presence of phytoplankton in their stomachs. Consistent larval growth occurred throughout the 20-day experiment, and it is likely that the consumption of phytoplankton foods contributed to positive growth in bisected posterior portions of larvae fed all concentrations of both single and mixed diets. Growth rates were similar to nonbisected, well-fed control larvae. Resultant larvae were similar in size (larval axial length) to undamaged control larvae; most similarly possessed adult rudiments, indicative of approaching metamorphic competency. Quantitative effects of food availability on larval growth were detected as those posterior portions of larvae fed the medium and high concentrations of single or mixed phytoplankton foods generally grew larger than those fed low concentrations of foods or starved. Fenaux *et al.* (1994) similarly detected food-limited growth in echinoid echinoplutei. Moreover, Basch (1996) exam-

ined the effects of algal density on larval development and survival in sea star larvae of *Asterina miniata* and found that larval development and survival depended directly on food availability.

Perhaps the most compelling evidence that the absorption of DOM and/or resorption of larval body components may occur during regeneration in the larvae of *P. ochraceus* is the finding that starved posterior portions of larvae did not decrease in size and showed a trend for increased mean size over the last 2 weeks of the experiment. Manahan (1990) has shown that the nutrient requirements of a variety of marine invertebrate larvae, including the planktotrophic larvae of echinoderms, can be attributed in significant part to direct absorption of DOM from seawater. Chia and Burke (1978) suggest that metamorphosing echinoderm larvae utilize the larval body as an energy source for the construction of adult body structures. To resolve the question of the degree to which DOM and/or resorption of nutrients from larval body components meet the nutrient and energy demands of regeneration, two approaches might be undertaken. Recent work in our laboratory has demonstrated that it is possible to detect specific genes involved in regenerative processes in planktotrophic sea star larvae (Vickery, 2001; Vickery *et al.*, 2001a, 2001b). Similarly, it may be possible to identify genes turned on during larval regeneration that are involved in the breakdown and/or reallocation of materials within larval body components. Alternatively, measurements of the metabolic costs of regeneration coupled with quantification of DOM absorbed during regeneration would make it possible to model how nutrient and energy demands may be offset by the absorption of DOM.

In contrast to posterior portions of sea star larvae, starved anterior portions, while undergoing complete regeneration, decreased in size over the experimental 20-day period. A similar pattern of negative growth observed in regenerative anterior portions of larvae fed single or mixed diets reinforces the conclusion that negative growth is likely related to the considerable investment in energy required to regenerate a functional digestive system postbisection and not to the availability of phytoplankton food.

When considering reproductive modes of marine invertebrates from a life history perspective, generally two distinct patterns emerge (Strathmann, 1985; Pearse *et al.*, 1991; Wray, 1995). One pattern involves the production of copious numbers of small feeding larvae that spend relatively long periods in the plankton, while the other involves the production of small numbers of large yolky nonfeeding larvae that spend comparatively shorter periods in the plankton or are brooded (Vance, 1973; Strathmann, 1978). One would predict that regenerative capacity is selected for in planktotrophic larvae because mortality in the plankton is presumably extremely high. Lecithotrophic larvae that are brooded and protected by a parent are thus unlikely to be under selective pressure for regenerative capacity. Nonetheless, lecithotrophic larvae that are broadcasted may be vulnerable to the same suite of variables as planktotrophic larvae and, while generally spending less time in the plankton, may be even more vulnerable to predation as evidenced by their high incidence of chemical defenses (McClintock and Baker, 2001). Thus broadcasted lecithotrophic larvae may have also evolved the ability to regenerate. Should future studies demonstrate this capacity, it will be interesting to compare and contrast the utilization of energy and acquisition of nutrients in larvae of marine invertebrates with planktotrophic and lecithotrophic modes of reproduction.

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Figure 1. Growth after 20-day experimental period of anterior portions of bisected larvae of the sea star *Pisaster ochraceus* presented three concentrations of a single diet of phytoplankton consisting of either *Chaetocerous calcitrans*, *Dunaliella tertiolecta*, or *Isochrysis galbana*, or a mixed diet consisting of a combination of equal amounts of these three species of phytoplankton. H, high food concentration; M, medium food concentration; L, low food concentration. * over the histograms denotes that data are significantly different from indicated food level.

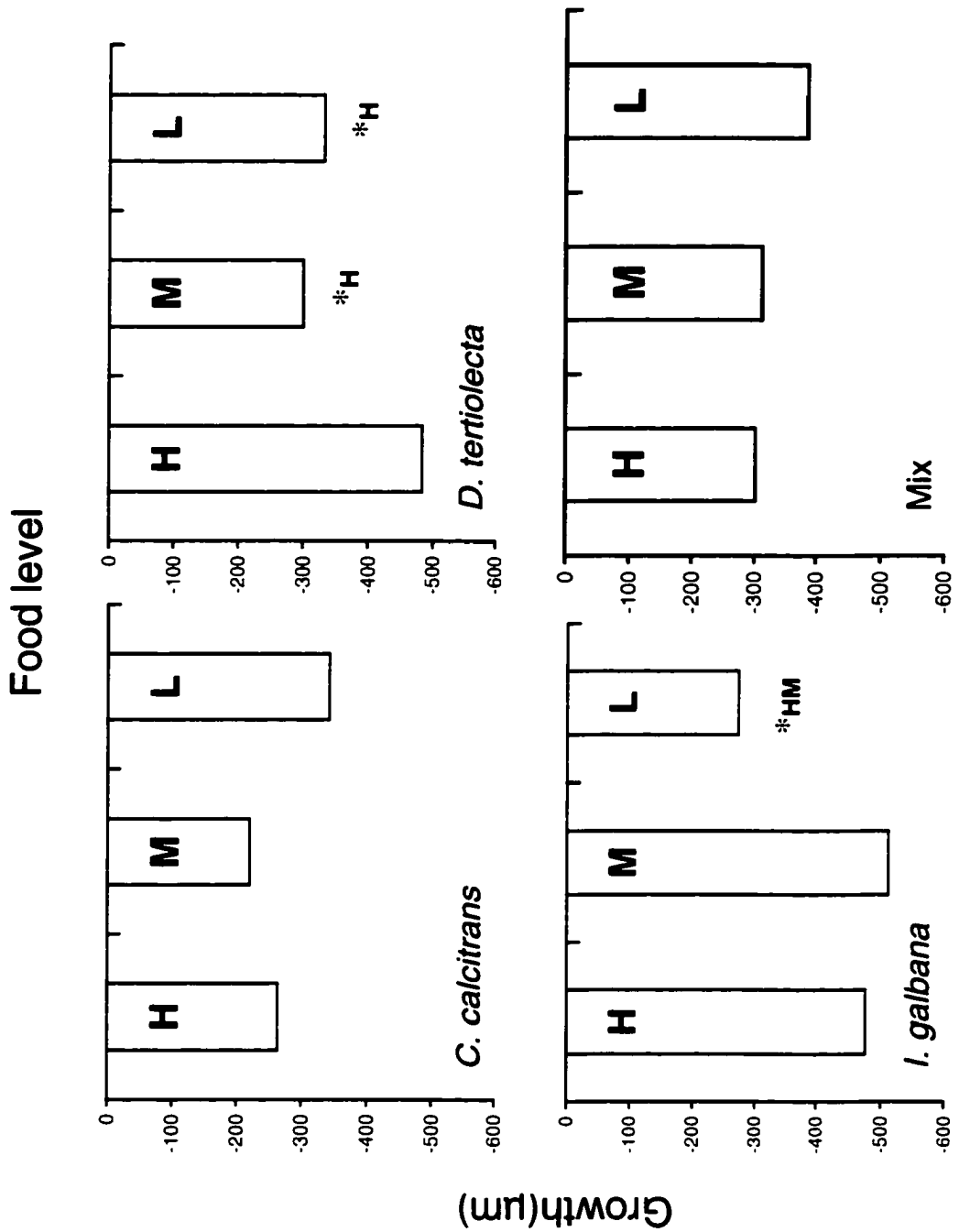


Figure 2. Growth after 20-day experimental period of posterior portions of bisected larvae of the sea star *Pisaster ochraceus* presented three concentrations of a single diet of phytoplankton consisting of either *Chaetocerous calcitrans*, *Dunaliella tertiolecta*, or *Isochrysis galbana*, or a mixed diet consisting of a combination of equal amounts of these three species of phytoplankton. H, high food concentration; M, medium food concentration; L, low food concentration. * over the histograms denotes that data are significantly different from indicated food level.

