THE COMPLETE DEVELOPMENT OF THE DEEP-SEA CIDAROID URCHIN *CIDARIS BLAKEI* (AGASSIZ, 1878) WITH AN EMPHASIS ON THE HYALINE LAYER

by

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A THESIS

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Alan Shanks

Living echinoids comprise two major sister clades, the Euechinoidea and the Cidaroidea. Cidaroids first appeared during the lower Permian (~255 mya) and are considered to represent the primitive form of all other living echinoids. The present study of *Cidaris blakei*, a deep-sea planktotrophic cidaroid urchin, provides a description of development from fertilization through early juvenile stages and is the first report of a deep-sea organism reared through metamorphosis. *Cidaris blakei* resembles other cidaroids in its lack of a cohesive hyaline layer, the absence of an amniotic invagination for juvenile rudiment formation, and the presence of a single spine morphotype at metamorphosis. *Cidaris blakei* differs from other cidaroids in the presence of an apical

tuft, the extent of fenestration of postoral skeletal rods, the shape of juvenile spines and an extended (14 day) lecithotrophic stage prior to development of a complete gut.

This study includes my co-authored materials.

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CHAPTER I

GENERAL INTRODUCTION

In May of 2008 I had the good fortune to take part in a deep-sea course, the majority of which was taught at sea in the Bahamas. The course was offered through the Oregon Institute of Marine Biology (OIMB), and the cruise was part of a grant obtained by University of Oregon faculty, including Drs. Craig Young and Richard Emlet, to study vertical distributions of larvae within the water column and potential food sources for larvae of bathyal invertebrates. Twice daily a submersible, the Johnson Sea Link II, was hoisted from the deck and put into the sea. Twice during the cruise I was aboard the sub, watching as the sunlight faded during our descent and the water became bright with bioluminescent organisms. More than 2000 feet below the surface of the ocean we explored a foreign landscape and saw places that no humans had seen before. Back on board the ship, students and scientists formed an efficient crew, unloading the organisms collected by the sub and quickly putting them into a cold room. One of the organisms collected by the submersible was *Cidaris blakei*, an urchin unlike any I had seen before. It had three different types of spines, one a wide paddle shape, and all of them covered by epibionts (Fig. 1). For my own class project, and as part of the larger grant objectives, I helped to spawn *Cidaris blakei* and rear and describe the larvae.

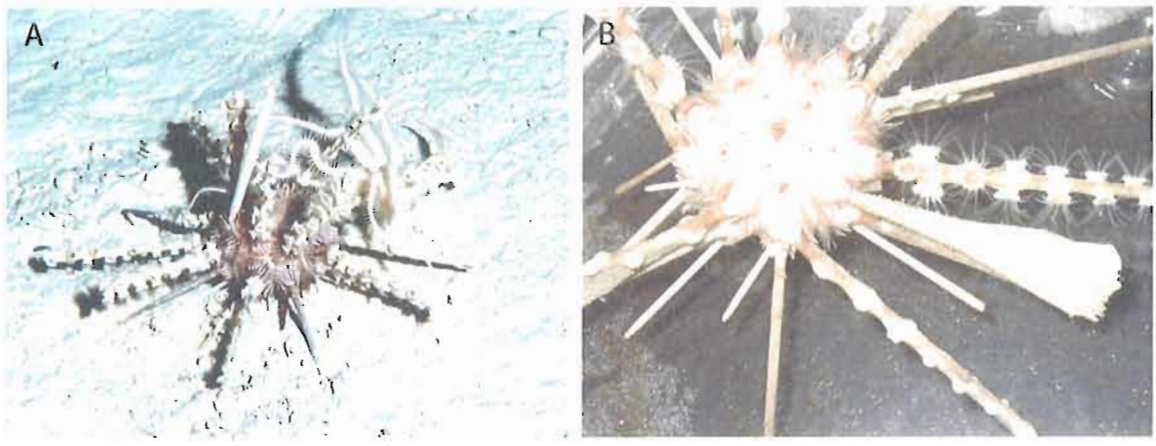

Figure 1. Two images showing *Cidaris blakei* adults. (A) an image taken *in situ* at 586 m using a camera mounted on the front of the submersible. (B) A close-up showing the three different spine morphotypes and epibiotic zoanthids.

When we returned from the Bahamas, I resumed the master's project I'd begun before the cruise, but found that I was spending much of my time at the microscope observing *C. blakei*, and even more of my time reading the scientific literature that helped me put my observations into a larger context. Eventually this side project began to dominate my thoughts and time, and I was fortunate that my advisor, Alan Shanks, was flexible enough to allow me to follow my interests and switch topics and that Richard Emlet and Craig Young were willing to let me take on a piece of that project as my own.

To study a single organism is not to have a narrow focus. Attendant to that organism are questions of reproduction, ecology, embryology, evolution and physiology, among others. In this thesis, I address questions of *Cidaris blakei* in the context of an echinoid with a biphasic life history, an inhabitant of the deep sea, and a cidaroid.

Most echinoids have a biphasic life history, the majority of which is spent on the bottom in a familiar adult form. During the first part of their life, however, they are microscopic larvae that bear little morphological resemblance to the adult form. The environments they inhabit during each of these life stages are also dramatically different. As adults, echinoids live upon the ocean's substratum, exploiting algal and animal food sources. As larvae, echinoids are planktonic, adrift in the water column, and those that feed during this stage do so by capturing minute particles of organic matter using a ciliated band.

We collected *Cidaris blakei* from the ocean's bottom, far below the reach of sunlight and the abundant photosynthetic organisms of the euphotic zone. Deep-sea larvae have different challenges than their shallow water counterparts (e.g. Young and Eckelbarger, 1994). Some deep-sea larvae have been shown to migrate from depth into the productive euphotic zone before they descend through the water column to settle on the benthos. Direct evidence for ontogenetic vertical migration by deep-sea gastropods has been collected in the form of isotopic analyses of the protoconchs of bathyal and abyssal species, and by capture and identification of the veligers of deep-sea organisms in surface water tows (reviewed by Bouchet and Warén, 1994; Van Gaest, 2006; Arellano, 2008).

The larva of a deep-sea echinoid, *Aspidodiadema jacobyi*, whose range of distribution overlaps with *C. blakei*, has an extended pre-feeding stage of almost 21 days prior to the opening of the mouth and completion of the gut. Young *et al.* (1989)

hypothesized that the extended pre-feeding period of *A. jacobyi* could be an adaptation to deal with patchy food resources in the deep-sea, or alternatively might provide the larva with sufficient yolk reserves to undertake vertical migration to the euphotic zone.

Cidaris blakei is a cidaroid sea urchin. Cidaroids are an ancient lineage, and are considered to be "living fossils" of all other living echinoids. There are 124 species of echinoids known from the Paleozoic, more than 250 million years ago. Of these, only one is known to have survived the Permo-Triassic extinction: the small, opportunistic generalist *Miocidaris* that persisted into the Triassic (Smith, 1984). By the early Upper Triassic (250 mya), there were 16 known species of echinoids. This was when the first true cidaroids appeared (Kier, 1977). Then echinoids underwent a significant adaptive radiation (there are 3,672 known from the Mesozoic, compared to the 124 of the Paleozoic), and all major lineages of post-paleozoic echinoids appeared—probably in response to a new food source that they learned to exploit and few predators or competitors (Smith, 1984). From this post-Paleozoic radiation persist the two lineages of extant urchins: the Cidaroidea and the Euechinoidea (Smith, 1984).

In Chapter II of this thesis, which includes unpublished co-authored materials, I describe developmental features of the embryos, larvae and juveniles of *Cidaris blakei* and also provide preliminary evidence for a thermal barrier against ontogenetic vertical migration. In Chapter III, I use monoclonal antibodies and confocal microscopy techniques to describe a single embryonic structure, the hyaline layer. In Chapters II,

III and IV, I discuss these developmental characteristics in the context of echinoid phylogeny and the deep sea.

CHAPTER II

LARVAL DEVELOPMENT AND METAMORPHOSIS OF THE DEEP-SEA CIDAROID URCHIN *CIDARIS BLAKEI*

This chapter includes unpublished co-authored material. I was the principle investigator of the work presented in this chapter, and Drs. Richard Emlet and Craig Young assisted in larval culture and the description of the embryos and larvae.

Introduction

Extant echinoids are divided into two major sister clades, the Euechinoida and the Cidaroida. Euechinoids are diverse, encompassing nine of the ten major groups of echinoids and comprising more than 800 living species, including both regular and irregular echinoids (Smith, 1984). There is abundant literature on the embryonic and larval development of euechinoids (reviewed by Guidice, 1986; reviewed by Pearse and Cameron, 1991). Cidaroida, the order of "pencil urchins", is much less diverse, comprising 27 genera and 148 living species and subspecies (Mortensen 1938). There

have been relatively few studies detailing cidaroid development. The only planktotrophic cidaroid reared through metamorphosis and fully described is *Eucidaris thouarsi*, a shallow-water urchin collected in the Bay of Panama (Emlet, 1988). Other species for which some portion of development has been described include *E. metularia* and *E. tribuloides* (Mortensen, 1937; Schroeder, 1981), *Cidaris cidaris* (Prouho, 1887) and *Prionocidaris bacculosa* (Mortensen, 1938). Development in two lecithotrophic species, *Phyllacanthus parvispinus* and *P. imperialis* (Parks *et al.*, 1989; Olson *et al.*, 1993) has also been described. Cidaroid urchins are often regarded as "living fossils". Since they first appeared in the late lower Permian (lower Zechstein, ~255 mya) cidaroids have undergone few morphological changes and are often regarded as "living fossils". (Kier, 1977; Smith and Hollingworth, 1990; Smith, 2005; Smith *et al.*, 2006).

The spawning, reproduction and early larval development of *Cidaris blakei* were studied more than 20 years ago by C.M. Young, J.L. Cameron and P.A. Tyler, but none of the cultures survived to metamorphosis and only the egg size, breeding behavior and reproductive periodicity have been reported in the literature (Young, 1992, 2003). In the present study, we extend and complete this preliminary work by describing the complete embryonic and larval development through metamorphosis and into the juvenile stage. This is the first description of a deep-sea echinoid reared through metamorphosis. Previous studies of the morphology, larval duration, physiological tolerances and developmental timetables of deep-sea larvae from the bathyal zone have been reviewed by Young, 2003. The long-held belief that brooding is the dominant mechanism of reproduction in the deep sea (Thorson, 1950) has been summarily disproved (Pearse,

1994; Young, 1994). It is now known that deep-sea organisms employ a wide range of developmental modes (reviewed by Young, 2003). Although planktotrophy is not uncommon in the deep sea (e.g. Bouchet, 1994; Young *et al.* 1998; Van Gaest, 2006), there is still the question of what planktotrophic larvae might eat as they begin to develop far below the productive waters of the euphotic zone. Evidence for ontogenetic vertical migration by deep-sea gastropods has been collected in the form of isotopic analyses of the protoconchs of bathyal and abyssal species (Rex and Warén, 1982), and by capture and identification of the veligers of deep-sea organisms in surface water tows (reviewed by Bouchet and Warén, 1994; Van Gaest, 2006; Arellano and Young, 2009). Other studies have shown that physiological tolerances might present a barrier to migration through the wide range of temperatures and salinities in the water column (Young and Cameron, 1989; Young *et al.*, 1996; Young *et al.*, 1998).

We compare development of *C. blakei* to that of other cidaroids and also other deep-sea echinoids. We discuss which characteristics are common to cidaroids, and potentially attributable to phylogenetic constraints, and which adaptations may be specific to the deep sea environment. Additionally, we describe morphological features not previously observed in a planktotrophic echinoid, and provide preliminary evidence for physiological barriers to ontogenetic vertical migration.

Materials and Methods

Collection and husbandry

Adult specimens of the deep-water echinoid *Cidaris blakei* (A. Aggasiz, 1878) were collected from 12 sites in the northern Bahamas using the manned submersible Johnson Sea-Link II (Harbor Branch Oceanographic Research Institute, Fort Pierce, FL, U.S.A.). *Cidaris blakei* was collected at depths ranging from 540 m-685 m between May 14, 2008, and May 22, 2008. *In situ* temperatures at the collection sites ranged from 11-13 °C. Organisms were brought from depth to the ship in closed, seawater-filled containers and immediately transferred into aquaria in a 13°C cold room. At the cessation of the cruise, on May 25, adult specimens were transported to the Oregon Institute of Marine Biology (OIMB) in Charleston, Oregon, and placed in seawater tables with recirculating filtered seawater maintained at 11°C and salinity of 32. Adult *C. blakei* were fed algae (e.g. *Ulva sp.* and *Sargassum muticum*) as well as encrusting organisms including the bryozoan *Membranipora membranacea*, the sponge *Halichondria panacea* and the gorgonian *Leptogorgia* sp., all collected from the intertidal and subtidal zones of Oregon.

Spawning and culturing

On June 12, 2008, we induced spawning of *Cidaris blakei* with an intracoelomic injection of 1.0-5.0 ml of 0.55 M KCL in 0.45 μm filtered seawater (FSW). Adults of *C. blakei* were again spawned on June 23, 2008 and June 25, 2008, using intracoelomic injections of 0.5-1 mL of 0.1 M acetylcholine in FSW. Sperm from seven different males

(one on June 12, four on June 23 and two on June 25) were used to fertilize eggs. Within hours of fertilization, embryos were transferred to 1-L beakers of 0.45 millipore filtered seawater. Cultures were stirred constantly using swinging paddles (ca. 12 cycles per minute) that hung from a rack similar to that described by Strathmann (1987). The embryos and larvae resulting from the June 12 spawning event were kept in a 16 °C incubator. These did not survive, so subsequent cultures were maintained between 11 and 13°C by partial submersion in flowing seawater tables. Larval cultures were cleaned every three days by reverse filtration with a 100 μm-mesh sieve. Once larvae developed complete digestive tracts, they were fed a combination of *Chaetoceros gracilis*, *Dunaliella tertiolecta* and *Rhodomonas lens* at a total concentration of 2000 cell/ml. Algae were cultured in f/2 media (Guillard, 1975). Algal cells were removed from the culture media by centrifugation and decanting, then re-suspended in FSW prior to feeding to the larvae.

Juveniles were kept in culture dishes maintained at 11 °C and fed the algae *Ulva spp.* which were macerated with a razor blade. We also fed the macerated and whole colonies of the arborescent bryozoan *Bugula pacifica.* Individuals were seen on the bryozoan and with bryozoan fragments in their guts.

Microscopy

Embryos and larvae were photographed using a Nikon Coolpix 4500 camera mounted on either an Olympus BH-2 compound microscope or an Olympus SZH110 dissecting microscope. Absolute measurements were determined with calibrated ocular

micrometers or by photographing a stage micrometer at the same magnification as that of a larva or embryo of interest.

For scanning electron microscopy (SEM), samples were fixed in 10% buffered formalin, then washed and stored in Millonig's phosphate buffer. Samples were post-fixed in 2% osmium tetroxide, washed in phosphate buffer and dehydrated through an alcohol series before critical point drying, mounting, and sputter coating with gold/palladium. All SEM micrographs were taken on a Tescan VEGA TC microscope.

Temperature Tolerances

We tested the ability of *Cidaris blakei* larvae to survive a range of temperatures that they might experience during an ontogenetic vertical migration to the upper water column. We exposed them to temperatures representing the thermal profile of the Bahamas during their spawning season (Young *et al.*, 1998). Fifty days after fertilization, we placed ten 8-arm plutei in each of six 20 ml scintillation vials. These vials were then placed in an aluminum temperature gradient block (Sewell and Young, 1999) with one recirculating water bath at each end, that maintained a thermal gradient from 11 ºC-22 ºC. Three replicate vials, each containing 10 plutei, were moved up through the thermal gradient with a 12-hour acclimation period for every two-degree temperature increase. As a control, three additional vials containing 10 larvae each were held in the thermal block at 11 °C. At every temperature transfer we checked the larvae for survivorship and noted morphological changes.

Results

Development of *Cidaris blakei* from fertilization to settlement took four months (Table 1). Three different females shed relatively large, opaque eggs after injection on June 12, 23 and 25 (a single female on each date). Eggs of the female spawned on June 12, 2008 were 160.6 μm (± 3.3 μm SD, *n*=20) in diameter. Eggs of the females spawned on June 23 and 25, 2008 had mean diameters of 153.1 μm (± 5.0 μm SD, *n*=20) and 158.1 (± 4.9 μm SD, *n*=20), respectively (Fig. 1A).

Table 1. Developmental timetable of *Cidaris blakei*, cultured at 11-13 °C, from fertilization to metamorphosis.

Time since fertilization	Developmental stage
2.5 h	Two cell embryos
6 h	Four cell embryos
7.5 h	Eight cell embryos
9 h	16 cell embryos
23 h	Unhatched blastulae. Wrinkled with a clear blastocoel
47 h	Hatched blastulae. Apical tuft visible.
73 h	Initiation of gastrulation
4 days	Mid-stage gastrulae. Archenteron about ½ way into
7 days	Gastrulae that are compressed. First spicules visible with cross-polarized light.
8 days	Prisms
13 days	Two-arm plutei with open mouths
1 month 3 days	Four and six-arm plutei
1 month 8 days	Formation of lobes begins
2 months 17 days	Juvenile rudiments with podial buds and pedicellariae
3 months 29 days	Metamorphosis. Juvenile spine count ranges from 5 to 23.

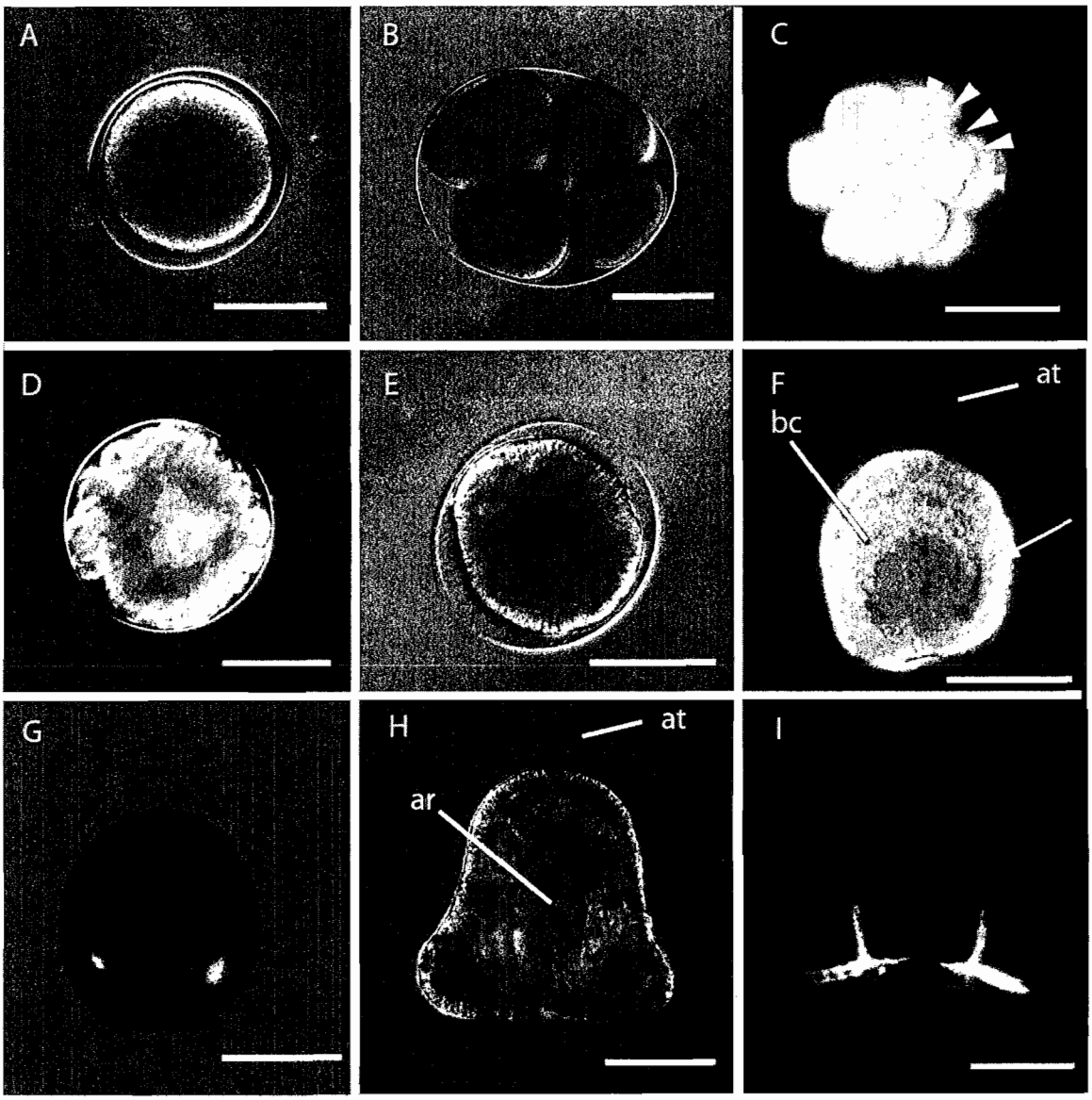

Figure 1. Light micrographs of *C. blakei* from immediately after fertilization to nine days after fertilization. All scale bars= 100 μm. (A) Fertilized egg; (B) four-cell stage undergoing 3rd cleavage; (C) 16-cell stage with micromeres labeled; (D) late morula; (E) unhatched blastula; (F) blastula with ball of cells in blastula and an apical tuft; (G) pigmented gastrula with ventro-lateral spicules illuminated with cross-polarized light; (H) prism; (I) prism with tripartite, fenestrated skeletal rods illuminated with cross-polarized light. Labels: arrowheads, micromeres; arrow, ball of cells in blastocoel; ar, archenteron; at, apical tuft; bc, blastocoel.

The blastomeres of early embryos were widely spaced (Fig. 1B, C, D). Attempts to visualize the hyaline layer on the embryos of *C. blakei* using light microscopy were unsuccessful, suggesting that the hyaline layer was either very reduced or absent. The blastomeres appeared to be constrained only by the perimeter of the fertilization envelope, and multi-cell and morula stages were disorganized and amorphous (Fig. 1 C, D, E). In many but not all cases after the fourth cleavage, there were smaller cells present at one end (presumed vegetal) of *C. blakei*, numbering anywhere from zero to five (Fig. 1 C). Primary mesenchyme was not specifically noted in the blastulae of *C. blakei*, although we observed a mass of cells similar to that figured by Prouho (1887) and labeled as primary mesenchyme (Fig. 1 F). *Cidaris blakei* blastulae were wrinkled and often irregularly shaped. Because of the opacity of the embryos, we were unable to note coelom formation. Seven days after fertilization, during the gastrula stage, calcified spicules were evident with cross-polarized light (Fig. 1 H). A pair of these skeletal elements progressed to tri-radiate spicules (Fig. 1 I) and, eventually, the fenestrated post-oral rods characteristic of all cidaroid larvae that have skeletons. Fenestration began at the base of the skeletal rods and continued to the tip (Fig. 1 I).

Later embryos of *Cidaris blakei* were different from other cidaroids in several ways. Hatched embryos were yellow-white and very opaque with numerous red pigment cells. A long apical tuft (83 μm long on a 165 μm long embryo) first became evident in recently hatched blastulae and persisted through the early two-arm pluteus stage (Figs. 1 F, 3 A). The ectoderm of 4-day old gastrulae had "pits" or invaginations (Fig. 2 A, B).

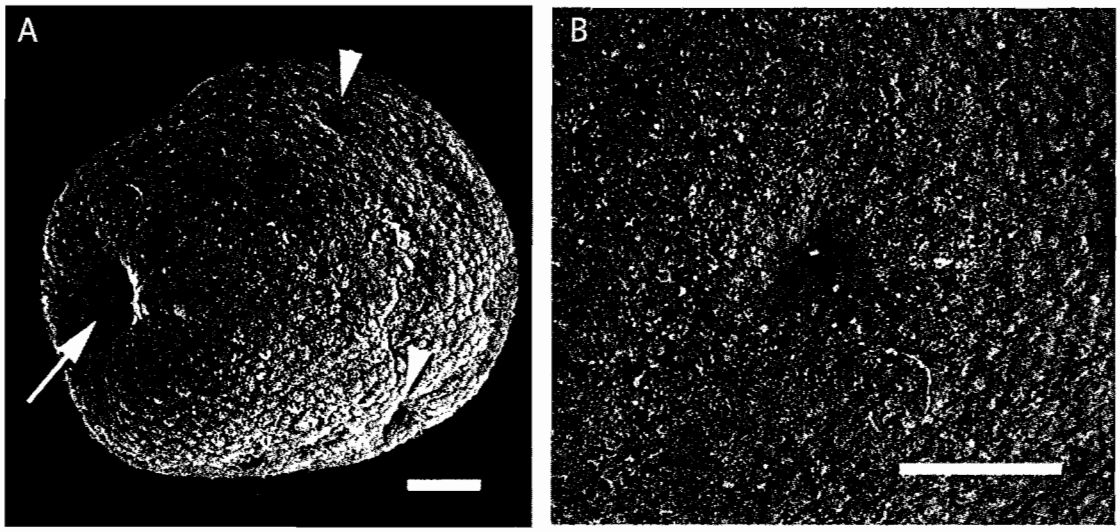

Figure 2. Scanning Electron Micrographs of a 4 day-old *C. blakei* gastrula showing ectodermal pits. (A) Gastrula with blastopore and cylindrical invaginations in the ectoderm labeled; (B) close-up of cylindrical pit; scale bars = 20 μm. Labels: arrowheads, invaginations; arrow, blastopore.

The mouth of *C. blakei* did not open until well into the two-arm pluteus stage, 14 days after fertilization (Fig. 3 A, B). Fifteen days after fertilization, the preoral arms began to elongate from the preoral hood and the postoral arms were 484 μm. At this stage, the fenestrated posterodorsal arms also began to lengthen (Fig. 3 B). The postoral arms of 25 day-old plutei were 680 μm long, and the first pair of epidermal lobes appeared at the base of the posterodorsal arms 38 days after fertilization (Table 1, Fig. 3C); the larvae subsequently formed the five pairs of lobes as described for *E. thouarsi* (Fig. 3 D) (Emlet, 1989).

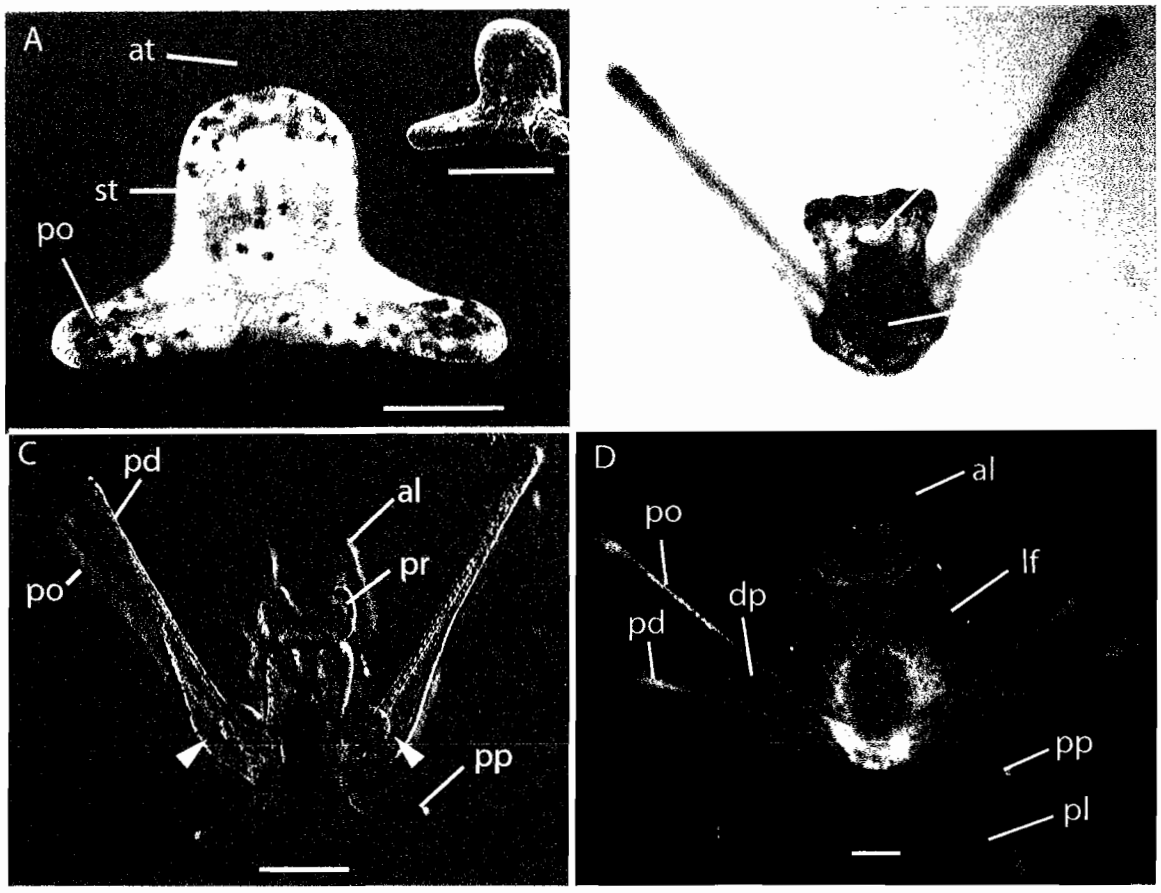

Figure 3. Light micrographs of *C. blakei* plutei from 10 days to 79 days after fertilization. (A) Ventral view of a two-arm pluteus with post-oral arms, an apical tuft and unopened mouth (scale bar=100 μm); (B) dorsal view of an advanced two-arm pluteus with a complete gut (scale bar=200 μm); (C) dorsal view of an eight-arm pluteus showing early lobe development (scale bar=200 μm); (D) ventral view of an advanced, multi-lobed pluteus (scale bar=100 μm). Labels: arrowheads, dorso-posterior lobes; al, anterolateral arm; at, apical tuft; gt, gut; mo, mouth; pd, posterodorsal arm; po, right post-oral arm; st, stomodeum.