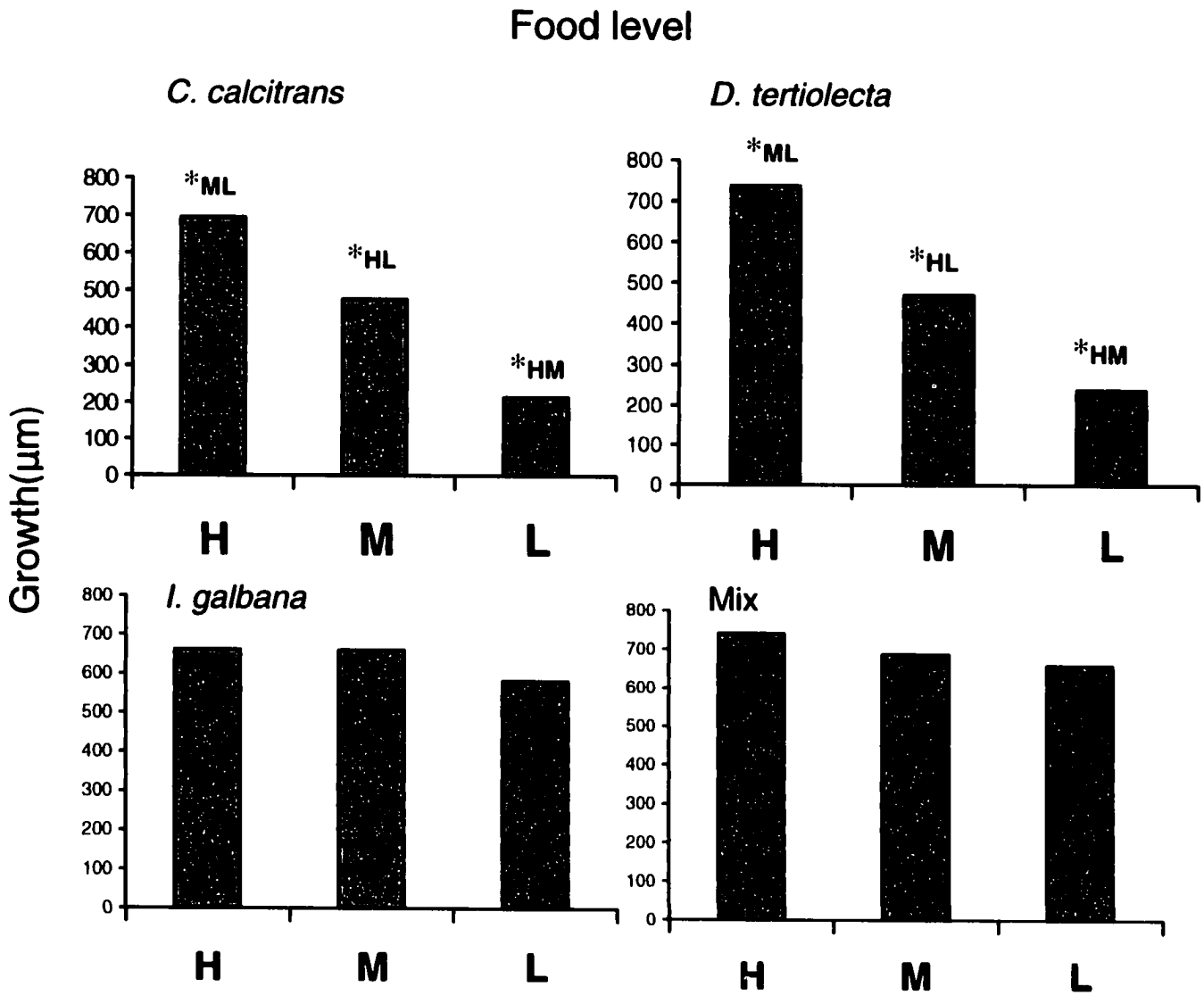


Figure 3. Photomicrographs showing a representative regenerated anterior (A) and posterior (B) portions of larvae of the sea star *Pisaster ochraceus* starved for 10 days after being bisected on Day 39 of their development (study Day 50 shown). Also shown is a representative nonbisected control larva raised on a diet of mixed phytoplankton over a similar period (study Day 50 shown) (C). ar, adult rudiment; bra, brachiolar apparatus; c, coelom; e, esophagus; m, mouth; rc, regenerated coelom; re, regenerated esophagus; rm, regenerated mouth; rs, regenerated stomach. Scale bar in A = 100 μm, and in B and C = 250 μm.

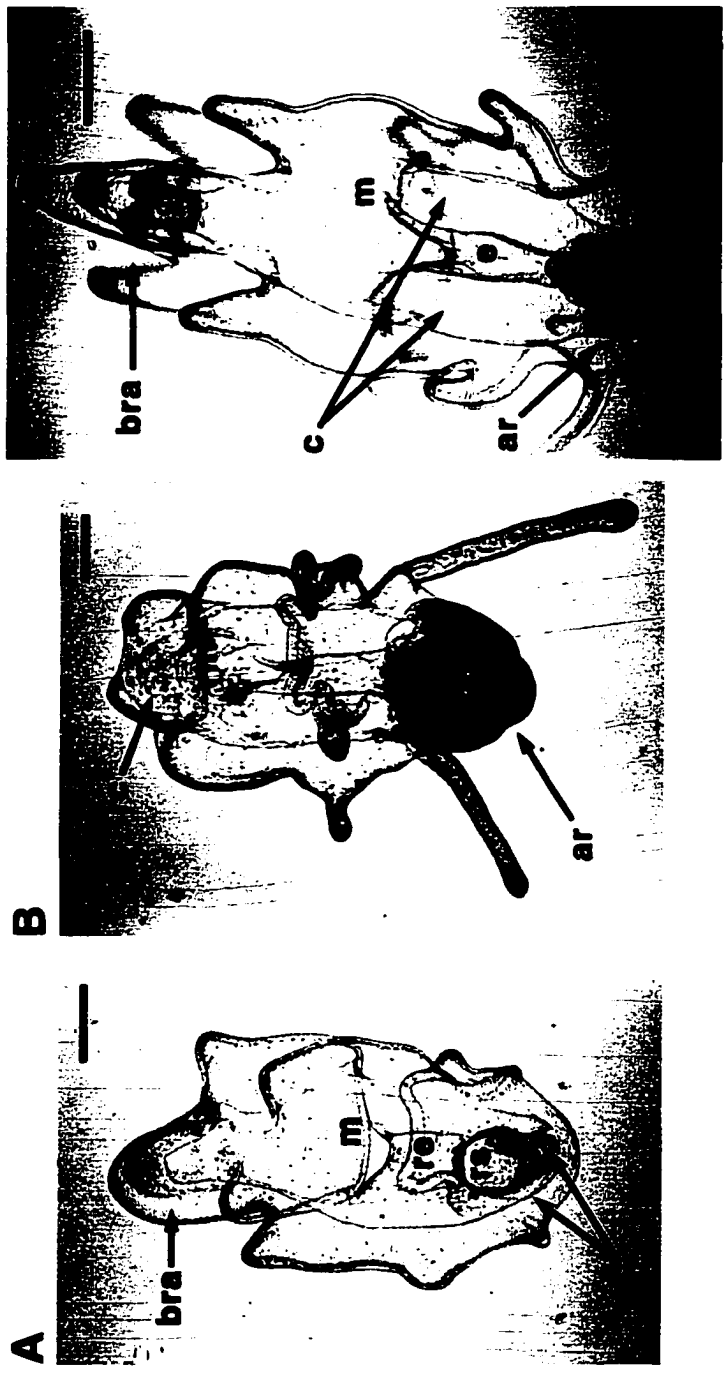


Figure 4. Growth after 20-day experimental period of starved anterior and posterior portions of bisected larvae of the sea star *Pisaster ochraceus*. Clear histograms represent the growth of anterior portions (A) and shaded histograms represent the growth of posterior portions (P). * over the histograms denotes that data for Day 20 are significantly different from Day 0.

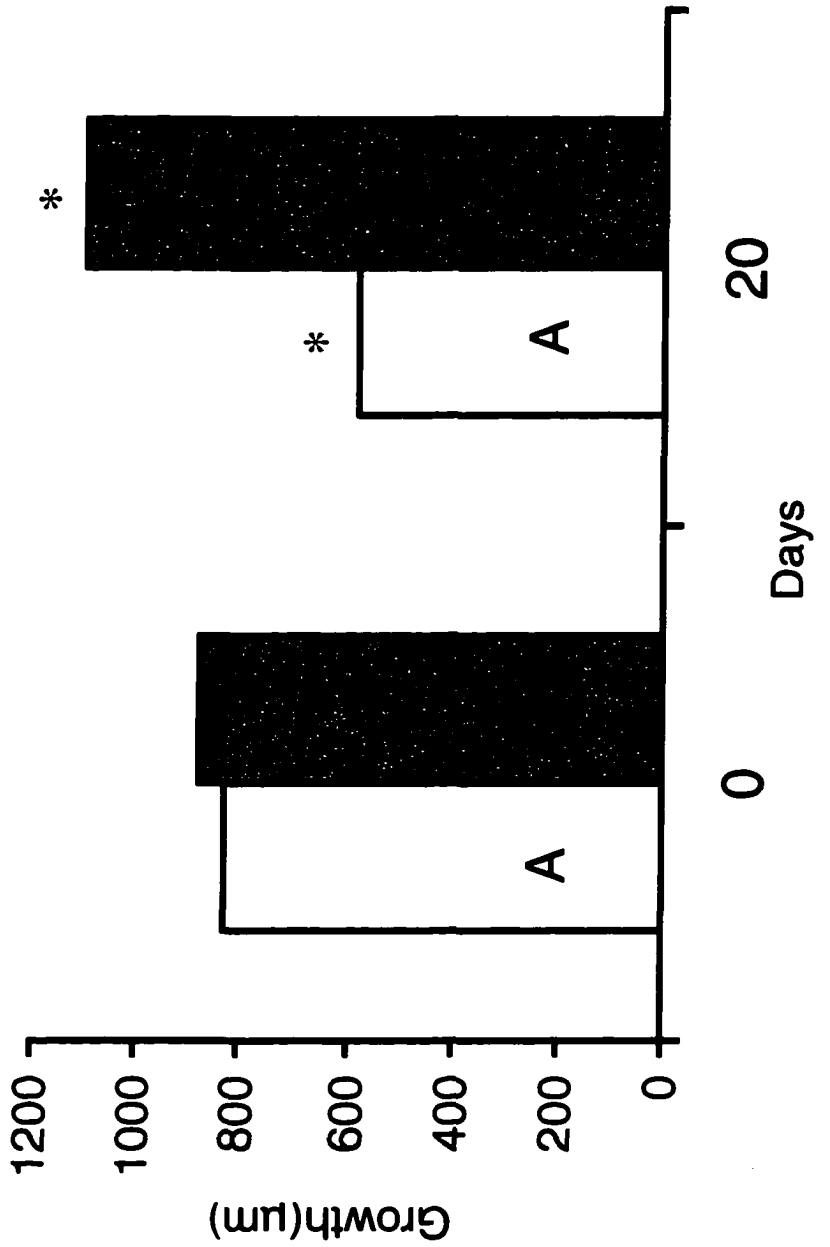
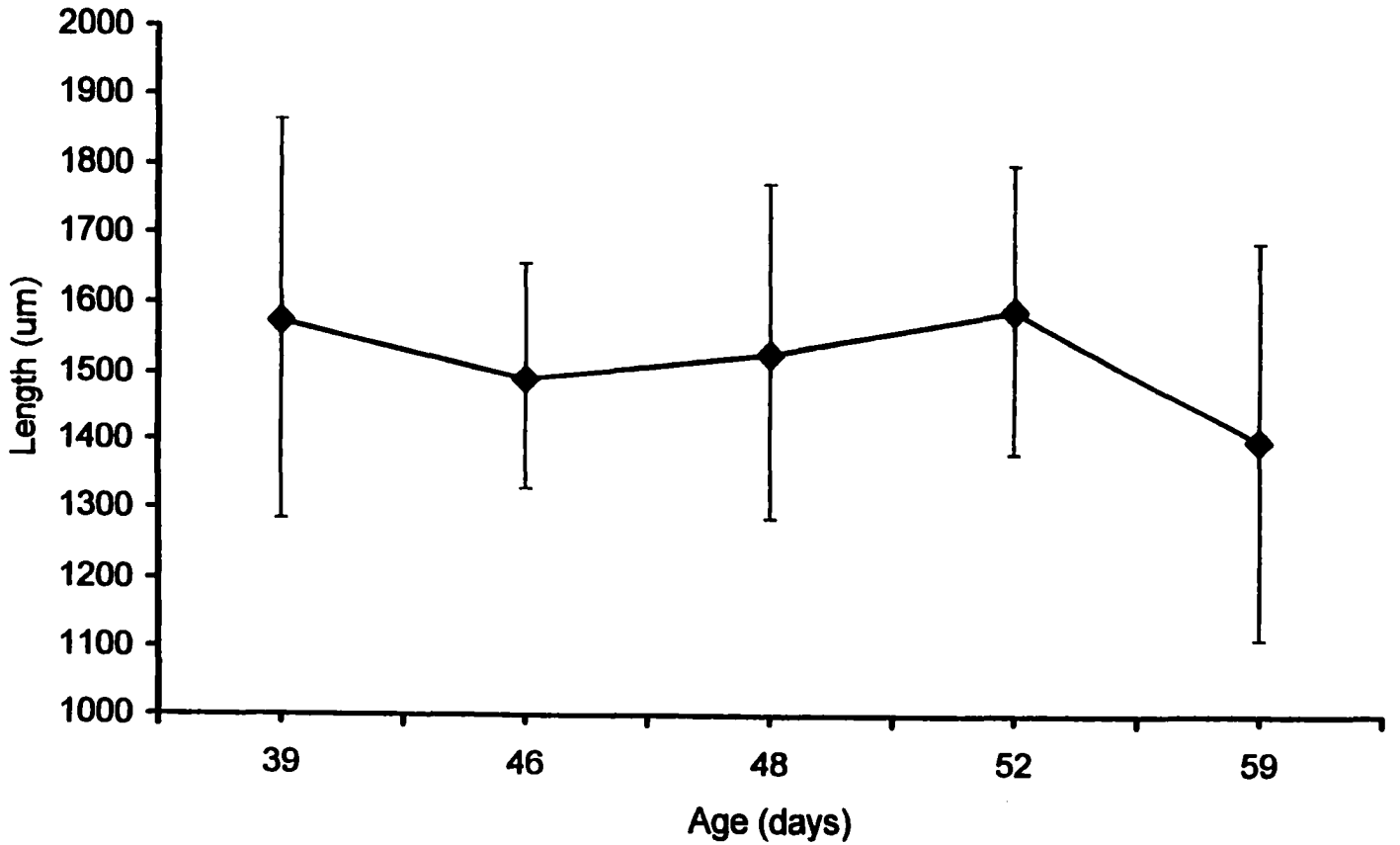