The echinus rudiment did not develop within a vestibule, but instead grew out of the left hydrocoel of the larval body. Juvenile structures first became evident 77 days after fertilization (Table 1) in the form of podial buds on the left side of the larva and a single pedicellaria at the base of the posterodorsal arms (Fig. 4A, B).

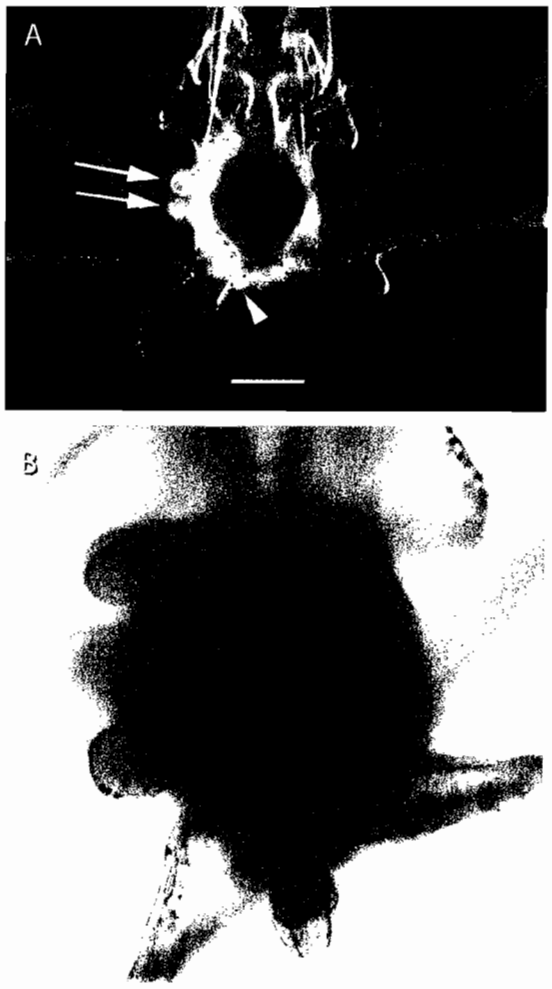

Figure 4. Light micrographs of advanced larvae of *C. blakei* with juvenile structures evident on the larval body. All scale bars = 200μm. (A) Close up of a 79-day larva with podial buds and pedicellariae labeled; (B) 98-day larva with well-developed podia and pedicellaria, image taken with compound scope. Labels: arrowheads, pedicellariae; arrows, podial buds.

Juvenile structures continued to develop for 43 days before the first larva underwent metamorphosis (Table 1). Unfortunately, most cultures became infected with bacteria at ~80 days after fertilization, and only nine larvae survived to metamorphosis. Of these, all metamorphosed with a set of five primary podia growing from the left hydrocoel and between one and three pedicellariae, one at the dorsal arch, and two posterior on the left side—one dorsal and one ventral (Fig 4A, B). In all competent larvae, there was always a pedicellaria on the dorsal arch. All spines present at metamorphosis were of the same type and they varied widely in number between 5 and 23 (Fig 5A, B.). We observed competent larvae using their podia to adhere to the bottoms of dishes. In many larvae, it appeared as if metamorphosis had taken place, but the larval arms sometimes remained for days after the tube feet had attached and the oral side of the juvenile had oriented toward the substratum (5A).

Juveniles

One day after metamorphosis, juvenile test diameter was 471 μm (±49 μm SD, n=4). Juvenile spines after metamorphosis were all identical in morphology to one another, (Fig. 5B, C). Adult spines developed 14 days after metamorphosis, and the first set of secondary podia appeared 20 days after metamorphosis (Fig. 5 C, D). At thirty-five days after metamorphosis, the two remaining juveniles had test diameters of 769 μm and 762 μm, respectively, and had buccal plates and calcified teeth (Fig. 5 D).

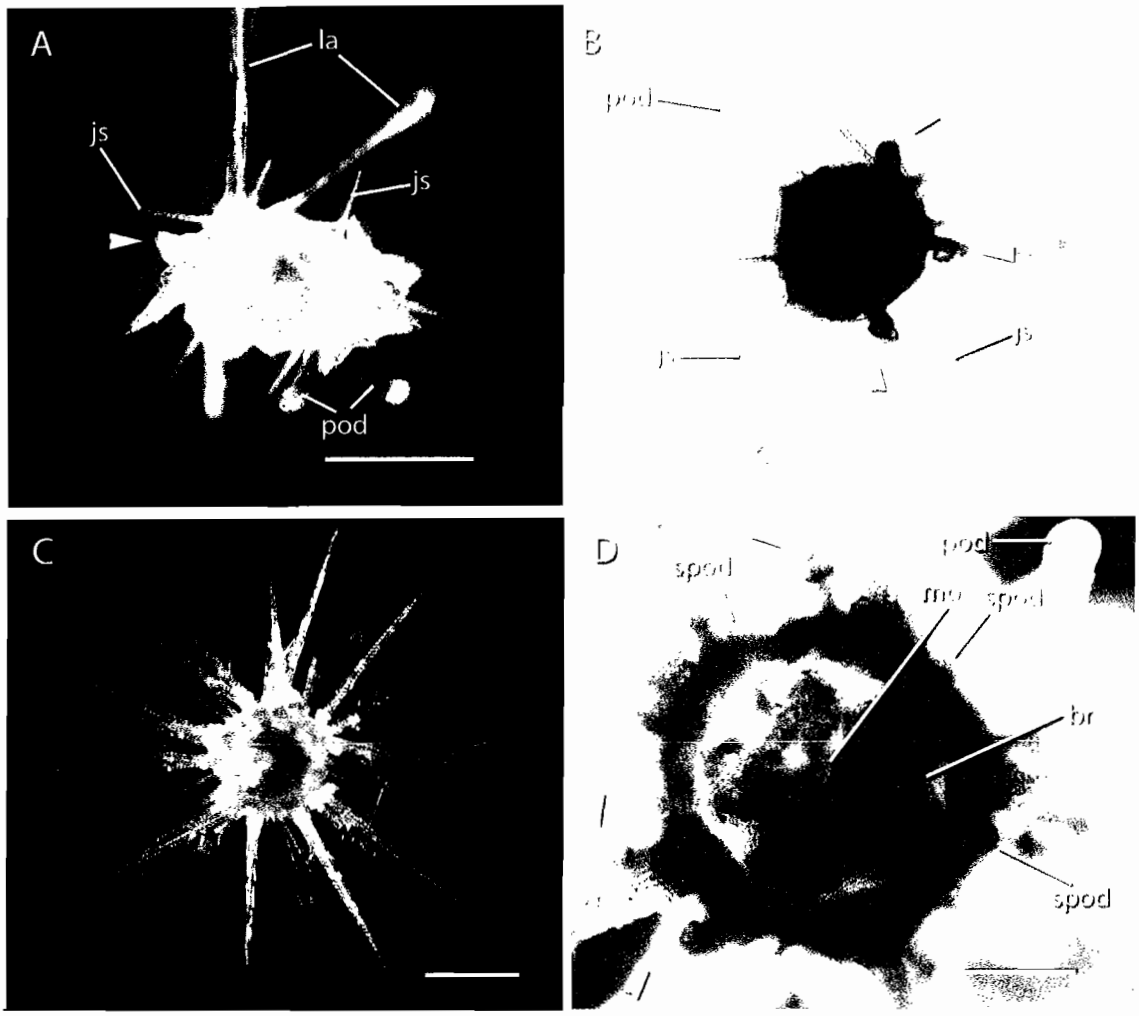

Figure 5. Light micrographs of a competent larva and juveniles of *C. blakei*. (A) Competent larva with 10 juvenile spines and well-developed pedicellaria and podia, but unmetamorphosed; scale = 500 μm; (B) aboral view of juvenile one day after metamorphosis; scale = 500 μm; (C) aboral view of juvenile 40 days after metamorphosis; scale = 500 μm; (D) oral view of 40-day juvenile using dark field, the test and teeth are calcified; scale = 200 μm. Labels: arrowheads, 2° podia; as, adult spine; bcp, buccal plate; js, juvenile spine; la, larval arms; mo, mouth; ped, pedicellariae; pod, podia.

Temperature

We were unable to culture *C. blakei* larvae at temperatures higher than 12 ºC. Cultures placed in incubators and maintained at 16 ºC did not survive. Attempts to acclimate larvae to increased temperatures using the thermal block were also unsuccessful. After 24 hours and a temperature increase to 15 ºC, 100% of plutei in all vials became morphologically abnormal. At 22 C, all larvae had resorbed arms, deformed mouths and reduced or lost lobes compared to control plutei held at 11 ºC. (Fig. 6).

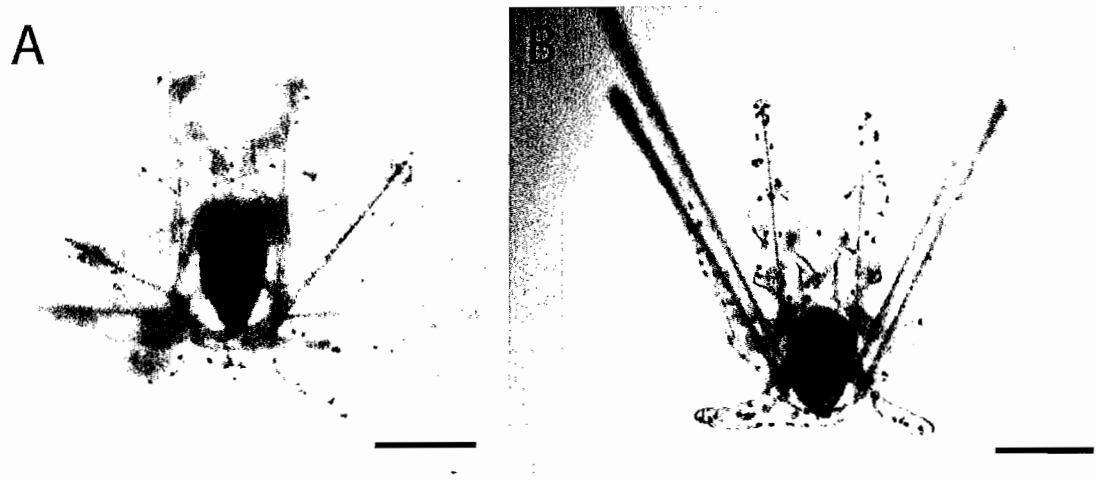

Figure 6. Light micrographs of *C. blakei* larvae from different temperature treatments. Plutei were maintained in scintillation vials and moved up a thermal gradient, from 11 °C to 22°C, over a four day period. As a control, plutei were kept in scintillation vials for four days at 11 °C. (A) Pluteus after four-day temperature increase to 22 °C; (B) pluteus after four days maintained at 11 °C.

Discussion

Characteristic embryology and larval form of cidaroids

In many respects, the development of *Cidaris blakei* resembles that described for other cidaroids. In early development there is an apparent lack of a hyaline layer, disorganized blastomeres, variable numbers of micromeres, and an absence of primary mesenchyme. Later in development there are five pairs of lobes arising from the ciliated band (Prouho, 1887; Mortensen 1937, 1938; Schroeder, 1981). As in *Eucidaris thouarsi*, a vestibule for juvenile rudiment formation is lacking (Emlet 1988).

For all species in which early development has been described, it has been noted that the blastomeres of cidaroid embryos are widely spaced. In *Prionocidaris baculosa*, Mortensen (1938) observed that the cells following first cleavage were so separate that they formed twins from a single egg (pg. 14). Mortensen also noted that a large percentage of embryos were of an unusual shape that ultimately went on to form a normal blastula. Schroeder (1981) noted the same phenomenon, and cited a "virtual absence" of the hyaline layer (p. 145). Though we did not notice any instances in which two embryos developed within the same fertilization envelope, our observations of relatively large distances between the cells of developing embryos, our inability to see a hyaline layer, and irregularly shaped embryos after fourth cleavage that later formed regular blastulae, certainly concur with those of Mortensen (1938) and Schroeder (1981).

In euechinoids, there are two types of mesenchyme cells. The first wave of mesenchyme cells to ingress are called primary mesenchyme, and are derived from the

small micromeres that arise at the vegetal pole of the embryo during an unequal fourth cleavage. These primary mesenchyme cells produce the larval skeleton. The secondary mesenchyme cells ingress later, and are associated with pigment cells and musculature (Horstadius, 1973; Okazaki, 1975). Although cidaroids appear to lack primary mesenchyme, it has been shown in *E. tribuloides* that the skeletogenic cells derive from the 16-cell micromeres, just as they do in euechinoids, and that cells with skeletogenic fates do ingress earlier than other mesenchyme cells but not as early as in euechinoids (Wray and McClay, 1988). In experiments on cell lineage in *E. tribuloides* (Wray and McClay, 1988), blastomeres were size-fractionated using a sucrose gradient, then micromeres from donor embryos were combined with host blastomeres to form chimaeras. The small donor cells went on to form the larval skeleton. In a much earlier study, Horstadius (1939) showed that different numbers of micromeres added to host embryos from which micromeres had been removed, resulted in vastly different outcomes. For instance, the outcome of adding a single micromere to isolated rings of animal or vegetal cells was a ciliated blob, whereas the addition of four micromeres resulted in a normal pluteus. It would be interesting to know whether cidaroids, with their varied number of micromeres, have similarly varied results.

Some embryos of *Cidaris blakei* were observed without any micromeres, or at least no size distinction was evident between cells. In euechinoids it has been shown that embryos from which micromeres have been excised still produce a larval skeleton, but the skeleton is derived from the veg2 cells (Horstadius, 1975). It is possible that the same is true for those embryos of *C. blakei* that lack micromeres. Embryos from which

micromeres have been removed have delayed initiation of gastrulation and a slower rate of archenteron elongation compared to embryos with micromeres (Ishizuka *et. al*, 2001). Alternatively, the size distinction may be volumetric, and cells with a skeletogenic fate may not always be smaller than other cells. It is possible that the extended time before spicules first appear in *C. blakei* (e.g. eight days compared to 72 hours in *Strongylocentrotus droebachiensis*, which has similar egg size and was reared at a lower temperature (Stephens, 1972), is due to many of the embryos having few or no micromeres.