Figure 5. Mean larval lengths ±1 SD of nonbisected control larvae fed a high concentration of a mixed diet consisting of three species of phytoplankton.



EFFECTS OF FOOD CONCENTRATION AND AVAILABILITY ON THE INCIDENCE OF CLONING IN PLANKTOTROPHIC LARVAE OF THE SEA STAR *PISASTER OCHRACEUS*

by

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Abstract

A decade ago, cloning was first observed in the planktotrophic larvae of sea stars obtained from plankton tows. However, no controlled experimental studies have investigated what factors may regulate this remarkable phenomenon. In the present study we offer the first documentation of cloning in the planktotrophic larvae of *Pisaster ochraceus* from the northern Pacific coast. This species was used as a model system to investigate three factors that may influence the incidence of asexual reproduction (cloning) in planktotrophic sea star larvae. In an initial experiment, larvae were reared under nine combinations of three temperatures and three food (phytoplankton) concentrations. Larvae reared at 12-15°C and fed the highest food concentrations grew larger than the other larvae and produced significantly more clones. In a second experiment, qualitatively different algal diets were fed to larvae reared under the conditions found to be optimal in the initial experiment. Up to 24% of the larvae consuming a mixed phytoplankton diet of *Isochrysis galbana*, *Chaetocerous calcitrans*, and *Dunaliella tertiolecta* cloned, and significantly more clones were produced by these larvae than by those fed monospecific diets. Our experiments indicate that cloning generally occurs after larvae have attained asymptotic body length and only when food is abundant and of high quality. Since larval mortality is considered to be extremely high for marine invertebrates with planktotrophic larvae, production of clones under optimal conditions of temperature and food may serve to increase larval populations when the environment is most conducive to larval growth.

Introduction

Asexual reproduction and regeneration of missing body parts are well-known phenomena in adult sea stars (Anderson, 1956; Emson and Wilkie, 1980; Shirai and Walker, 1988; Mladenov *et al.*, 1989; Mladenov and Burke, 1994). The capacity for asexual reproduction (cloning) in sea star larvae was first suggested over 60 years ago from observations of the bipinnaria larvae of *Luidia sarsi* (Tattersall and Sheppard, 1934), but subsequent laboratory experiments indicated that these larvae were incapable of asexual reproduction (Wilson, 1978). Only recently has cloning and regeneration of missing body parts been confirmed in planktotrophic larvae of sea stars (Bosch *et al.*, 1989; Rao *et al.*, 1993; Jaeckle, 1994; Vickery and McClintock, 1998, Dissertation Manuscript 1; Kitazawa and Komatsu, 2000).

Cloning in the bipinnaria larvae of the sea star *Luidia* sp. was first documented from plankton samples collected in the Sargasso Sea in which clones were observed to develop from buds that formed on the larval arms (Bosch, 1988; Bosch *et al.*, 1989). Shortly thereafter, two additional reports confirmed the occurrence of cloning in the natural environment in two unidentified species of planktotrophic sea star larvae also obtained from plankton samples (Rao *et al.*, 1993; Jaeckle, 1994). In addition to the budding process described above (Bosch, 1988; Bosch *et al.*, 1989), autotomy of the anterior portions of larvae was also observed in one of these studies (Jaeckle, 1994). Recently, brachiolaria larvae of the sea star *Distolasterias brucei* have been reported to undergo cloning in laboratory cultures (Kitazawa and Komatsu, 2000) in a manner identical to that described in previous reports (budding and autotomy). Moreover, cloning in laboratory cultures of the planktotrophic larvae of the brittle star *Ophiopholis aculeata* from the

northern Pacific has been reported (Balser, 1998). We reported regeneration in planktotrophic larvae of the sea stars *Luidia foliolata* and *Pisaster ochraceus* (Vickery and McClintock, 1998). In addition, we observed similar regenerative capacity in planktotrophic echinopluteus larvae of the sea urchins *Dendraster excentricus* and *Lytechinus variegatus* (Vickery and McClintock, in press, Dissertation Manuscripts 2 and 5). Thus, among Echinodermata, regenerative capacity in planktotrophic larvae has been demonstrated in sea stars, brittle stars, and sea urchins.

The existence of cloning and regenerative capacity among echinoderms with planktotrophic modes of reproduction suggests that there may be selective and adaptive advantages associated with such life history traits. These processes presumably operate under a suite of energetic constraints and trade-offs, whereby only larvae exposed to the most appropriate conditions would be expected to undergo clonal and regenerative events.

To date, no experimental studies have investigated the factors that regulate cloning in planktotrophic marine invertebrate larvae. Larval development, growth, and survivorship are particularly influenced by temperature and food availability (*e.g.*, George, 1994; Fenaux *et al.*, 1994). These may also be important factors affecting rates of larval cloning (Levitan, 1995; Morgan, 1995). In the present study we offer the first confirmed report of cloning in the planktotrophic larvae of the sea star *P. ochraceus*. We also examined the effects of temperature and both food concentration and availability on growth and cloning in *P. ochraceus* larvae.

Materials and Methods

Larval culturing

Pisaster ochraceus is commonly found in intertidal and shallow subtidal habitats of the U.S. Pacific Northwest. The breeding season of *P. ochraceus* is in the late spring and early summer months in the vicinity of Puget Sound, Washington (Strathmann, 1987). Adult specimens were collected during late spring months in 1997 (for temperature-food experiment) and 1998 (for food availability experiment) from rocky substrates along the shore of East Sound, Orcas Island, and transported to Friday Harbor Laboratories, San Juan Island, Washington. Ovaries and testes were dissected from sexually mature specimens (a single female and a single male). Fertilizable ova were obtained by treating excised ovaries with 1-methyladenine (10^{-4} *M*) (Kanatani, 1969), and sperm were diluted in filtered sea water prior to fertilization. During fertilization, ova were rinsed in filtered seawater to remove excess sperm. Embryos and larvae were reared in 2.5-liter glass jars. The cultures were gently stirred and the seawater was changed every 3 days following the methods outlined by Strathmann (1987). The single-celled algae *Chaetocerous calcitrans*, *Dunaliella tertiolecta*, and *Isochrysis galbana* were selected for larval diets (Strathmann, 1987). Observations and photographs of larvae were made with both a Wild M-5 dissecting microscope and a Nikon Optiphot-2 compound microscope.