The only cidaroid for which primary mesenchyme has been noted is *Cidaris cidaris* (Prouho, 1887). Wray and McClay (1988) suggested that Prouho's drawing of primary mesenchyme on *C. cidaris*, "may actually represent the very early tip of the invaginating archenteron, which in *Eucidaris* is loosely organized and could be mistaken for ingressing cells" (p.313). We observed *C. blakei* at a similar stage (Fig. 1F), and noted a cluster of cells very similar to that figured by Prouho. This stage was soon followed by elongation of the archenteron and further investigation is warranted to determine the origin of these cells.

Juvenile structures developed on *Cidaris blakei* in the absence of a vestibule, as has been described for the planktotrophic cidaroid *E. thouarsi* and the direct developing cidaroid *P. parvispinus* (Emlet, 1988; Parks *et al.*, 1989). In euechinoids, the vestibule is formed from an invagination of the larval epidermis that comes into contact with the left hydrocoel. In the absence of an invagination, the structures developed on the larval

epidermis, resulting in an apparently simpler metamorphosis, compared to the eversion of the vestibule in euechinoids. This type of avestibular rudiment has now been described for a lecithotrophic and two planktotrophic cidaroids in three different genera and from both shallow and deep-water habitats; it seems likely, therefore, that this character is conserved in the entire order.

Differences among cidaroids

Cidaris blakei differs from other planktotrophic cidaroids in several ways that distinguish them from other species or genera. These include: egg size, the length of larval life, the presence of an apical tuft (present only in the congener *C. cidaris*), pits on the ectoderm of gastrulae, a mouth that opens late in the two-arm pluteus stage, and juvenile spine morphology.

The eggs of *Cidaris blakei* are among the largest reported for planktotrophic cidaroids (Table 2). They represent an almost five-fold increase in volume over the eggs of *E. thouarsi*, *E. tribuloides* and *E. metularia* (Table 2). In general, echinoids with larger egg sizes (and therefore more maternal investment in materials and energy) have shorter developmental times to metamorphosis than those with smaller eggs (Emlet et al., 1987; Emlet, 1995; Levitan 2000). Mortensen (1938) reported an "enormous" difference in the rate of the developmental processes of *P. baculosa* and *E. metularia* and postulated that the difference was due to the smaller egg of *E. metularia* (pg. 15). Though the extended developmental period of *C. blakei* (a four-fold time increase over the smaller-egged *Eucidaris* species) seems somewhat anomalous in this regard, it cannot be

discussed without addressing the large differences among the rearing temperatures of *C. blakei* and those of other cidaroid larvae that have been studied (Table 2).

Temperature affects the rate at which larvae develop, with increased temperatures shortening time to metamorphosis (e.g. Pearse and Cameron, 1991; Emlet *et al.* 1987; Emlet 1995). *Eucidaris thouarsi* metamorphosed after only 30 days at 28 °C (Emlet, 1987). *Prionocidaris bacculosa* also metamorphosed 30 days after fertilization, at temperatures above 23 °C (Mortensen, 1937). The only cidaroid with a developmental timetable similar to that of *C. blakei* is the congeneric *C. cidaris* (Prouho, 1887). Although *C. cidaris* was not reared to metamorphosis (and Prouho does not note the appearance of any juvenile structures), his final observations were of a larva cultured for three months. In his study of *C. cidaris*, Prouho (1887) does not include the temperature at which cultures were reared, but he does note (somewhat surprisingly) that the urchins spawned without chemical stimulation in the month of February at the Arago Laboratory, which is situated on the Southeastern coast of France in the Mediterranean Sea, and where surface water temperatures in February have been recently recorded as 11.5 °C (Charles et al., 2005). Based on this evidence, the congeners *C. cidaris* and *C. blakei* have a similar developmental timetable at similar temperatures. We cautiously suggest that prolonged development at relatively cool temperatures is characteristic of this genus, assuming adequate nutrition and culture conditions.

Table 2. Comparison of known cidaroid larvae with respect to egg size, developmental time and morphology.

	Depth Collected (m)	Egg diameter (μm)	Egg volume (nl)	Apical tuft	Time to metamorphosis (days)	Temperature (°C)	Juvenile test diameter (μm)	Source
Cidaris cidaris		170	2.57	P	(reared for 3 months)	11*		Prouho (1887)
Cidaris blakei	450-685	157	2.03	P	120	11	471	This study
Eucidaris metularia		90	0.38	A		23**		Mortensen (1937)
Eucidaris thouarsi	2-3	90	0.38	A	30	28	510	Emlet (1988)
Eucidaris tribuloides	3-5	95	0.45	A		26		Schroeder (1981)
Prionocidaris baculosa	73	150	1.77	A	30	23**	375	Mortensen (1938)

Labels: P=present; A=absent; in instances where no data are available, the cell is left blank.

* Prouho did not note the temperature at which larvae of *C. cidaris* were reared. This value is the temperature of surface water at the location and season where the study was completed

** Mortensen did not regulate the exact temperatures at which he reared *E. metularia* and *P. bacculosa*. These values are a rough estimate of culture temperature based on notes in the text.

In order to examine whether the extended larval duration of *C. blakei* is a functional adaptation to life in the deep-sea or if it is a result of phylogenetic relationships, we must first try to normalize for the wide variation in temperatures at which cidaroid larvae have been raised. Previous studies have used Q_{10}, the factor by which rate of a process changes with a 10 °C increase in temperature, to compensate for differences in temperature when comparing larvae (Emlet, 1995). Previous studies on echinoid Q_{10} values have shown a range of 3.0-3.6 for the tropical urchins *Lytechinus variegata* and *Echinometra lucunter*, and for the sand dollar *Dendraster excentricus* (Cameron, 1985; McEdward, 1985). If we apply this range of Q_{10} values to *E. thouarsi* and *C. blakei*, and normalize the temperature to 20°C, there is still a wide difference in developmental times, but in this case *C. blakei* would reach metamorphosis twice as fast as *E. thouarsi* (Table 3). This supports previously demonstrated evidence that smaller eggs have a longer pre-metamorphic stage. Other factors, such as depth of distribution and resource availability, have also been shown to affect developmental duration. Low food concentrations and increased depth can increase developmental times (Strathmann, 1987; Emlet, 1995), and we might expect to see even longer developmental times of *C. blakei in situ*.

Table 3. Days to metamorphosis of *C. blakei* and *E. thouarsi* when temperature is adjusted to 20 °C using the equation for Q_{10}.

Q_{10} values	Days to metamorphosis: *C. blakei*	Days to metamorphosis: *E. thouarsi*
3.0	45	72
3.2	42	76
3.4	40	80
3.6	38	84

Extended developmental periods are not unknown for deep-sea larvae. The bathyal echinoid *Aspidodiadema jacobyi* was reared in culture for five months (Young and George, 2000). The cold- seep gastropod *Bathynerita naticoidea* may spend up to one year in the water column before settlement (Van Gaest, 2006) and the deep-sea mussel "*Bathymodiolus*" *childressi* has been estimated to spend between 9 and 13 months in the plankton. This extended larval period may result in long-range dispersal and a wider geographic range (Arellano and Young, 2009). *Cidaris blakei* has a relatively small geographic range, and has been found in the North Atlantic only between the Bahamas and Barbados (Mortensen, 1928; Young, 1994). In an analysis of 215 species of regular echinoids, including 24 species of cidaroids, larval duration was not related to geographic range (Emlet, 1995).

The bathyal congeners *Cidaris cidaris* and *C. blakei* were reared at similar temperatures and had similar developmental timetables. A notable difference, however,

is in the completion of the gut, which occurs in *C. cidaris* at eight days (it may have opened before this, but Prouho (1887) first notes an open mouth in a figure of an eight day-old gastrula), but not until 14 days in *C. blakei* (this study). It is difficult to discuss this difference in the context of habitat, because Prouho does not note the depth at which he collected his specimens. *Cidaris cidaris* occurs at a broad range of depths, extending from 50-1000 m (Mortensen, 1928). There is, however, an example of a deep-sea euechinoid with a prolonged non-feeding stage. The deep-sea diadematid *Aspidodiadema jacobyi*, which has eggs only 98 μm in diameter, was shown to have an extended lecithotrophic stage prior to development of a complete gut. The mouth of *A. jacobyi* did not open until late in the two-arm pluteus stage, 21 days after fertilization. Young et al. (1989) hypothesized that the extended pre-feeding period of *A. jacobyi* could be an adaptation to deal with patchy food resources in the deep-sea. Alternatively, the female might provide the larva with sufficient yolk reserves to undertake vertical migration to the euphotic zone (Young *et al.*, 1996). These two hypotheses may apply to *C. blakei* as well. Vertical migration, however, is unlikely in this species (see section on thermal tolerance below). We must therefore concur with Young *et al.* (1989) that delayed mouth formation may allow deep-sea larvae to conserve energy in a food patchy environment, but in the case of *C. blakei* the delay probably does not facilitate migration to productive upper strata of the water column.

Ectodermal pits have been described previously only on the direct developing, lecithotrophic echinoids *Asthenosoma ijimai* (an echinothurioid) and *Phyllacanthus parvispinus* (a cidaroid) (Amemiya and Emlet, 1992; Parks *et al.* 1989). Amemiya and

Emlet (1992) postulated that the ectodermal pits could be associated with the deep wrinkles in lecithotrophic blastulae, but Parks *et al.* (1989) noted that position and number of pits did not coincide with location of the wrinkles of *P. parvispinus*. *Cidaris blakei* did form a wrinkled blastula, likely attributed to the widely spaced blastomeres of the early embryo. We do not know the function or derivation of these invaginations in *C. blakei*, but their presence on three different species of echinoids warrants further investigation.

When we compare the descriptions of the six planktotrophic cidaroids for which a portion of development has been described, two distinct morphological forms emerge. The three species of *Eucidaris* have eggs that are 90-95 μm in diameter (Table 2). The prism form of the *Eucidaris* congeners is triangular, transparent and has numerous pigment spots. There is no apical tuft present at any stage. Eggs of the two species of *Cidaris* are 150 μm in diameter or larger. Larvae have an apical tuft, and the prism is elongate and very opaque. Drawings by Mortensen (1938) of the early stages of *P. baculosa* show that it closely resembles the two species of *Cidaris*, although it does not have an apical tuft. Additionally, Mortensen (1938) notes that the post-oral rods of *P. bacculosa* are fenestrated from base to tip, which is the same as the post-oral rods of *C. blakei*, but differs from *E. metularia*, in which the fenestration begins farther from the base. In phylogenies constructed using adult morphological characteristics, *C. blakei* is in the subtribe Cidarina, but *Prionocidaris*, *Eucidaris* and *Phyllacanthus* are all in the sister subtribe Phyllacanthina (Smith and Wright, 1989). Within the Phyllacanthina, Prionocidaris is a sister taxa of the clade containing both Eudicaris and Phyllacanthus If

this interpretation is correct, then among the planktotrophic species, large egg size, apical tuft and opaque, elongate gastrula may represent the more primitive condition.

Juveniles

The juveniles of *Cidaris blakei* differ from other cidaroid juveniles in the shape of their juvenile and adult spines. The 471-μm test diameter of *C. blakei* juveniles falls within the expected range of echinoids (Emlet et al., 1987) and between the reported 350 μm test of newly settled *Prionocidaris baculosa* and the 510 μm tests of *Eucidaris thouarsi* (Mortensen, 1938; Emlet, 1988). The morphology of the juvenile spines of *C. blakei* is unique among cidaroids. All other juvenile cidaroids have spines with a tri-radiate tip (Fig. 7) (Mortensen, 1938; Emlet, 1988; Parks *et al.*, 1989).

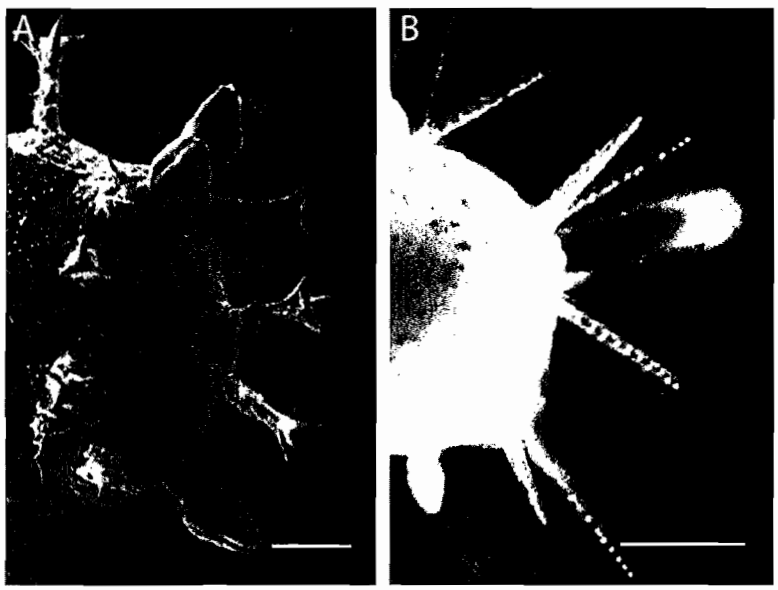

Figure 7. Two micrographs showing the difference between the juvenile spine shape of *Eucidaris thouarsi* and *Cidaris blakei*. All scale bars=100 μm. (A) Scanning electron micrograph of a juvenile *Eucidaris thouarsi* (image courtesy of R. Emlet). (B) Light micrograph of a juvenile *Cidaris blakei*, taken one day after metamorphosis.

Thermal tolerance and Ontogenetic Vertical Migration

Larvae in the deep-sea may migrate to the euphotic zone. Direct evidence for ontogenetic vertical migration of deep-sea organisms comes from capture of larvae in surface water plankton tows (reviewed by Bouchet and Warèn, 1994; Van Gaest, 2006; Arellano, 2008). In the laboratory, potential for vertical migration can be explored indirectly by testing the physiological tolerances of embryos and larvae (Van Gaest *et al.*, 2007). If larvae migrate up through the water column, then they must be able to withstand a wide range of temperatures and salinities. Water temperatures for the northern Bahamas during the spawning season of *C. blakei* range from 10 °C at the deepest location that *C. blakei* occur to 25 °C at the water's surface (Young, 1994). Our preliminary results suggest that larvae of *C. blakei* are unable to withstand temperatures above 15 °C (Fig. 6), and therefore are unlikely to successfully migrate into the photic zone.

Cidaris blakei is the first deep-sea echinoid reared through metamorphosis. Its development differs in several ways from shallow-water cidaroids. Characteristics such as embryonic and larval morphology, ectodermal invaginations at gastrula stage, presence of an apical tuft and juvenile spine morphology may represent the ancestral form of more derived echinoids, including cidaroids and euechinoids. Other characteristics, such as an extended larval pre-feeding stage, may be adaptations to the deep-sea habitat.

This chapter provided a description of the embryology, larva and metamorphosis of *Cidaris blakei*. The next chapter will use monoclonal antibody staining techniques and

confocal microscopy to look more closely at a single embryonic structure: the hyaline layer.

CHAPTER III

THE CIDAROID HYALINE LAYER DIFFERS IN FORM AND FUNCTION FROM THE EUECHINOID HYALINE LAYER

Introduction

The sea urchin embryo is considered a model developmental system, and the events directly following fertilization of sea urchin eggs have been extensively studied (reviewed by Giudice, 1986; Eisen *et al.*, 1984; Foltz and Lennarz, 1993). Immediately after fertilization the sea urchin egg undergoes a number of structural changes. When the fertilizing sperm first enters the egg, secretory vesicles called cortical granules undergo exocytosis and release the protein components of the fertilization envelope, which rises from the egg and prevents further insemination. The cortical granules then release a large glycoprotein, hyalin, into the perivitelline space. This glycoprotein is the main component of the hyaline layer, an extracellular matrix that forms around the sea urchin embryo in the perivitelline space (Stephens and Kane, 1970; Citkowitz, 1971; Gray *et al.*, 1986). Early studies show that when the hyaline layer is removed from an embryo the blastomeres dissociate, suggesting that hyaline layers play a role in cell adhesion in early embryos (Kane, 1970). More recently, the hyaline layer has been shown to be essential

to normal morphogenesis and archenteron elongation of gastrulating embryos (McClay and Fink, 1982; Razinia *et al.*, 2007; Contreras *et al.*, 2008). Although the hyaline layer is well-described in echinoids, and its form and function have been the subject of many studies, the hyaline layer appears reduced or absent in the Order Cidaroidea.

Extant echinoids are divided into two major sister clades, the Euechinoida and the Cidaroida. Since they first appeared in the late lower Permian (lower Zechstein, ~255 mya), cidaroids have undergone few morphological changes (Kier, 1977; Smith, 1984; Smith and Hollingworth, 1990; Smith *et al.* 2006). Thus, cidaroids are considered "living fossils," examples of the modern form of all other living echinoids. Studies have shown some notable differences between the early developmental patterns of the basal cidaroids and the more derived euechinoids (Mortensen, 1937; Schroeder, 1981; Emlet, 1988). Chief among these differences is the apparent lack of a coherent hyaline layer. It has been noted for a number of species of cidaroids (Mortensen, 1938; Schroeder, 1981; this study) that the blastomeres of early embryos are widely spaced. Mortensen (1938) described the two-cell stage of *Prionocidaris bacculosa* in which blastomeres were so segregated from one another that twin embryos formed inside a single fertilization envelope. When Schroeder (1981) removed the fertilization envelope of *Eucidaris tribuloides*, the blastomeres dissociated, suggesting that the envelope was the only extracellular component holding the blastomeres together. Scanning electron micrographs of *E. tribuloides* revealed microvilli and a "virtual absence of a cohesive hyaline layer," (p. 145). Widely spaced blastomeres and subsequent irregularly shaped blastulae were also observed in the deep-sea cidaroid *Cidaris blakei* (this study).

The goal of this study is to determine whether a hyaline layer is present during the development of two cidaroid species, *Cidaris blakei* and *Eucidaris tribuloides*. To test this, we removed the fertilization envelopes of fertilized eggs and early embryonic stages and observed specimens using both light and confocal microscopy. Additionally, this study uses the monoclonal antibody (McA Tg-HYL) specific for sea urchin hyalin (Adelson and Humphreys, 1988) for immunofluorescent staining. McA Tg-HYL has been shown to recognize hyalin in three different families of sea urchins, and has been used to stain the hyaline layer of embryos and also for immunoblot staining of the hyaline protein band (Adelson and Humphreys, 1988). This study also uses McA Tg-HYL on the fertilized eggs and early embryos of a clypeasteroid, *Dendraster excentricus*, which is more closely related to cidaroids than the other urchins it has been previously used on. The monoclonal antibody was also reacted with the hyaline layer of *S. purpuratus*, on which it has previously been shown to work (Adelson and Humphrey, 1988) as a positive control for technique.

Materials and Methods

Collection, spawning and removal of the fertilization envelope

The purple urchin *Strongylocentrotus purpuratus* was collected from Middle and South Cove, Cape Arago, Oregon, between the months of May and August 2009. The sand dollar *Dendraster excentricus* was collected from the North Spit, Coos Bay, Oregon in July 2009. The cidaroid urchin *Eucidaris tribuloides* was purchased from Gulf Specimen Marine Lab (Panacea, FL, USA) in July 2009. Adult specimens of the deep-

water cidaroid urchin *Cidaris blakei* were collected in the northern Bahamas using the manned submersible Johnson Sea-Link II (Harbor Branch Oceanographic Institute at FAU, Fort Pierce, FL, U.S.A.) between May 14, 2008, and May 22, 2008. Animals were induced to spawn using an intracoelomic injection of 0.5-1 mL of 0.1 M acetylcholine in filtered seawater (FSW). Gametes of *S. purpuratus* and *E. tribuloides* were washed several times in 0.45 millipore FSW then fertilized in a solution of the peroxidase inhibitor, 3-ATA (3-amino-1,2,3-triazole, Sigma Co.), which prevents the fertilization envelope from hardening (G. von Dassow, pers. comm.). Gametes of *D. excentricus* were also washed several times in 0.45 μm FSW, but did not require 3-ATA to prevent hardening of the fertilization envelope, and were fertilized in FSW. To remove their fertilization envelopes about 30 minutes after fertilization, eggs of all species except *C. blakei* were forced through a nylon mesh sieve slightly smaller than the fertilized egg (70 μm, 80 μm and 130 μm mesh for *S. purpuratus*, *E. tribuloides* and *D. excentricus*, respectively) (Strathmann, 1987). At this point some embryos were fixed, and others continued to develop in FSW at 11 °C for *S. purpuratus* and *D. excentricus*, and 20 C for *E. tribuloides*. Fertilization envelopes of *C. blakei* gametes were removed post-fixation, by rupturing the vitelline with sharpened forceps and then gently passing the embryo through a pipette with a diameter roughly the size of the embryo, about 150 μm.

Fixation

Embryos of *S. purpuratus*, *E. tribuloides* and *D. excentricus* were fixed in 4% paraformaldehyde in freshwater for one hour then washed several times with 1X

Phosphate Buffered Saline (PBS). After fixation, embryos stored in PBS were kept at 4 °C for no more than two weeks before staining. *Cidaris blakei* embryos were fixed in 10% buffered formalin and stored in PBS.

Hyaline layer removal

To show specificity of the McA Tg-HYL antibody for the hyaline layer I removed the hyaline layer from *S. purpuratus* embryos by rinsing them several times in Calcium and Magnesium-free seawater (Strathmann, 1987) prior to fixation. Embryos were then stained following the same methods as for all other samples (described below).

Antibody Stain

All samples were washed with 1X PBS with 0.1% Triton X-100 (PBT) and allowed to settle. To block non-specific binding, the PBT was then replaced with 5% normal goat serum in PBT for 30 minutes to 1 hour at room temperature. Embryos were then rinsed with PBT and allowed to settle. Embryos were placed in a solution of the primary antibody, diluted 1:10 in PBT for at least two hours at room temperature, washed three times in PBT and then left for two hours in a solution of the secondary antibody, Goat Anti-Mouse with the fluorescent conjugate Alexafluor 488 (Molecular Probes, Inc.) diluted 1:500 in PBT. At this stage Phalloidin was also added at a dilution of 1:1000 to stain the cell boundaries. Samples were then washed three times in PBT and immediately mounted in Vectashield (Vector Labs, Inc.) on poly-L-lysine coated slides for confocal fluorescence microscopy. Confocal stacks were acquired with an Olympus 1X81

microscope and Fluoview 1000 confocal system. Image stacks were analyzed using Image-J.

Results

A hyaline layer analogous to the hyaline layer of euechinoids was not visible at any developmental stage of the cidaroid embryos. At 16-cell stage and in later stages, however, a cohesive extracellular layer could be seen surrounding the blastomeres of *C. blakei* and *E. tribuloides* after the fertilization envelope had been removed (Fig. 1A, B). Except for its location in the perivitelline space, this layer did not resemble the euechinoid hyaline layer.

The primary antibody McA Tg-HYL reacted with hyalin on embryos of *S. purpuratus*. In *S. purpuratus* embryos that were fixed within 10 minutes of fertilization the hyalin was evident as a coating of small spots on the exterior of the fertilized egg (Fig. 2A). This spotted appearance is consistent with the exocytosis of hyalin from cortical granules soon after fertilization. In *S. purpuratus* embryos fixed more than 10 minutes after fertilization a thick, cohesive layer had formed around the surface of the fertilized egg (Fig. 2B). The antibody McA Tg-HYL did not react with *Eucidaris tribuloides* embryos (Fig. 3A, B). There was no visible layer surrounding the fertilized egg. A ring of brighter fluorescence in the Phalloidin channel around the periphery of the fertilized egg is evident in cross-section (Fig. 3B), but might be due to entrainment of the Phalloidin stain in the microvilli that cover the fertilized egg's surface rather than an extracellular matrix.

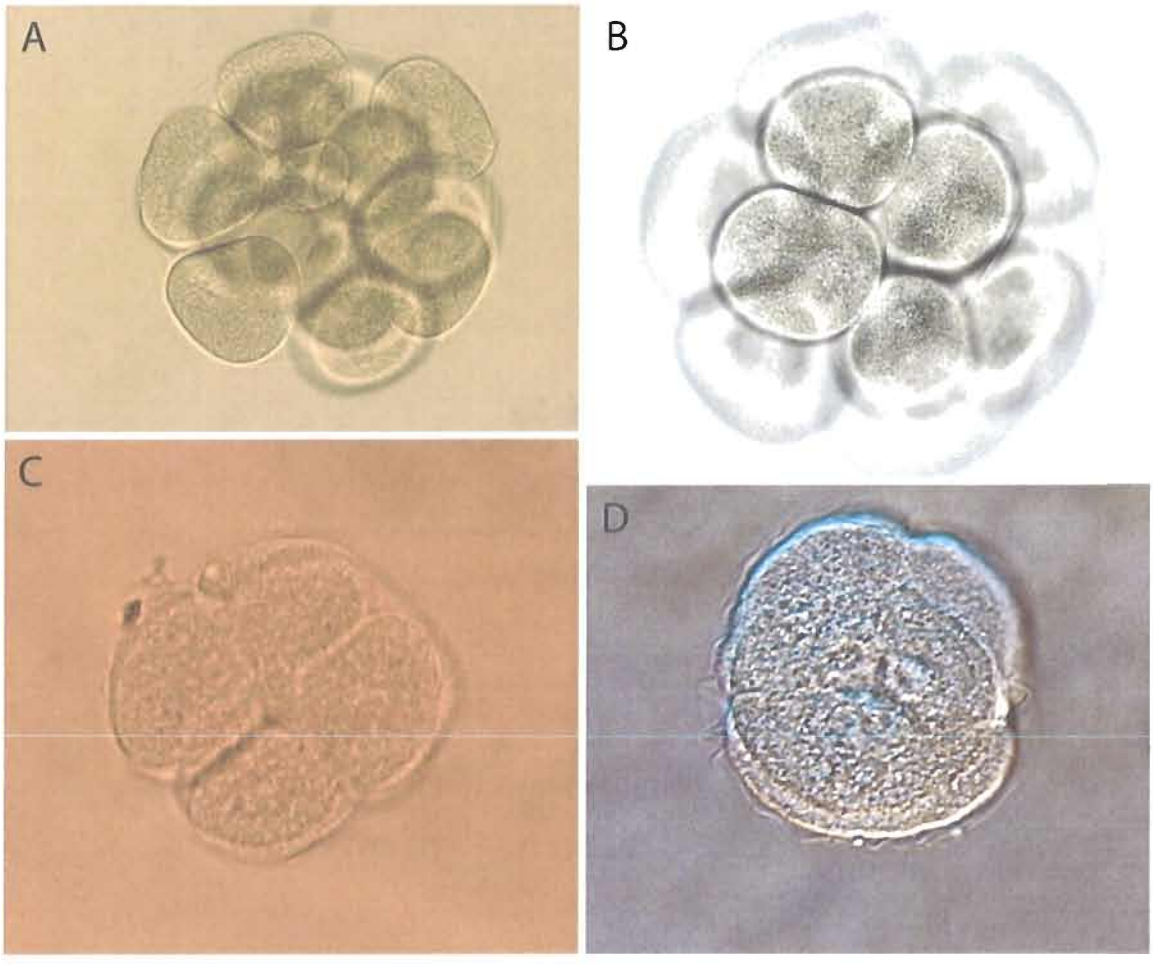

Figure 1. Light micrographs of multi-cell stages of cidaroid sea urchins *Cidaris blakei* and *Eucidaris tribuloides* showing a thin extracellular layer surrounding the blastomeres. (A, B) Embryo of *C. blakei*. (C, D) Embryo of *E. tribuloides*.