Combined temperature and food-level experiments

Once the bipinnaria larvae developed functional digestive systems they were separated into nine experimental treatments (3 x 3 factorial design) to examine the effects of temperature and food level on rates of cloning. Each experimental treatment combin-

ing temperature and food level was replicated three times, and each consisted of about 2400 larvae held in 2.5l of seawater in a glass jar (approx. 1 larva/ml). Ambient spring seawater temperatures (12-15°C) were bracketed in increments of about 5°C to yield treatment groups of low (7-10°C), medium (12-15°C), and high (17-20°C) temperature. Ambient seawater temperatures along the northern Pacific coast generally fall within the middle temperature range (12-15°C) throughout the year, but it is likely that larvae encounter lower temperatures (7-10°C) at the northern limit of their biogeographic range and in deeper water, or during the late winter months (Cannon, 1978). Similarly, larvae may encounter higher temperatures (17-20°C) at the southern limits of their biogeographic distribution, or in surface waters during the early summer months (Cannon, 1978; Strathmann, 1987).

Three levels of food composed of equal cell numbers (as determined using a hemocytometer) of the phytoplankton species *C. calcitrans*, *D. tertiolecta*, and *I. galbana* were proffered to larvae in each treatment. The three concentrations of mixed algal cells were 5×10^2, 5×10^3, and 5×10^4 cells/ml (modified after Basch, 1996; Fenaux *et al.*, 1994). The nine experimental treatments combining temperature and food level were therefore as follows: high temperature and high food (HT-HF), high temperature and medium food (HT-MF), high temperature and low food (HT-LF), medium temperature and high food (MT-HF), medium temperature and medium food (MT-MF), medium temperature and low food (MT-LF), low temperature and high food (LT-HF), low temperature and medium food (LT-MF), and low temperature and low food (LT-LF). Subsamples of larvae in each experimental treatment ($n = 200$) were examined every 3 days under a dissecting microscope. The numbers of clones and of larvae undergoing clonal reproduction

as evidenced by budding were recorded. The lengths of the larvae in each subsample of each experimental treatment and of all larvae in the process of cloning were measured along the larval axis (George, 1994). Clones and larvae undergoing cloning were placed in separate containers and monitored every 3 days to determine whether clones successfully developed into normal functional larvae and metamorphosed into juveniles. Larval lengths were compared within temperature and food-level experiments using analysis of variance. Only probability levels where $P \leq 0.05$ were considered statistically significant.

Food-availability experiment

To examine the effects of food availability (phytoplankton type) on the rate of cloning, simulating conditions in which nutrient diversity might be limited, larvae of *Pisaster ochraceus* were obtained as described above using ova and sperm from a different set of parent sea stars than those used for the combined temperature and food-level experiment. The larvae were cultured at 12-15°C at a density of about 2400 larvae per 2.5l of seawater. Larvae were fed 5×10^4 cells/ml of either a monospecific diet of either *C. calcitrans*, *D. tertiolecta*, or *I. galbana* or a mixed diet composed of an equal cell number of these three algae. Each of the four experimental treatments was replicated three times. Subsamples of larvae ($n = 200$) were examined every 3 days under a dissecting microscope and analyzed using the same methods as for the combined temperature and food-level experiment. Larval lengths were compared within food availability experiments using analysis of variance. Only probability levels where $P \leq 0.05$ were considered statistically significant.

Results

Combined temperature and food-level experiments

Bipinnaria larvae exposed to seawater temperatures of 12-15°C and fed the highest food level (MT-HF) attained lengths significantly greater than those of larvae in any other experimental treatment (Fig. 1). Moreover, cloning occurred only in this experimental treatment (Fig. 2). Thirty-four days after fertilization, larval clones were first observed in the MT-HF bipinnaria culture (1.2%, Fig. 2). Subsequently, small numbers of additional larval clones were observed. Once bipinnaria in this treatment had doubled in length while developing into brachiolaria larvae (at about day 45), a fivefold increase was observed in the incidence of cloning (6%, Fig. 2).

Larval clones obtained from the MT-HF cultures were isolated and their development was followed through metamorphosis. Clones produced resulted from the regeneration of anterior and posterior portions of bipinnaria and brachiolaria larvae (Fig. 3A, B) by processes that closely resembled those described in detail by Vickery and McClintock (1998). After about 2 weeks, fully developed clonal larvae were functionally and morphologically indistinguishable from larvae in the cultures from which they were originally isolated. A number of bipinnaria and brachiolaria larvae in the MT-HF cultures had missing larval arms or were missing small fragments of the larval body. Some larvae with missing larval arms were in the process of cloning, as evidenced by the development of projections or buds, which later became functional larvae, at the site of the missing fragment (Fig. 3C). However, some larvae with missing larval arms did not form projections or buds, but instead regenerated the larval arm. In addition, some small fragments of larval body parts, including severed larval arms, were observed in the MT-HF

cultures, presumably the result of damage incurred when the water in the larval culture was changed. A number of these fragments, including severed arms, were separated from the cultures and observed for 2 weeks. During this time the fragments neither grew nor formed clones, although no mortality was observed.

Those bipinnaria larvae exposed to the highest temperature treatments (HT-LF, HT-MF, and HT-HF) all died within one week; no cloning occurred in the short period before mortality. Unlike larvae held at mid-range temperatures, bipinnaria larvae reared at low temperatures (7-10°C) and fed any of the three levels of food (LT-LF, LT-MF, and LT-HF) never developed into brachiolaria larvae over a 70-day period. They remained morphologically identical to early-stage bipinnariae. Moreover, no clones were produced. Larvae presented any food level at low temperature did not increase in length. In fact, they were slightly reduced in length by the end of the 70-day observation period (Fig. 4). Considerable larval mortality occurred throughout the experiment in all low-temperature treatment groups.

Food availability experiment

Bipinnaria larvae in experimental treatments fed a monospecific diet of *Isochrysis galbana* or an equivalent density of a mixture of equal cell numbers of *Chaetocerous calcitrans*, *Dunaliella tertiolecta*, and *I. galbana* grew significantly larger than larvae fed monospecific diets of *C. calcitrans* or *D. tertiolecta* (Fig. 5). The incidence of cloning was greatest (24%) on a mixed diet during and after the transformation from the bipinnaria to the brachiolaria stage (Fig. 6). Although temperature and food concentration were similar in this treatment and in the MT-HF treatment group of the combined tem-

perature and food-level experiment (Figs. 1, 2), growth was more rapid, and cloning began earlier and was more frequent. This might be attributed to the high variability in larval development rate in batches of larvae of *Pisaster ochraceus* (Strathmann, 1978). Also, larvae used for this experiment were the offspring of a different set of parents than those used in the previous experiments.

Discussion

Cloning and regenerative capacity in echinoderm larvae has only recently been documented (Bosch, 1988; Bosch *et al.*, 1989; Rao *et al.*, 1993; Jaeckle, 1994; Balser, 1998; Vickery and McClintock, 1998, in press, Submitted, Dissertation Manuscripts 1, 2 and 5; Kitazawa and Komatsu, 2000). In the present study we provide the first documentation of cloning in the planktotrophic larvae of *Pisaster ochraceus*. The results of our bifactorial analysis indicated that both seawater temperature and food level are important factors affecting growth and survival-and therefore cloning-of *P. ochraceus* larvae. Simulated environmental conditions that produced normal development and optimal larval growth generated the greatest number of clones. Larvae reared at temperatures (12-15°C) with the most abundant food exhibited normal development and positive growth, which resulted in the highest survival, with the greatest incidence of cloning. Although we observed rapid larval growth in the high-temperature treatment (15-17°C), none of the larvae survived beyond one week. One possible explanation for the high mortality is that the higher temperatures triggered an increase in bacterial and microalgal growth in the cultures.

In contrast, sea star larvae reared in the low-temperature treatments (7-10°C) showed no net positive growth, and in most cases, decreased in length, regardless of food availability. These larvae also failed to attain the brachiolaria stage of development. It is unlikely these larvae would eventually become clonal because they continued to shrink in length over the course of the experiment. Decreased rates of growth and development at low temperatures may be related to decreased rates of larval metabolism (Boidron-Métairon, 1995). While low seawater temperatures have been suggested as an indirect cause of mortality in marine invertebrate larvae (Thorson, 1950), no studies of larval culturing have shown that low temperature can actually lead to a decrease in larval length as seen in the present study.

The production of larval clones was greatest during phases of rapid larval growth in MT-HF condition. As *P. ochraceus* in the North Pacific spawns in the late spring, larvae typically encounter moderate seawater temperatures (12-15°C) and high phytoplankton availability (Cannon, 1978). Such conditions could be expected to enhance *in situ* rates of larval cloning. Further analysis indicated that presenting larvae with different levels and types of food under an optimal regime of seawater temperature had a pronounced effect on the initiation and rate of larval clone production.

The greatest numbers of clones were produced by larvae in cultures presented a mixture of three single-celled algae. Although monospecific patches of single-celled algae are unlikely to exist in the natural environment, our use of monospecific algal diets simulated conditions in which nutrient diversity might be limited. Thus some of the observed differences in growth (and cloning) rates among the larvae fed monoalgal diets may have resulted from differences in the nutrient content of the food rather than in the

type of food, since the larvae were fed equal cell numbers of algae, not an equal nutritional content (Pechenik and Fisher, 1979). However, the amount of nutrients actually consumed by the larvae does not necessarily have any correlation with the nutrient content of the food presented, as some food types may be more palatable to the larvae than others. Future studies may shed more light on this subject. The important information gained from the food-availability experiment is that nutrient availability may be an important factor affecting larval growth and therefore the rate of cloning, as evidenced by the fact that growth rates among larvae fed a monoalgal diet of *Isochrysis galbana* were similar to those fed a diet of mixed algae, yet the larvae fed the mixed diet produced far more clones.

In adult echinoderms, cloning (fission) is common and has been well described (Emson and Wilkie, 1980). Seasonal fluctuations in the incidence of cloning in adult sea stars, especially a high incidence in summer months, have been related to periods of maximum growth (Emson and Wilkie, 1980). This suggests that suitable biotic and environmental conditions such as abundant food and moderate temperatures may trigger cloning processes in adults just as they did in the larvae studied here. In some instances, more than 50% of the adults in a population were observed undergoing cloning (fission) when conditions were optimal (Emson and Wilkie, 1980).