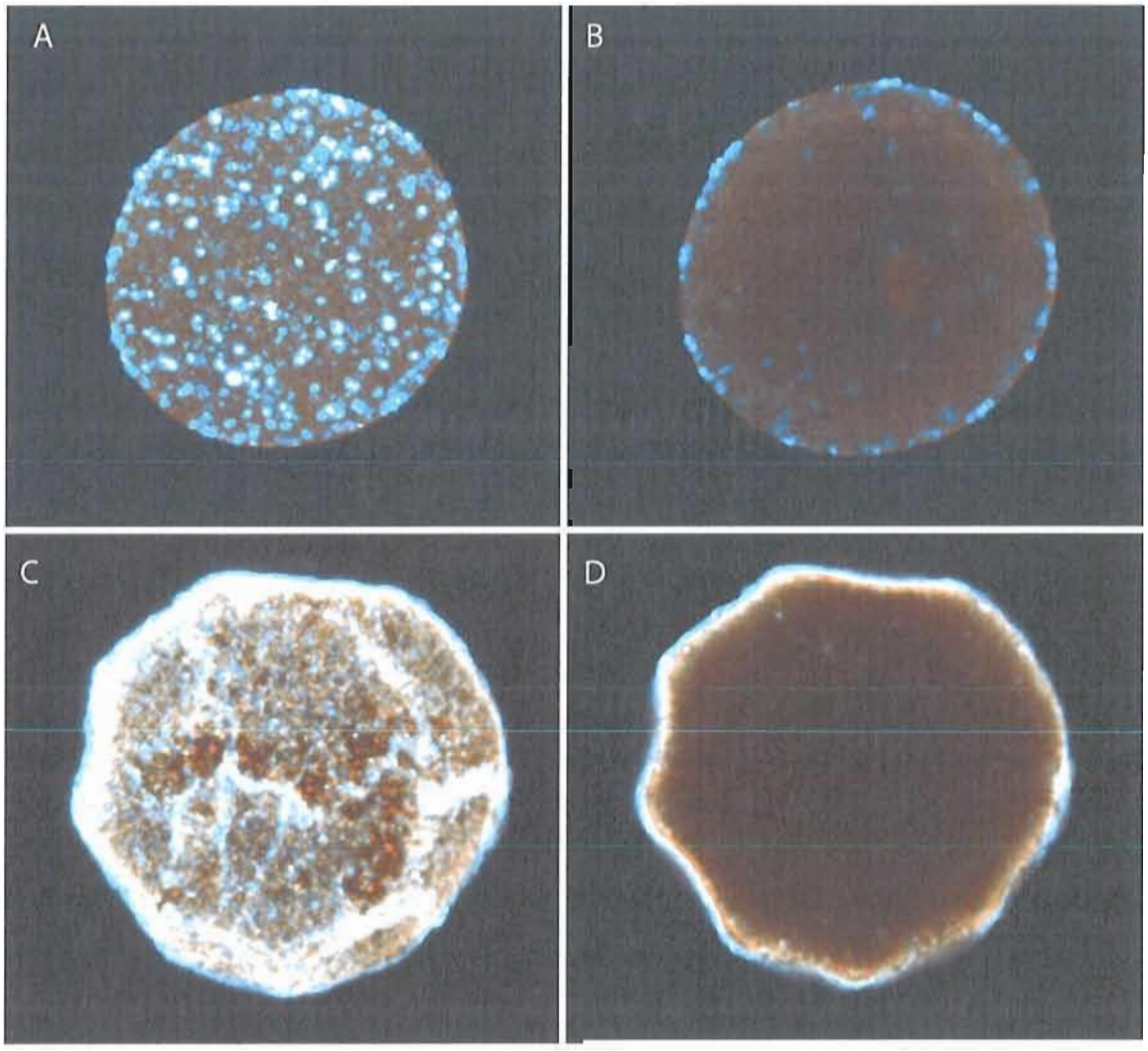

Figure 2. Confocal projections of fertilized eggs of *Strongylocentrotus purpuratus* labeled with phalloidin (orange) and the hyalin-specific monoclonal antibody McA Tg-HYL (blue). (A) Confocal z-projection of an egg less than 10 minutes after fertilization. McA Tg-HYL is reacting with hyalin as it releases from the cortical granules. (B) A cross-section of the same egg showing localization of hyalin on the exterior of the fertilized egg. (C) Confocal z-projection of an egg more than 10 minutes after fertilization showing a cohesive hyaline layer. (D) A cross-section of the same egg as C showing a uniform coat of hyalin around the fertilized egg.

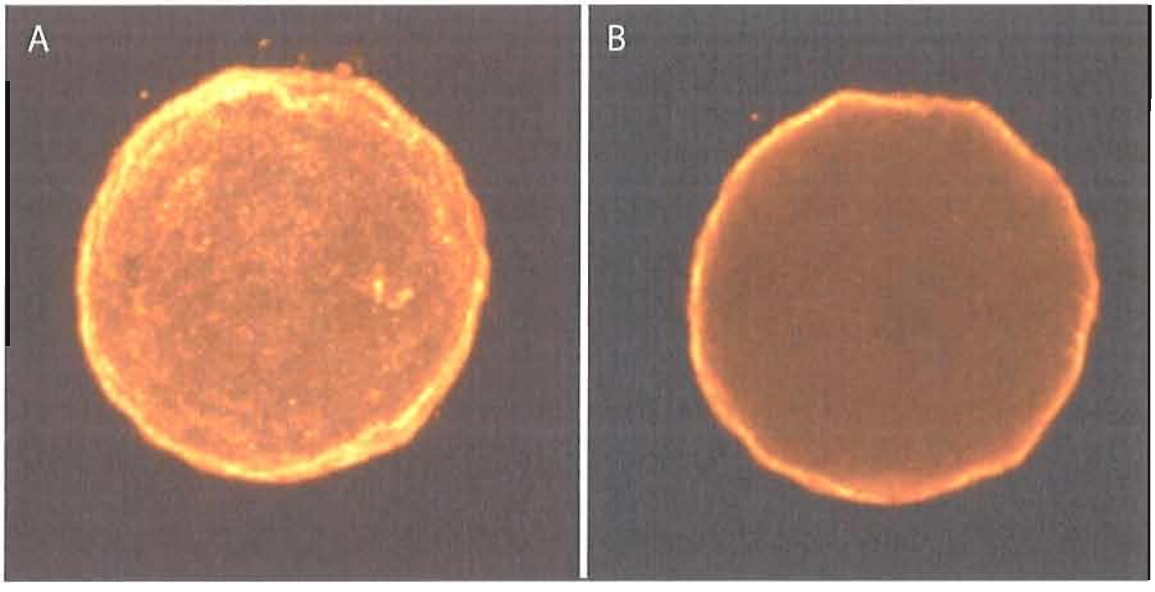

Figure 3. Confocal projections of a fertilized egg of *Eucidaris tribuloides* labeled with phalloidin (orange). This fertilized egg was also treated with the monoclonal antibody McA Tg-HYL, but it did not react. (A) Confocal z-projection of an egg more than 10 minutes after fertilization showing no labeling by McA Tg-HYL. (B) A cross-section of the same egg.

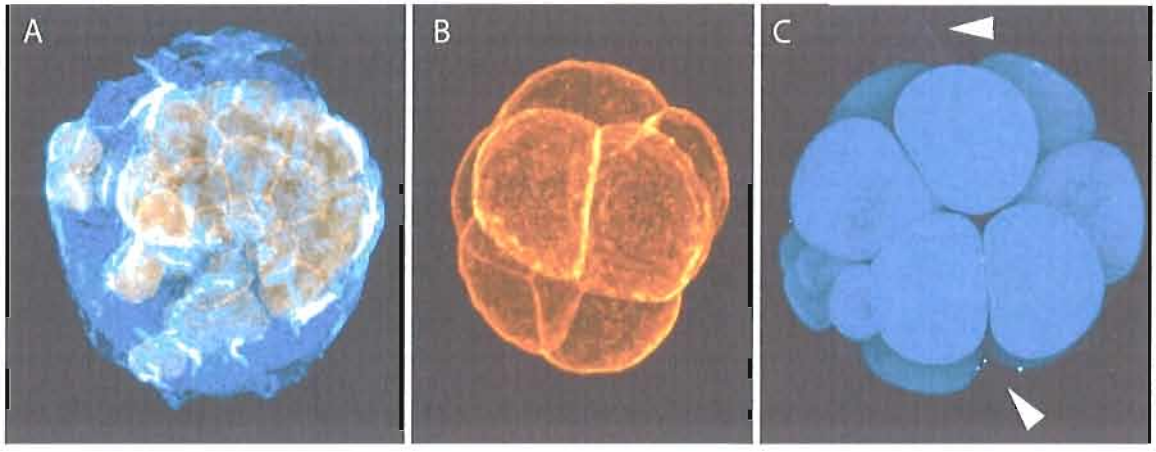

Figure 4. Confocal projections of multi-cell stages of three species of sea urchin labeled with phalloidin (orange) and the hyalin-specific monoclonal antibody McA Tg-HYL (blue). (A) Confocal z-projection of *Strongylocentrotus purpuratus* showing a stiff hyaline layer surrounding phalloidin-labeled blastomeres. (B) Confocal z-projection of *Eucidaris tribuloides* showing no reaction of McA Tg-HYL. (C) Confocal z-projection of *Cidaris blakei* showing non-specific labeling of the entire embryo with McA Tg-HYL. Phalloidin did not stain the *C. blakei* embryo because it was fixed in a solution containing methanol. A cohesive extracellular layer is visible around the entire embryo (arrowheads).

In the multi-cell stages of *S. purpuratus*, *E. tribuloides* and *C. blakei*, McA Tg-HYL reacted only with the hyaline layer of *S. purpuratus* (Fig. 4A, B, C). On the *S. purpuratus* embryo, a thick coat of hyaline surrounds the blastomeres, and seems stiff in structure, maintaining its shape even though the blastomeres are falling apart. There was no evidence of anti-hyaline immunofluorescence in *E. tribuloides* (Fig. 4B), but the primary antibody reacted with the entirety of the *C. blakei* embryo showing no specificity (Fig. 4C). The stain also illuminated a thin and ruptured layer around this stage of *C. blakei* (Fig. 4C). The lack of stain specificity may be due to the buffered formalin fixative used on *C. blakei*, which contains methanol, and which was almost certainly why

Phalloidin didn't work (G. von Dassow, pers. comm.). However, McA Tg-HYL stained only the hyaline layer on an *S. purpuratus* embryo fixed in the same buffered formalin solution (Fig. 5).

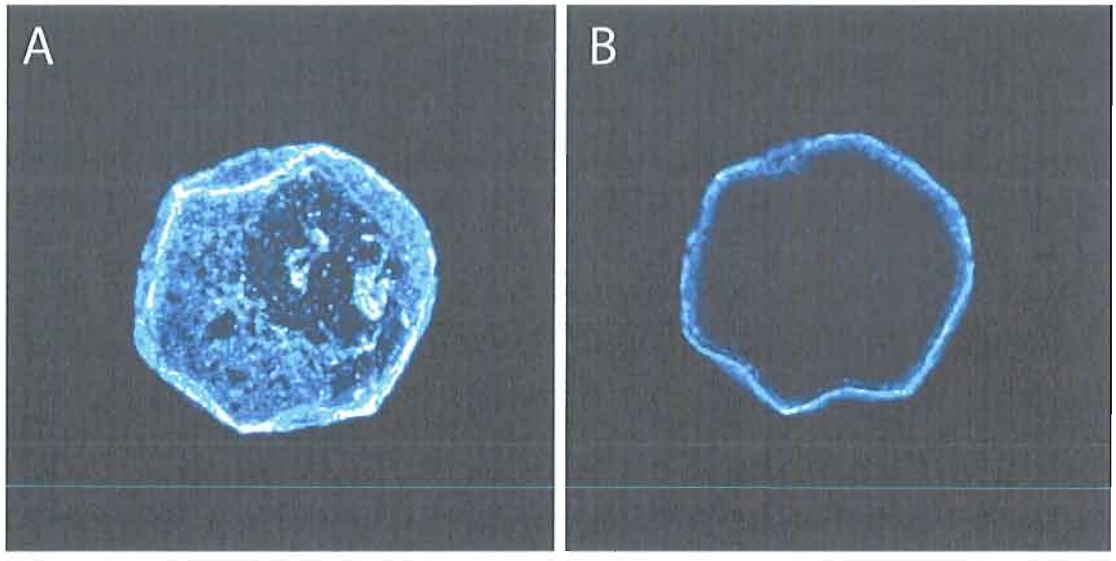

Figure 5. Confocal projection of a fertilized egg of *S. purpuratus* that has been fixed in the same 10% buffered formalin solution as *C. blakei* showing a hyaline layer clearly labeled with McA Tg-HYL. (A) Z-projection and (B) cross-section of the same fertilized egg.

McA Tg-HYL reacted with the hyaline layer of *S. purpuratus* blastulae (Fig. 6A, B), and when blastulae were washed in Mg/Ca-free seawater prior to fixation, the hyaline layer was almost entirely removed (Fig. 6C, D). Again, *C. blakei* shows no specific hyaline layer stain, but is stained in its entirety (Fig. 6E, F). McA Tg-HYL reacted with the surface of *D. excentricus* embryos (Fig. 7), but the layer was not cohesive and thick, as it appears around the embryos when examined with light microscopy.

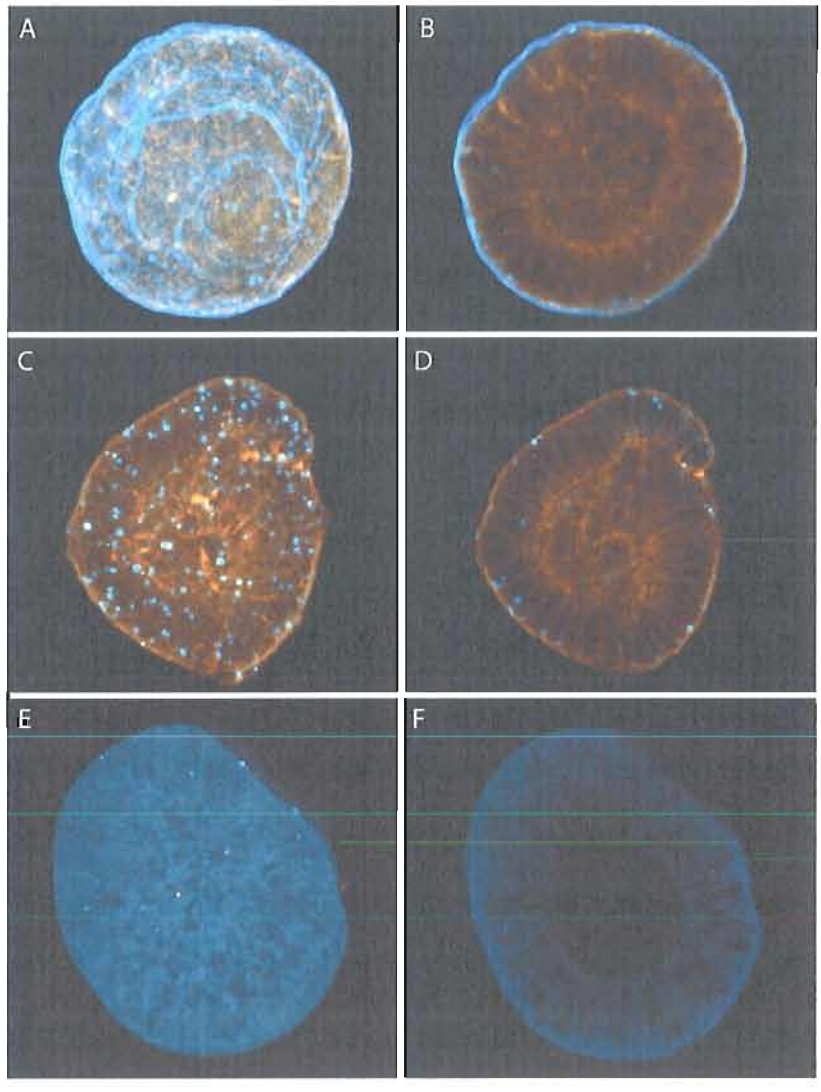

Figure 6. Confocal projections of blastula-stage embryos labeled with phalloidin (orange) and the hyalin-specific monoclonal antibody McA Tg-HYL (blue). (A) Confocal z-projection of a *Strongylocentrotus purpuratus* blastula showing a cohesive hyaline layer around the embryo. (B) The same blastula as A in cross-section. (C) A blastula of *S. purpuratus* that has been treated with MgCa-free seawater, which removes the hyaline layer. It's possible that the spots of hyalin are from the second wave of hyalin release that occurs in the blastula stage. The blue spots visible in the cross-section (D) of the same blastula may be cortical granules that have migrated to the surface where they will release the hyalin. (E) Confocal z-projection and cross-section (F) of a *C. blakei* blastula showing non-specific staining with McA Tg-HYL.