Cloning may serve as a mechanism to enhance recruitment in *P. ochraceus* and perhaps in other marine invertebrates with planktotrophic modes of reproduction. Larvae dispersed across significant distances are likely to encounter a variety of environmental and biotic conditions, and our results suggest that those larvae encountering favorable conditions may be stimulated to reproduce by cloning, thereby possibly increasing the

probability of successful larval metamorphosis and juvenile recruitment. Future studies of the effects of larval cloning on larval survivorship and recruitment will provide more insight into the true impact of this phenomenon on the life history of sea stars with planktotrophic larvae.

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Figure 1. Growth of larvae of *Pisaster ochraceus* (measured as changes in length) reared at 12-15°C and fed low, medium, and high levels of mixed phytoplankton. M indicates stage at which brachiolaria larvae developed an adult rudiment and a decrease in length of the larval body (indicating metamorphic competence). Larvae that reached metamorphic competence were later observed to complete the metamorphosis to juveniles. Error bars represent mean
values ± 1SD.

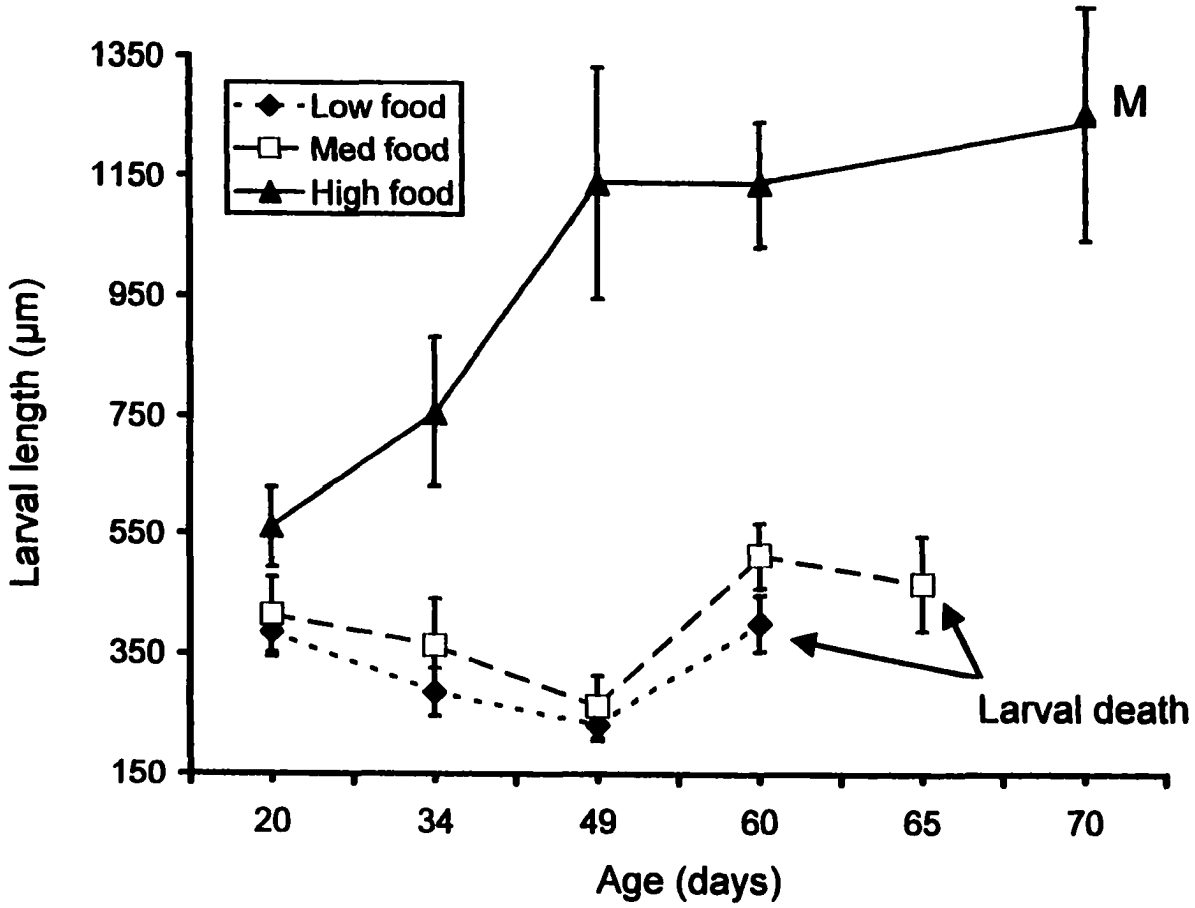


Fig. 2. Percentages of larvae of *Pisaster ochraceus* undergoing cloning when reared at 12-15° C and fed high levels of mixed phytoplankton. Diagram indicates approximate onset of brachiolaria stage. Error bars represent mean values ±1 SD.

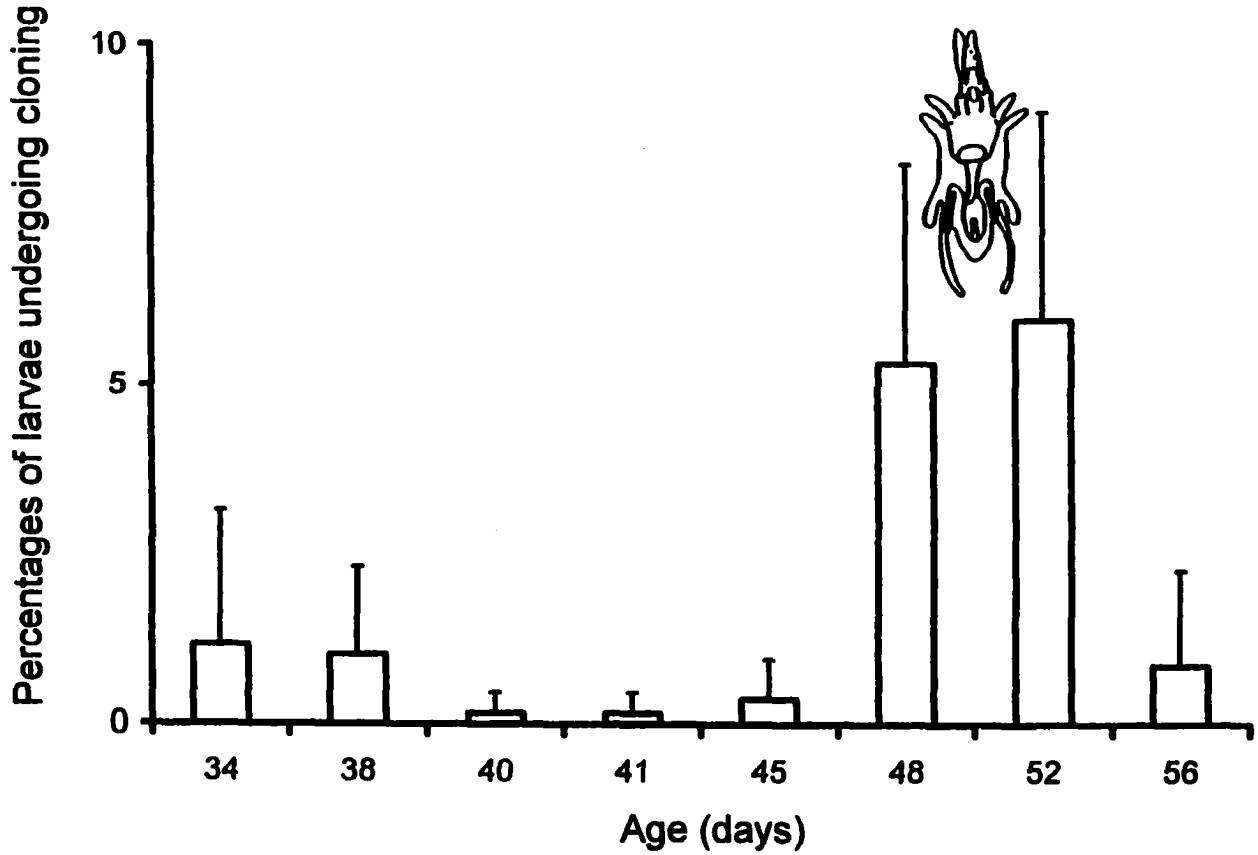


Figure 3. Light photomicrographs of clonal larvae of *Pisaster ochraceus* including the anterior (A) and posterior (B) portion of bipinnaria larvae. (C) Bipinnaria larva in the process of budding (see arrow). The bud later formed an early bipinnaria stage larva and subsequently detached (similar to that described by Bosch *et al.*, 1989). (D) Bipinnaria larva in the process of autotomization by fission. This larva was observed for about 2 weeks, during which the secondary larva (see arrow) detached and became functionally and morphologically indistinguishable from the primary larva in a manner similar to that described by Jaeckle (1994). We have observed this type of autotomy in several other species of planktotrophic larvae (pers. Obs., M. Vickery). Scale bars = 200 μm.