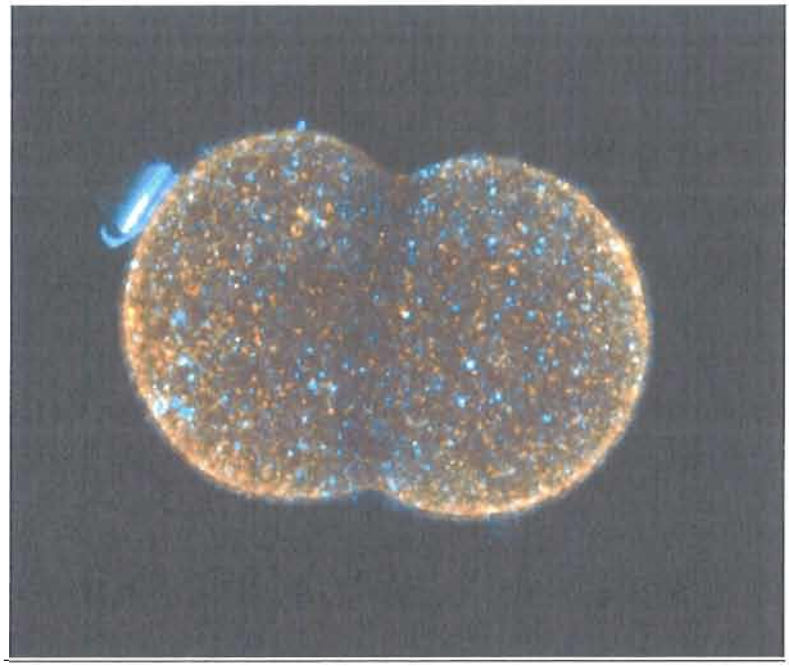

Figure 7. Confocal z-projection of a two-cell embryo of a clypeasteroid, *Dendraster excentricus*, labeled with phalloidin (orange) and the hyalin-specific monoclonal antibody McA Tg-HYL (blue) showing some reaction of McA Tg-HYL, but not a cohesive hyaline layer.

Discussion

Hyaline layer in euechinoids

The hyaline layer of euechinoids has been well described (e.g. Kane, 1991; Citkowitz, 1971, 1972). The main protein component of the hyaline layer, generically called hyalin (Gray *et al.*, 1986), has been characterized as a large glycoprotein secreted from the cortical granules soon after fertilization (Stephens and Kane, 1970), and again later in development at the mesenchyme blastula stage (McClay and Fink, 1982). The

hyaline layer becomes less important for cellular integrity of the embryo as development proceeds, and cellular affinity for hyalin is germ layer-specific (McClay and Fink, 1982). The function of the hyaline layer is not completely understood, but has been considered, "an absolute requirement for the adhesion of the blastomeres in early cleavage stages," (Citkowitz, 1971, p. 357) and "required for sea urchin morphogenesis," (Adelson *et al.*, 1992, p. 1288). A series of recent papers found that exogenous hyalin inhibits archenteron elongation and attachment to the blastocoel roof in sea urchin embryos, and suggested that this is direct evidence for a specific adhesive function of hyalin (Razinia *et al.*, 2007; Alvarez *et al.*, 2007; Contreras *et al.*, 2008).

Hyaline layer in cidaroids

All of the studies on the sea urchin hyaline layer have been on euechinoids. Although very difficult to see, there is a layer present in at the 16-cell and later stages of the cidaroid urchins *Cidaris blakei* and *Eucidaris tribuloides*. Although this layer is in same location as the euechinoid hyaline layer, it appears to be different in both form and function. The layer surrounding cidaroid embryos is very thin, and does not follow the contours of the blastomeres, as the hyaline layer does in euechinoids. It is virtually impossible to see without first removing the fertilization envelope. This difficulty in discerning this extracellular layer of cidaroids is due in large part to the minute perivitelline space of the cleaving embryos. Cells of early cidaroid embryos appear to be in direct contact with the fertilization envelope, or vitelline, in contrast with the large perivitelline space in *S. purpuratus* embryos. The extracellular layer surrounding *C.*

blakei and *E. tribuloides* embryos does not appear to contribute to cell adhesion prior to the formation of cell junctions, as it does in euechinoids (reviewed by Wray, 1997). The integrity of early cidaroid embryos instead seems to be maintained by the fertilization envelope, as evidenced by the dissociation of *E. tribuloides* blastomeres when the envelope was removed (Schroeder, 1981).

Monoclonal antibody

The monoclonal antibody McA Tg-HYL was generated against the toxopneustid echinoid *Tripneustes gratilla*, and has been shown to react to hyaline on three distantly related families of sea urchins (Adelson and Humphreys, 1988). Most notably, McA-Tg-HYL reacted with the hyaline layer of *Arbacia punctulata*, a member of the family Arbaciidae, which is thought to have diverged from other euechinoids more than 200 million years ago (Smith *et al.*, 2006). Adelson and Humphreys (1988) considered the immunological cross-reactivity of McA Tg-HYL to embryos from divergent groups of sea urchins as an indication that, "the recognized epitope is strongly conserved" (p. 400). McA Tg-HYL did not react with either *C. blakei* or *E. tribuloides*, suggesting that this protein is either absent or significantly different in cidaroids than in euechinoids. The entirety of the *C. blakei* embryos reacted to McA Tg-HYL, but this non-specific staining may be an artifact of prolonged storage after fixation.

The non-reactivity of euechinoid-generated antibodies with cidaroid cells is not always the case. Four of five monoclonal antibodies that recognize mesenchyme cells in euechinoids reacted with mesenchyme cells in *E. tribuloides*, and the fifth monoclonal

antibody reacted with pigment cells, a subset of mesenchyme cells (Wray and McClay, 1988). If McA Tg-HYL recognizes an evolutionarily conserved epitope, as Adelson and Humphrey (1988) assert, but does not recognize that epitope in either *C. blakei* or *E. tribuloides*, then we might conclude that the epitope is significantly different in basal echinoids. With the aid of light microscopy, the hyaline layers of *D. excentricus* and *S. purpuratus* look much the same. In this study, McA Tg-HYL reacted only partially with the surface of *D. excentricus* embryos, but did not react with the entirety of the hyaline layer, as it did with *S. purpuratus*. Further investigation is warranted to determine whether *D. excentricus* might have an intermediate form of the hyalin epitope.

Conclusion

Cidaroids are basal to all living urchins. They are thought to have diverged from euechinoids more than 255 million years ago (Smith *et al.*, 2006). Living cidaroids closely resemble the cidaroid ancestor of modern echinoids, and represent the primitive, or ancestral form of euechinoids (Schroeder, 1981; Smith, 1984). Previous studies on the development of cidaroids have described differences in development between the basal cidaroids and the more highly derived euechinoids. An irregular number of micromeres, a lack of early mesenchyme ingression (Schroeder, 1981) and the development of juvenile structures without a vestibule are all characteristics of Order Cidaroidea that might be considered primitive compared to the more highly derived euechinoids (Emlet, 1988; Parks *et al.*, 1989; Olson *et al.*, 1993).

Recent studies of the hyaline layer in sea urchins cite the importance of sea urchins as a model system, and the importance of their position within the deuterostome clade (Razinia *et al.*, 2007; Alvarez *et al.*, 2007; Contreras *et al.*, 2008). A hyaline layer analogous in structure to the hyaline layer in euechinoids is present in the lancelet *Branchiostoma floridae* (Phylum Chordata) (Holland and Holland, 1989) and the hyalin repeat domain has been identified in proteins from organisms as diverse as bacteria and humans (Contreras *et al.* 2008). Additional insight into the evolution of the hyalin protein and the hyaline layer might be gained by studying the differences in its form and function within Class Echinoidea, in particular between the basal cidaroids and the more derived euechinoids.

CHAPTER IV

CONCLUDING SUMMARY

This thesis provides a description of the embryology, larval development and metamorphosis of the deep-sea cidaroid urchin *Cidaris blakei*. Although there is inherent interest in this description on its own, its greatest value comes from placing it in the broader contexts of phylogenetic relationships and deep-sea biology.

Many echinoid phylogenies are based upon adult morphological characteristics and use a combination of fossil and neontologic evidence in their construction (Smith and Wright, 1989). Because of the disparate habitats of echinoid adults and larvae and the subsequently different selective pressures, it has been argued that larval forms may be of little value for understanding phylogenetic relationships (Smith, 1997). Larval and adult forms of echinoids have been shown to evolve independently, with a disproportionate number of changes in adult morphology relative to larval morphology (Smith *et al.*, 1996). Smith (1997) noted that life history strategy is a better predictor of larval morphology than either adult morphology or phylogenetic history. He refers to the congeneric echinoids *Heliocidaris erythrogramma* and *H. tuberculata* as an example of two urchins with very similar adult morphologies, but different larval forms (*H. erythrogramma* has a direct-developing larva and *H. tuberculata* has a planktotrophic

larva). It must be noted, however, that in non-feeding larvae that still have vestigial pluteal structures, there is often evidence of phylogenetic relationships. For instance, the direct-developing cidaroid, *Phyllacanthus parvispinus*, develops juvenile structures without an amniotic invagination (Parks *et al.*, 1989), as has been described only for planktotrophic larvae within the cidaroid clade (Emlet, 1988; this study). This avestibular development is characteristic of the order Cidaroidea, and is considered representative of the ancestral condition of all other extant urchins (Emlet, 1988; Parks *et al.*, 1989).

The embryos, larvae and juveniles of the deep-sea cidaroid urchin *Cidaris blakei* differ from other cidaroids in a number of ways. The egg size of *C. blakei* is larger than most other planktotrophic cidaroids. An apical tuft is present on the blastula stage of *C. blakei* and was also described in the congeneric *Cidaris cidaris* (Prouho, 1878). An apical tuft has not been described in other cidaroids and it is likely that this trait was lost among later lineages (Fig. 1). The ectodermal pits on *C. blakei* gastrulae have only been described in one other cidaroid species, the lecithotrophic *Phyllacanthus parvispinus* (Parks *et al.*, 1989). The postoral and posterodorsal arms of *C. blakei* are fenestrated all the way to their base, as are those of larvae of the genus *Prionocidaris*, but larval skeletal rods in the genus *Eucidaris* are fenestrated only part way to the base. It follows that partially fenestrated skeletal rods are the more derived trait (Fig. 1). The juvenile spines of *C. blakei* are different from those of all other cidaroids.

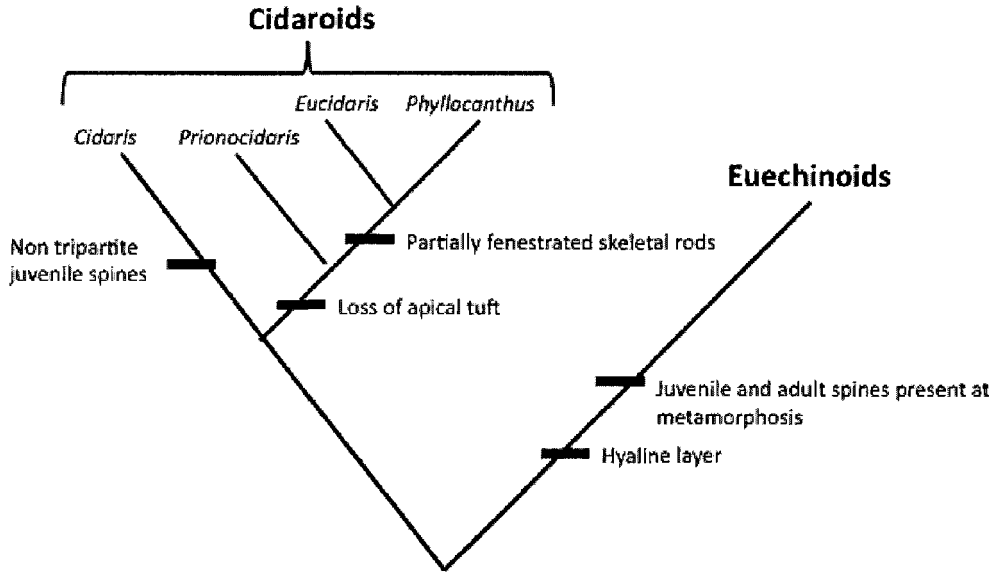

Figure 1. A phylogenetic tree showing the two main branches of the echinoid clade: the cidaroids and the euechinoids. Developmental traits described in this study, including skeletal rod fenestration, presence of an apical tuft, juvenile spine morphology and presence of a hyaline layer are mapped on the tree using the most parsimonious approach. The branches of the cidaroid clade are placed based on a phylogenetic tree constructed using adult morphological characteristics (Smith and Wright, 1989).

A record of the development of *C. blakei* is of value not only for comparisons among cidaroids, but also for sharpening the image of a characteristic cidaroid larva and how it differs from the more highly derived larvae of euechinoids. Traits described for one or more species of cidaroid, including this description of *C. blakei*, that differ from euechinoids include widely spaced blastomeres during cleavage stages (Mortensen, 1938; Schroeder, 1981); a virtual absence of hyaline layer (Schroeder, 1981); varied numbers or a lack of micromeres (Mortensen, 1937; Schroeder, 1981); primary mesenchyme that is

homologous to euechinoids, but differs temporally (Wray and McClay, 1988, in reference to *E. tribuloides*); the presence of five pairs of vibratile lobes arising from the ciliated band (Prouho, 1887; Mortensen 1937, 1983; Emlet, 1988); juvenile structures that develop in the absence of a vestibule (Emlet, 1988; Parks *et al.*, 1989); and juveniles that have only juvenile spines at metamorphosis, in contrast to euechinoid juveniles which have both juvenile and adult spines (Mortensen, 1938; Emlet, 1988). This investigation has provided further evidence that the hyaline layer is absent in cidaroids, suggesting that it is a derived trait among euechinoids (Fig. 1). The data presented in Chapter III further suggest that the form and function of the hyaline layer is also different among different clades of euechinoids.

This record of the development of *C. blakei* is a unique opportunity to further our understanding of the biology and ecology of deep-sea echinoids. Two traits that *C. blakei* shares with another deep-sea urchin, *Aspidodiadema jacobyi*, which was reared in culture for 5 months, are a prolonged larval life and an extended lecithotrophic stage prior to development of a mouth (Young and George, 2000). Whether these traits might be adaptations to life in the deep-sea certainly warrants further investigation. The inability of *C. blakei* to tolerate temperatures above 15 °C provides preliminary evidence that this species would be unable to migrate to the surface waters, and therefore must be feeding on an alternative food source that is available at depth.

BIBLIOGRAPHY

Chapter I

Arellano, S. M. 2008. Embryology, larval Ecology, and recruitment of "*Bathymodiolus" Childressi*, a cold-seep mussel from the Gulf of Mexico. Ph.D. dissertation, University of Oregon, Eugene.

Bouchet, P., and A. Warén. 1994. Ontogenetic migration and dispersal of deep-sea gastropod larvae. Pp. 98-118 in *Reproduction, larval biology, and recruitment of the deep-sea benthos*, C. M. Young and K. J. Eckelbarger, eds. Columbia University Press, New York.