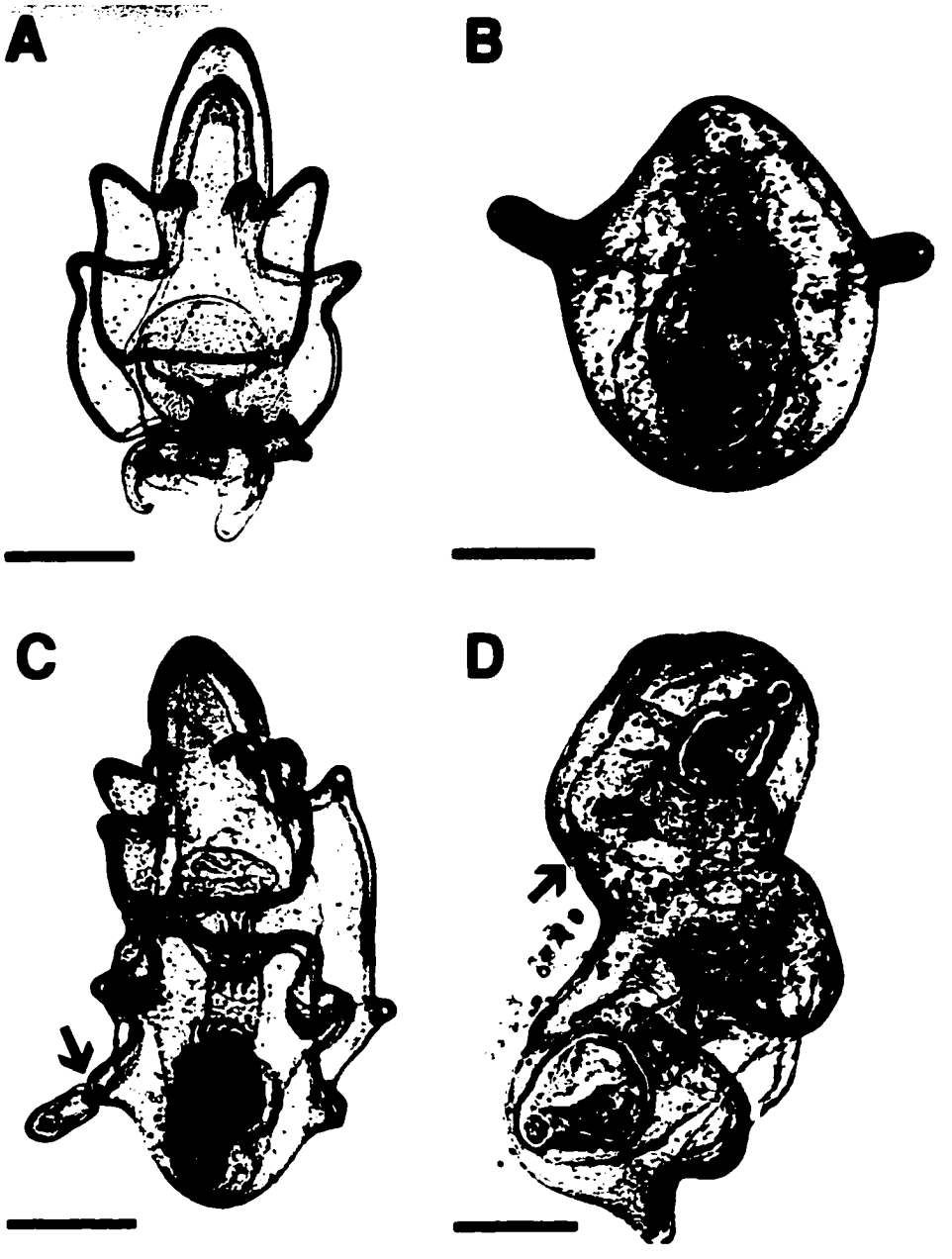


Figure 4. Growth of larvae of *Pisaster ochraceus* (measured as changes in length) reared at 7-10° C on low, medium, and high levels of mixedphytoplankton. Error bars represent mean values ±1 SD.

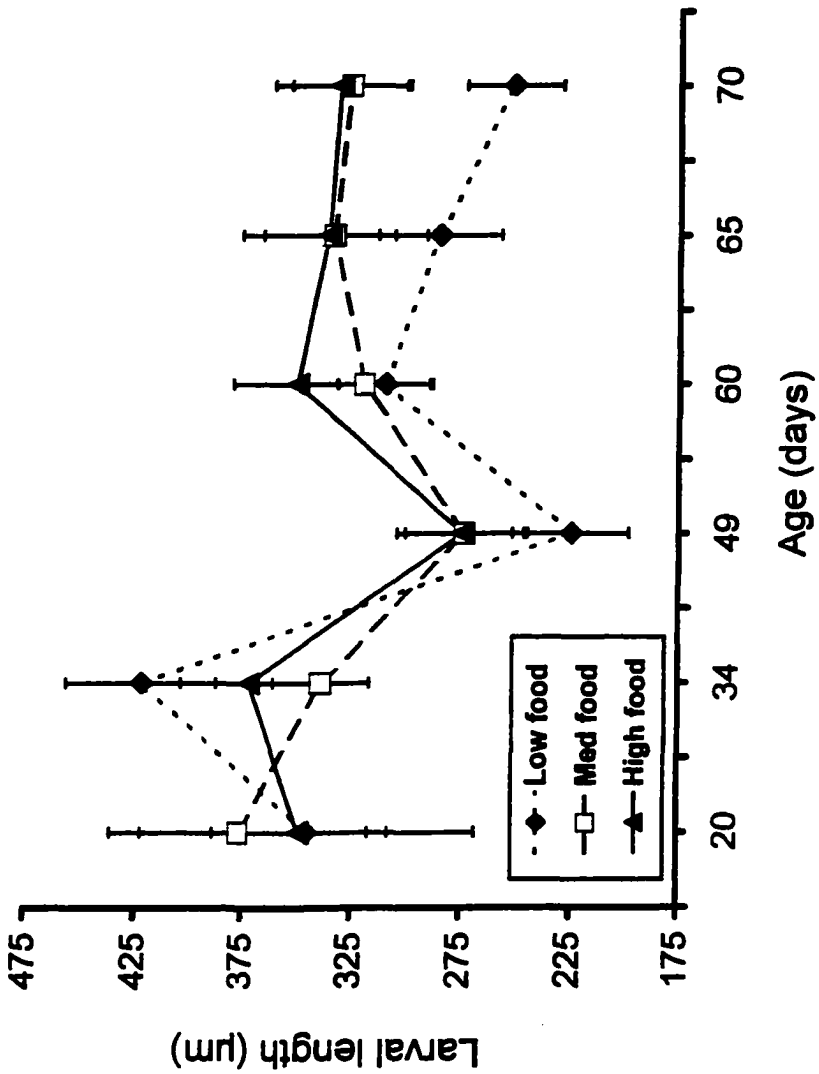


Figure 5. Growth of larvae of *Pisaster ochraceus* (measured as changes in length) reared at 12-15° C and fed high levels of four different phytoplankton diets. M indicates stage at which brachiolaria larvae developed an adult rudiment and a decrease in length of the larval body (indicating metamorphic competence). Larvae that reached metamorphic competence were later observed to complete the metamorphosis to juveniles. Error bars represent mean values ±1 SD.

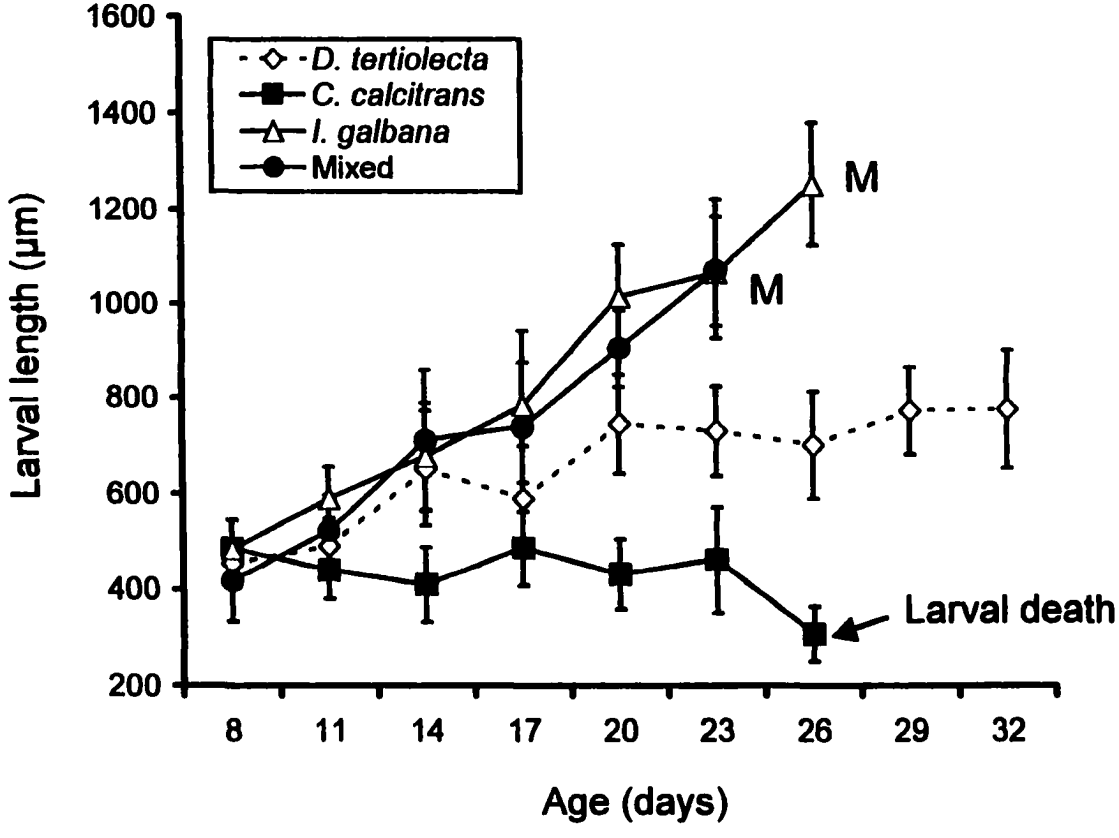
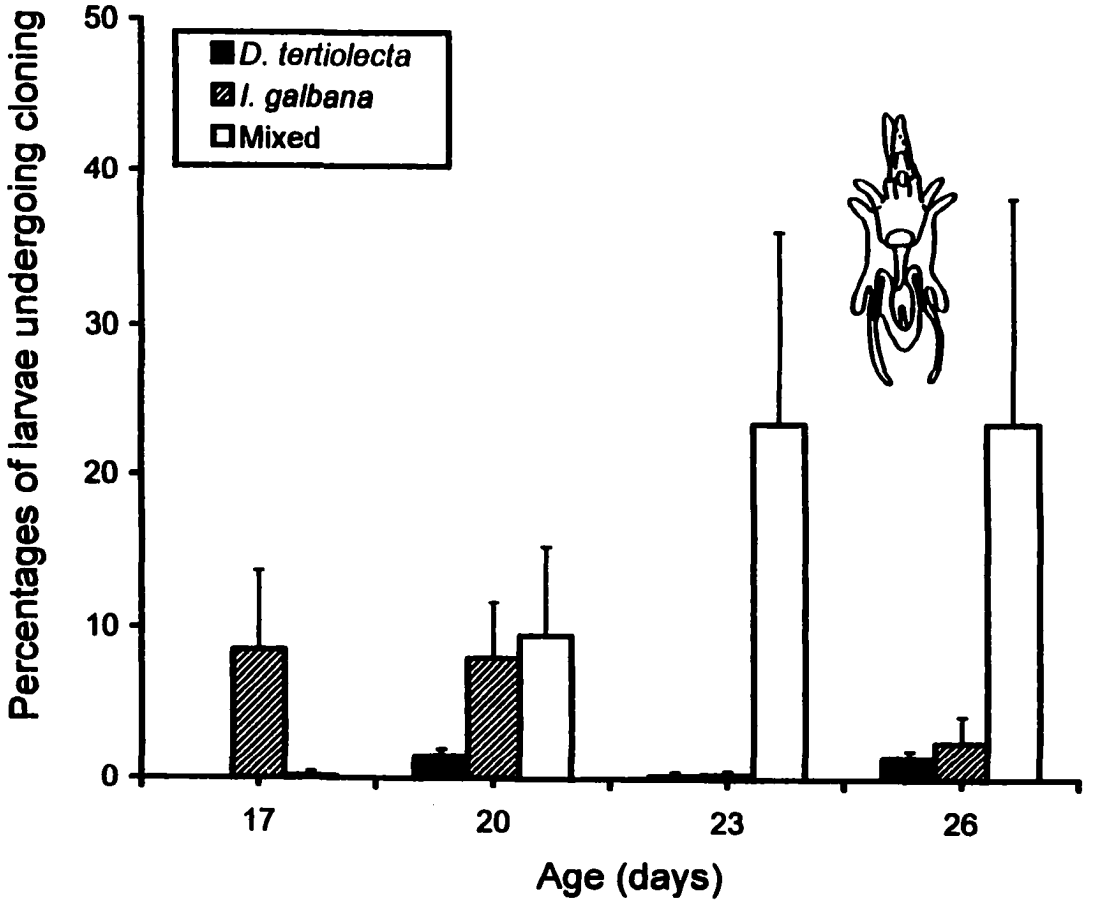


Figure 6. Percentages of larvae of *Pisaster ochraceus* undergoing cloning when reared at 12-15°C and fed high levels of four different phytoplankton diets. Error bars represent mean values ±1 SD.



REGENERATION IN PLANKTOTROPHIC LARVAE OF A REGULAR AND AN IRREGULAR ECHINOID

by

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Abstract

We recently reported that planktotrophic asteroid larvae have the ability to completely regenerate all body structures, tissues, and organs completely after surgical bisection (Vickery and McClintock, 1998, Dissertation Manuscript 1). In the present report we extend our studies of the regenerative capacity of planktotrophic echinoderm larvae to echinoid larvae. Planktotrophic pluteus larvae of the regular echinoid *Lytechinus variegatus* and the irregular echinoid *Dendraster excentricus* were surgically bisected across the esophagus into discrete and equal anterior and posterior portions. In *L. variegatus*, we observed complete regeneration of both larval portions into morphologically and functionally normal larvae indistinguishable from nonbisected control larvae. The regenerative processes appeared to be similar to those we observed in planktotrophic asteroid larvae. Larvae readily metamorphosed into normal juveniles. In *D. excentricus*, posterior portions of larvae completed regeneration and metamorphosis, while anterior portions displayed only partial regeneration during the course of the 2-week study period. The present study demonstrates that, similar to asteroid larvae, planktotrophic echinoid larvae are capable of rapid and complete regeneration. Regenerative capacity may be an important adaptation to increase survival when larvae suffer damage from biotic or physical sources in the plankton.

Introduction

Echinoderms are known to possess remarkable regenerative capacity and, like vertebrates, are deuterostomes, sharing developmental features that differentiate them from the protostome group (Lawrence, 1987; Willmer, 1990; Balser, 1998; Thorndyke *et al.*, 1999). We recently discovered that asteroid larvae have the ability to regenerate all

missing body structures, tissues, and organs after surgical bisection (Vickery and McClintock, 1998, in press, Dissertation Manuscripts 1 and 2; Vickery *et al.*, 2001b), with this being the first report of regenerative ability that included complete organogenesis in deuterostome larvae. In these studies, complete regeneration of anterior and posterior portions of surgically bisected planktotrophic bipinnaria larvae of the asteroid *Luidia foliolata* and of both bipinnaria and brachiolaria larvae of the asteroid *Pisaster ochraceus* were observed to occur over a 12- to 14-day period. The resulting larvae were morphologically and functionally indistinguishable from nonbisected control larvae. Moreover, no mortality caused by surgical bisection was observed and a second surgical bisection of regenerated larvae yielded identical results. Regenerated larvae subsequently metamorphosed into normal juvenile asteroids (Vickery and McClintock, 1998, in press, Dissertation Manuscripts 1 and 2; Vickery *et al.*, 2001b).

Detailed microscopic observations of early cellular regenerative processes in asteroid larvae of *L. foliolata* and *P. ochraceus* revealed aggregations of mesenchyme cells with pseudopodia at the site of bisection (Vickery and McClintock, in press, Dissertation Manuscript 2). These cellular aggregations are similar to the mesenchymal blastema that forms during regeneration in most adult echinoderms (Dolmatov, 1992; Candia Carnevali *et al.*, 1995; Bonasoro *et al.*, 1998; Van Den Spiegel *et al.*, 2000). In additional studies we have examined the effects of food quantity and quality on the regenerative capacity of asteroid larvae (Dissertation Manuscript 3). Moreover, we have developed asteroid larvae as a model system for the study of regeneration genetics in deuterostomes, identifying novel genes that are involved in early stages of larval regeneration in the sea star *L. foliolata* (Vickery, 2001; Vickery *et al.*, 2001a, submitted).

While early studies examined the ability of echinoid larvae to regenerate specific missing tissues (reviewed in Hörstadius, 1973, Vickery *et al.*, 2001b), no studies similar to those we have outlined above for planktotrophic asteroid larvae have examined similar regenerative characteristics in planktotrophic echinoid larvae. Thus it remains to be determined whether echinoid larvae are even capable of regeneration. Importantly, if regenerative capacity exists, then given our immense knowledge of aspects of echinoid molecular and cellular development, echinoid larvae could become a model system for studies of deuterostome regeneration. In the present study we examine the regenerative capacity of both the planktotrophic pluteus larvae of the regular echinoid *Lytechinus variegatus* and the irregular echinoid *Dendraster excentricus*.

Materials and Methods

Larval cultures

Adult *Lytechinus variegatus* were collected from shallow waters (approx. 0.3-2.0 m depth) from the Gulf of Mexico near Port Saint Joseph, FL, during late spring 1999. Individuals were maintained in 160-liter recirculating aerated aquaria containing artificial seawater (Instant Ocean, Aquarium Systems, Mentor, OH) and held at approximately 20-25°C. Larvae were obtained from a single male/female pair by artificial fertilization using gametes from spawn triggered via intracoelomic injection of 0.5 M KCl (Strathmann, 1987). Diluted seawater-suspended sperm were added to ova, after which the fertilized ova were held in glass finger bowls (250 ml) until hatching. Embryologic and larval development was monitored and photographed using an Olympus CO-11 binocular dissecting microsope and an Olympus CH binocular compound light microscope equipped with

a camera. Larvae were reared in artificial seawater using standard culturing protocols (Strathmann, 1987).

Upon hatching, the early pyrimidshaped larvae of *L. variegatus* were transferred into culture jars containing approximately 3.5l artificial seawater. Cultures were maintained with gentle stirring at approximately 20-23°C at a density of no greater than 0.8 larvae/ml, and the culture water was changed every 3 days. Once the larvae obtained functional digestive systems (2 days after fertilization), they were fed approximately 5×10^4 cells/ml of a diet comprised of equal amounts of the single-celled algae *Chaetocerous calcitrans*, *Dunaliella tertiolecta*, and *Isochrysis galbana* (Strathmann, 1987). Thirteen days after fertilization, the larvae had developed into early eight-armed pluteii. These larvae were cultured for an additional two days to allow them to attain the late eight-armed pluteus stage for use in the present experiment.

The eight-armed pluteus larvae of the irregular echinoid *Dendraster excentricus* were cultured at Friday Harbor Laboratories, University of Washington, during summer 1998. These larvae, which were obtained by artificial fertilization using similar methods to those described above, were cultured in glass finger bowls (250 ml) in natural seawater. Glass fingerbowls containing larvae were held in a circulating seawater system at 12-15°C. Larvae were fed the single-celled algae *Rhodomonas lens* (Strathmann, 1987). Larval regeneration was monitored and photographed using a Nikon Wild M-5 dissecting microscope and a Nikon Optiphoto-2 microscope equipped with a camera system. Only 15-day-old larvae were used in the present experiment.

Surgical bisection of larvae

Pluteus larvae of the regular echinoid *L. variegatus* were surgically bisected into anterior and posterior sections approximately equidistant between the anterior and posterior poles (Figs. 1A, 2A) (Vickery and McClintock, 1998, Dissertation Manuscript 1; Vickery *et al.*, 2001b). Prior to bisection, larvae of *L. variegatus* ranged from approximately 600-1100 μm in length (along the anterior to posterior larval axis), possessed a larval skeleton (Fig. 1A), and were observed to be actively swimming and feeding. The same procedure was used to surgically bisect pluteus larvae of the irregular echinoid *D. excentricus*. Larvae ranged from approximately 700-1000 μm in length (along the anterior to posterior larval axis) and also possessed a larval skeleton prior to bisection. All anterior and posterior portions of larvae were maintained in 250-ml glass finger bowls under the culture conditions described above for each species. Non-bisected control larvae were also simultaneously cultured under identical conditions for comparison. Anterior and posterior larval portions of both species were observed and photographed daily over a 14-day period.

Results and Discussion

Within 5 days, anterior and posterior portions of pluteus larvae of the regular echinoid *Lytechinus variegatus* regenerated missing body components, including a digestive system and a larval mouth, respectively (Fig. 1A-E). The morphogenic and organogenic processes appeared to be similar to those we observed in regenerating asteroid larvae, with aggregations of mesenchyme cells with pseudopodia evident at the site of bisection (Vickery and McClintock, in press, Dissertation Manuscript 2). Eight days after bisection

both larval portions had completely regenerated missing larval structures (Fig. 1 F, G). Elongation of the larval skeleton resulted in replacement of the larval arms (Fig. 1E). Two weeks after bisection regenerated larvae were morphologically and functionally indistinguishable from nonbisected control larvae, and displayed normal swimming and feeding behaviors. Regenerated larvae 18 days after bisection developed adult rudiments posteriorly and metamorphosed into normal juveniles.

In larvae of the irregular echinoid *Dendraster excentricus*, the regeneration processes were observed to be almost identical to those in larvae of *L. variegatus*. Six days after bisection, the digestive system of anterior portions of larvae had almost completed regeneration; however, the anus had yet to form (Fig. 2A, B). Elongation of the larval skeleton was observed to occur in the regenerating posterior larval portion, while the exposed larval skeleton was surrounded by newly regenerated epidermis and ciliary bands (Fig. 2A, C). While posterior portions of larvae were capable of complete regeneration, the anterior portions only partially regenerated during the course of the study (approx. 2 weeks). Additional studies are needed in order to determine whether bisected anterior portions of larvae of *D. excentricus* have the capacity to regenerate and metamorphose fully into juveniles. The inability of the anterior larval portion of *D. excentricus* to regenerate fully over a 2-week period may be related to the low ambient seawater temperatures at which these larvae were reared (12-15°C for *D. excentricus* vs. 20-23°C for *L. variegatus*). Lower temperature can suppress metabolic rates in developing marine invertebrate larvae (Pearse *et al.*, 1991; Boidron-Métairon, 1995; Marsh *et al.*, 2001).

Our study demonstrates that planktotrophic larvae of regular and irregular echinoids are capable of complete regeneration after surgical bisection, as we have observed

to occur in the planktotrophic bipinnaria and brachiolaria larvae of asteroids (Vickery and McClintock, 1998, in press, Dissertation Manuscripts 1 and 2; Vickery *et al.*, 2001b). The planktotrophic larvae of asteroids are morphologically similar to those of holothuroids (Smiley, 1986), while the planktotrophic larvae of echinoids are similar to those of ophiuroids (Levin and Bridges, 1995), which, like asteroid larvae, also have been shown to undergo asexual clonal reproduction (involving regeneration of a complete new larval body) (Balser, 1998). Therefore we feel it is likely that planktotrophic larvae of most if not all echinoderms generally have the capacity for regeneration. Regenerative capacity in planktotrophic echinoderm larvae may help offset their presumably high mortality rates in the plankton (Menge, 1975; Vickery and McClintock, 1998, Dissertation Manuscript 1).

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Figure 1. Surgically bisected pluteus larvae of the regular echinoid *Lytechinus variegatus*. (A) Nonbisected echinopluteus larva. (B) Regenerating anterior portion of larva immediately after bisection. (C) Regenerating posterior portion of larva immediately after bisection. (D) Regenerating anterior portion of larvae 5 days after bisection. (E) Regenerating posterior portion of larva 5 days after bisection. Missing structures from both the anterior and posterior larval halves (including the larval digestive system) are seen undergoing regeneration. (F) Regenerated anterior portion of larva 8 days after bisection. (G) Regenerated posterior portion of larva 8 days after bisection. ds, digestive system; la, larval arm; m, mouth; p, phytoplankton; pb, plane of bisection. Scale bars = 200 μm (A) and 100 μm (B-G, shown in B).

Figure 2. Surgically bisected pluteus larvae of the irregular echinoid *Dendraster excentricus*. (A) Nonbisected echinopluteus larva. (B) Regenerating anterior portion of larva 6 days after bisection. (C) Regenerating posterior portion of larva 6 days after bisection. ds, digestive system; m, mouth; r, rudiment; pb, plane of bisection. Scale bars = 200 μm (A), and 100 μm (B-C, shown in B).

GENERAL SUMMARY AND DISCUSSION

Several studies performed in the early 20th century and involving surgical bisection of various asteroid embryos, as well as bipinnaria larvae of the asteroid *Astropecten aranciacus*, suggested that larval asteroids possessed some (albeit limited) capacity for regeneration (Hörstadius, 1973; Vickery et al., 2001b). These early observations have not been questioned or further examined in detail until recently, with the studies presented in this dissertation. We have for the first time successfully demonstrated the complete regeneration of missing body parts after surgical bisection in two species of planktotrophic sea star larvae (*Luidia foliolata* and *Pisaster ochraceus*). The regenerated larvae were further bisected and again observed to regenerate completely. Furthermore, complete larval regeneration was confirmed to occur throughout all stages of larval development. Remarkably, no mortality caused by surgical bisection was ever observed throughout any of these experiments.

Surgically bisected larvae not only completely regenerated missing tissues but also were observed to develop new organs from existing tissues and cells. Moreover, the regenerative cellular processes observed in larvae were very similar to those that have been reported for many other organisms, including adult asteroids (Thorndyke *et al.*, 1999; Candia Carnevali and Bonasoro, 1994; Candia Carnevali *et al.*, 1995). Twenty-four to 48 hours after surgical bisection, mesenchyme cells with pseudopodia became visible at the site of bisection. It appeared that aggregations of these cells, combined with dedif-

ferentiation and redifferentiation of various existing cells and probable proliferation of existing epidermal cells, eventually formed a completely new set of coeloms and a new digestive system. Moreover, with or without food available, regeneration occurred after surgical bisection, which suggested the utilization of dissolved organic matter (DOM) as a nutrient source, with a rearrangement of existing cells and the proliferation of new cells from the existing cells appearing to occur in unfed larvae. These observations ultimately suggest versatility in larval cell fate, which brings into question the current hypothesis that cell fate in developing larvae is irreversible (Cameron *et al.*, 1987; Peterson *et al.*, 1997). Many questions regarding cell fate versatility need to be studied in greater detail in future research.

In late-stage larvae (close to metamorphosis), surgical bisection demonstrated clear differences between *L. foliolata* and *P. ochraceus* in the fate of larval posterior portions during the regeneration process. In *L. foliolata*, surgical bisection induced the posterior portions (which had an adult rudiment) to complete metamorphosis by absorbing the remnant of the larval body. No regeneration of the missing anterior portion was observed to occur in the posterior portions of late-stage *L. foliolata* larvae, while the anterior portion regenerated completely the missing posterior portion. In *P. ochraceus*, however, none of the surgically bisected posterior portions completed metamorphosis unless they first completed regeneration of the lost anterior portions, including the brachiolar apparatus, consisting of three projections with papillae and one sucking disc. The distinct differences seen between the larvae of these two species, one a bipinnaria type larval development (*L. foliolata*) and one a brachiolaria type larval development (*P. ochraceus*), might result from their differing evolutional traits.

The phylogeny and classification of the Asteroidea remain controversial. Traditionally, the Luidiidae (to which *L. foliolata* belongs) and the Astropectinidae have been considered to be ancestral as compared with the rest of the Asteroidea (Downey, 1973; Clark and Downey, 1992; McEdward and Janies, 1993). *L. foliolata* has a bipinnaria larva that later develops an adult rudiment on its left posterior and then absorbs the entire larval body as it completes metamorphosis while swimming in the water column. In contrast, *P. ochraceus* has a bipinnaria larva that later develops into a brachiolaria larva equipped with a brachiolar apparatus on its anterior. The brachiolar apparatus is thought to be employed by the larva in the recognition of a favorable substrate for settlement (Barker, 1978). When brachiolaria larvae possessing an adult rudiment on the left posterior were surgically bisected, all bisected posterior portions fully regenerated the missing anterior portions, including the brachiolar apparatus. This finding emphasizes the importance of the brachiolar apparatus in larval development in *P. ochraceus*. Since the Luidiidae and Astropectinidae inhabit sandy substrata, Blake (1988) hypothesized they do not require a brachiolar apparatus to sense suitable substrate for settlement and therefore lost the brachiolar apparatus as an adaptational trait.

As was previously mentioned, complete regeneration occurred in larvae of both asteroid species after surgical bisection, even when no food was available to the larvae. After surgical bisection, the anterior portions lost a functional gut and were unable to feed; however, they were capable of compete regeneration, which suggests the utilization of DOM as an energy source. Moreover, the anterior portions became reduced in length, which supports the hypothesis that they utilized existing cells and tissues to reconstruct missing body parts. In contrast, the posterior portions of larvae grew significantly larger

(in length) when they were fed high food concentrations since they retained their ability to feed. The energetics of larval regeneration remain to be studied.

In addition to our discovery of regenerative capacity in planktotrophic asteroid larvae, we have successfully demonstrated for the first time that planktotrophic echinoid larvae have a strong regenerative capacity. Echinoid pluteus larvae possess larval skeletons; therefore it might seem that regeneration would be more difficult to accomplish in these larvae. Despite this fact, we have observed complete regeneration after surgical bisection of both larval halves in *Lytechinus variegatus* and nearly complete regeneration in surgically bisected larvae of *Dendraster excentricus*. Historically sea urchins (Echinodermata, Echinoidea) have been extensively used for studies in developmental biology. There is far greater information available regarding development in echinoids than in asteroids. Thus, the implications of this study could be far reaching in the context of developmental biology. Also, once again cell fate has classically been thought to be irreversibly determined once embryos became larvae (Cameron *et al.*, 1987; Wray, 1995). However, our observations suggest that the cells of planktotrophic marine invertebrate larvae are able to dedifferentiate and then redifferentiate to regenerate missing portions. This result itself implies broad versatility in cell fate, with important implications to developmental biology.

Larval regeneration in asteroids and echinoids has not previously been considered within the field of echinoderm larval biology, including aspects of embryology, development, and ecology. A reexamination of our current framework of knowledge in these areas is worth while in light of the findings presented in this dissertation. Moreover, investigations using sea star and echinoid larvae as models for the study of regeneration

biology (including regeneration genetics) may provide greater insight into the phenomenon of regeneration in other invertebrates and vertebrates, including humans.

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I certify that I have read this document and examined the student regarding its content. In my opinion, this dissertation conforms to acceptable standards of scholarly presentation and is adequate in scope and quality, and the attainments of this student are such that she may be recommended for the degree of Doctor of Philosophy.

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