Kier, P. M. 1977. The poor fossil record of the regular echinoid. *Paleobiology* **3**: 168-174.

Smith, A. B. 1984. *Echinoid Palaeobiology*. Allen & Unwin, London. 190 pp.

Van Gaest, A. 2006. Ecology and early life history of *Bathynerita naticoidea*: evidence for long-distance larval dispersal of a cold seep gastropod. M.S. thesis, University of Oregon, Eugene.

Young, C. M. and K. J. Eckelbarger, eds. 1994. *Reproduction, Larval Biology, and Recruitment of the Deep-Sea Benthos*. Columbia University Press, New York.

Young, C. M., J. L. Cameron, and K. J. Eckelbarger. 1989. Extended pre-feeding period in planktotrophic larvae of the bathyal echinoid *Aspidodiadema jacobyi*. *J. mar. biol. Ass. UK* **69**: 695-702.

Chapter II

Amemiya, S., and R. B. Emlet. 1992. The development and larval form of an echinothurioid echinoid, *Asthenosoma ijimai*, revisited. *Biol. Bull.* **182**: 15-30.

Arellano, S. M. 2008. Embryology, larval Ecology, and recruitment of "*Bathymodiolus" Childressi*, a cold-seep mussel from the Gulf of Mexico. Ph.D. dissertation, University of Oregon, Eugene.

Arellano, S. M., and C. M. Young. 2009. Spawning, Development, and the duration of larval life in a deep-sea cold-seep mussel. *Biol. Bull.* **216**: 149-162.

Bouchet, P., and A. Warén. 1994. Ontogenetic migration and dispersal of deep-sea gastropod larvae. Pp. 98-118 in *Reproduction, larval biology, and recruitment of the deep-sea benthos*, C. M. Young and K. J. Eckelbarger, eds. Columbia University Press, New York.

Cameron, R. A., I. Boidron-Metairon, and O. Monterrosa. 1985. Does the embryonic response to temperature and salinity by four species of Caribean sea urchins parallel the reproductive synchrony? *Proceed. 5th Intern. Coral Reef Cong., Tahiti.* **5:** 273-278.

Charles, F., F. Lantoine, S. Brugel, M.J. Chrétiennot-Dinet, I. Quiroga and B. Rivière. 2005. Seasonal survey of the phytoplankton biomass, composition and production in a littoral NW Mediterranean site, with special emphasis on the picoplanktonic contribution. *Est. Coast and Shelf Sci.* **65:** 199-212.

Emlet, R. B. 1988. Larval form and metamorphosis of a 'primitive' sea urchin, *Eucidaris thouarsi* (Echinodermata: Echinoidea: Cidaroida), with implications for developmental and phylogenetic studies. *Biol. Bull.* **174:** 4-19.

Emlet, R. B. 1995. Developmental mode and species geographic range in regular sea urchins (Echinodermata: Echinoidea). *Evolution* **49**: 476-489.

Emlet, R. B., L. R. McEdward, and R. R. Strathmann. 1987. Echinoderm larval ecology viewed from the egg. Pp.155-136 in *Echinoderm Studies,* M. J. Jangoux and J. M. Lawrence, eds. Balkema, Rotterdam.

Giudice, G. 1986. *The Sea Urchin Embryo: a Developmental Biological System.* Springer-Verlag, Berlin.

Guillard, R. L. 1975. Culture of phytoplankton for feeding marine invertebrates. Pp. 26-60 in *Culture of Marine Invertebrate Animals*, W. L. Smith and M. H. Chanley, eds. Plenum, New York.

Horstadius, S. 1939. The mechanics of sea urchin development, studied by operative methods. *Biol. Rev. Camb. Philos. Soc.* **14**: 132-179.

Horstadius, S. 1973. *Experimental Embryology of Echinoderms.* Oxford University Press, London.

Ishizuka, Y., T. Minokawa, and S. Amemiya. 2001. Micromere descendants at the blastula stage are involved in normal archenteron formation in sea urchin embryos. *Dev. Genes Evol.* **211**: 83-88.

Kier, P. M. 1977. The poor fossil record of the regular echinoid. *Paleobiology* **3**: 168-174.

Levitan, D. R. 2000. Optimal egg size in marine invertebrates: theory and phylogenetic analysis of the critical relationship between egg size and development time in echinoids. *Amer. Natural.* **156**:175-192.

McEdward, L. R. 1985. Effects of temperature on the body form, growth, electron transport system activity, and developmental rate of an echinopluteus. *J. Exp. Mar. biol.* **147**: 95-102.

Mortensen, T. H. 1937. Contributions to the study of the development and larval forms of echinoderms, III. *Kgl. Dan. Vedens. Selsk. Skr. Naturvid. Math. Ser. 9* **7**(1): 14-20.

Mortensen, T. H. 1938. Contributions to the study ofthe development and larval forms of echinoderms, IV. *Kgl. Dan. Vedens. Selsk. Skr. Naturvid. Math. Ser. 9* **7**(3): 13-26.

Okazaki, K. 1975. Normal development to metamorphosis. Pp. 177-232 in *The Sea Urchin Embryo, Biochemistry and Morphogenesis,* G. Czihak, ed. Springer-Verlag, Berlin.

Okazaki, K. 1975. Spicule Formation by Isolated Micromeres of the Sea Urchin Embryo. *Am. Zool.* **15**: 567-581.

Olson, R. R., J. L. Cameron, and C. M. Young. 1993. Larval development (with observations on spawning) of the pencil urchin *Phyllacanthus imperialis*: a new intermediate larval form? *Biol. Bull.* **185**: 77-85.

Parks, A. L., B. W. Bisgrove, G. A. Wray, and R. A. Raff. 1989. Direct development in the sea urchin *Phyllacanthus parvispinus* (Cidaroidea): phylogenetic history and functional modification. *Biol. Bull.* **177**: 96-109.

Pearse, J. S. 1994. Cold-water echinoderms break "Thorson's Rule". Pp. 26-43 in *Reproduction, Larval Biology, and Recruitment of the Deep-Sea Benthos*, C. M. Young and K. J. Eckelbarger, eds. Columbia University Press, New York.

Pearse, J. S., and R. A. Cameron. 1991. Echinodermata: Echinoidea. Pp. 514-624 in *Reproduction in Marine Invertebrates: Echinoderms and Lophophorates*, A. C. Giese, J. S. Pearse and V. B. Pearse, eds. The Boxwood Press, Pacific Grove, CA.

Prouho, H. 1887. Recherches sur le *Dorocidaris papillata* et quelques autres echinides de al Méditerranée. *Arch. Zool. Exp. Gén. Ser. 2.* **5**: 213-380.

Rex, M.A., and A. Warén. 1982. Planktotrophic development in deep-sea prosobranch snails from the western North Atlantic. *Deep-sea Res.* **29**:171-184.

Schroeder, T. E. 1981. Development of a "primitive" sea urchin (*Eucidaris tribuloides*): irregularities in the hyaline layer, micromeres, and primary mesenchyme. *Biol. Bull.* **161:** 141-151.

Smith, A. B. 1984. *Echinoid Palaeobiology.* Allen & Unwin, London. 190 pp.

Smith, A. B. (ed). 2005. The Echinoid Directory. [online] Available: http://www.nhm.ac.uk/research-curation/research/projects/echinoid-directory/ [2008, September].

Smith, A. B., and N. T. J. Hollingworth. 1990. Tooth structure and phylogeny of the Upper Permian echinoid *Miocidaris keyserlingi. Proc. York. Geol. Soc.* **48**: 47-60.

Smith, A. B., and C. W. Wright. 1989. British cretaceous echinoids, part 1. General introduction and cidaroida. *Monogr. Palaeont. Soc.* **141:** 1-101.

Smith, A. B., D. Pisani, J. A. Mackenzie-Dodds, B. Stockley, B. L. Webster, and D. T. J. Littlewood. 2006. Testing the molecular clock: molecular and paleontological estimates of divergence times in the echinoidea (echinodermata). *Mol. Biol. Evol.* **23**: 1832-1851.

Stephens, R. E., and R. E. Kane. 1970. The calcium-insoluble protein of the hyaline layer of the sea urchin egg. *J. Cell Biol.* **44**:611-617.

Strathmann, M. F. 1987. *Reproduction and Development of Marine Invertebrates of the Northern Pacific Coast:Data and Methods for the Study of Eggs, Embryos and Larvae.* University of Washington Press, Seattle.

Thorson, G.L. 1950. Reproductive and larval ecology of marine bottom invertebrates. *Biol Rev.* **1:** 1-46.

Van Gaest, A. 2006. Ecology and early life history of *Bathynerita naticoidea*: evidence for long-distance larval dispersal of a cold seep gastropod. M.S. thesis, University of Oregon, Eugene.

Van Gaest, A., C. M. Young, J. J. Young, A. R. Helms, and S. M. Arellano. 2007. Physiological and behavioral responses of *Bathynerita naticoidea* (Gastropoda: Neritidae) and *Methanoaricia dendrobranchiata* (Polychaeta: Orbiniidae) to hypersaline conditions at a brine pool cold seep. *Mar. Ecol.* **28:** 199-207.

Wray, G. A., and D. R. McClay. 1988. The origin of spicule-forming cells in a 'primitive' sea urchin (*Eucidaris tribuloides*) which appears to lack primary mesenchyme cells. *Development* **103**: 305-315.

Young, C. M. 1994. A Tale of Two Dogmas: The Early History of Deep-Sea Reproductive Biology.Pp. 1-25 in *Reproduction, Larval Biology, and Recruitment of the Deep-Sea Benthos*, C. M. Young and K. J. Eckelbarger, eds. Columbia University Press, New York.

Young, C. M. 2003. Reproduction, development and life-history traits. Pp. 381-426 in *Ecosystems of the World*, Vol. 28, *Ecosystems of the Deep Oceans*, P.A. Tyler, ed. Elsevier, Amsterdam.

Young, C. M., J. L. Cameron, and K. J. Eckelbarger. 1989. Extended pre-feeding period in planktotrophic larvae of the bathyal echinoid *Aspidodiadema jacobyi. J. mar. biol. Ass. UK* **69**: 695-702.

Young, C. M., M. G. Devin, W. B. Jaeckle, S. U. K. Ekaratne, and S. B. George. 1996. The potential for ontogenetic migration by larvae of bathyal echinoderms. *Oceanol. Act.* **19**: 263-271.

Young, C. M., S. U. K. Ekaratne, and J. L. Cameron. 1998. Thermal tolerances of embryos and planktotrophic larvae of *Archaeopneustes hystrix* (A. Agassiz) (Spatangoidea) and *Stylocidaris lineata* (Mortensen) (Cidaroidea), bathyal echinoids from the Bahamian slope. *J. Exp. Mar. Biol. and Ecol.* **223**: 65-76.

Young, C. M., and M. A. Sewell. 1999. Temperature limits to fertilization and early development in the tropical sea urchin *Echinometra lacunter. J. Exp. Mar. Biol. And Ecol.* **236**: 291-305.

Chapter III

Adelson, D. L., M. C. Alliegro, and D. R. McClay. 1992. On the ultrastructure of hyalin, a cell adhesion protein of the sea urchin embryo extracellular matrix. *J. Cell Biol.* **116**: 1283-9.

Adelson, D. L., and T. Humphreys. 1988. Sea urchin morphogenesis and cell-hyalin adhesion are perturbed by a monoclonal antibody specific for hyalin. *Development* **104**: 391-402.

Alvarez, M., J. Nnoli, E. J. J. Carroll, V. Hutchins-Carroll, Z. Razinia, and S. B. Oppenheimer. 2007. Exogenous hyalin and sea urchin gastrulation. Part II, Hyalin, an interspecies cell adhesion molecule. *Zygote* **16**: 73-8.

Citkowitz, E. 1971. The hyaline layer: its isolation and role in echinoderm development. *Dev. Biol.* **24**: 348-62.

Citkowitz, E. 1972. Analysis of the isolated hyaline layer of sea urchin embryos. *Dev. Biol.* **27**: 494-503.

Contreras, A., J. Vitale, V. Hutchins-Carroll, E. J. J. Carroll, and S. B. Oppenheimer. 2008. Exogenous hyalin and sea urchin gastrulation. Part III: Biological activity of hyalin isolated from *Lytechinus pictus* embryos. *Zygote* **16**: 355-61.

Eisen, A., D. P. Kiehart, S. J. Wieland, and G. T. Reynolds. 1984. Temporal sequence and spatial distribution of early events of fertilization in single sea urchin eggs. *J. Cell Biol.* **99**: 1647-54.

Emlet, R. B. 1988. Larval form and metamorphosis of a 'primitive' sea urchin, *Eucidaris thouarsi* (Echinodermata: Echinoidea: Cidaroida), with implications for developmental and phylogenetic studies. *Biol. Bull.* **174:** 4-19.

Foltz, K. R., and W. J. Lennarz. 1993. The molecular basis of sea urchin gamete interactions at the egg plasma membrane. *Dev. Biol.* **158**: 46-61.

Gray, J., R. Justice, G. M. Nagel, and E. J. J. Carroll. 1986. Resolution and characterization of a major protein of the sea urchin hyaline layer. *J. Biol. Chem.* **261**: 9282-9288.

Giudice, G. 1986. *The Sea Urchin Embryo: a Developmental Biological System.* Springer-Verlag, Berlin.

Holland, N. D., and L. Z. Holland. 1989. Fine structural study of the cortical reaction and formation of the egg coats in a lancelet (=Amphioxus), *Branchiostoma floridae* (Phylum Chordata: Subphylum Cephalochordata=Acrania). *Biol. Bull.* **176**: 111-22.

Kane, R. E. 1970. Direct isolation of the hyaline layer protein released from the cortical granules of the sea urchin egg at fertilization. *J. Cell Biol.* **45:** 615-622.

Kier, P. M. 1977. The poor fossil record of the regular echinoid. *Paleobiology* **3**:168-74.

McClay, D. R., and R. D. Fink. 1982. Sea urchin hyalin: appearance and function in development. *Dev. Biol.* **92**: 285-93.

Mortensen, T. H. 1937. Contributions to the study ofthe development and larval forms of echinoderms. III. *Kgl. Dan. Vedens. Selsk. Skr. Naturvid. Math. Ser. 9* **7**:14-20.

Mortensen, T. H. 1938. Contributions to the study ofthe development and larval forms of echinoderms. IV. *Kgl. Dan. Vedens. Selsk. Skr. Naturvid. Math. Ser. 9* **7**:13-26.

Olson, R. R., J. L. Cameron, and C. M. Young. 1993. Larval development (with observations on spawning) of the pencil urchin *Phyllacanthus imperialis*: a new intermediate larval form? *Biol. Bull.* **185**: 77-85.

Parks, A. L., B. W. Bisgrove, G. A. Wray, and R. A. Raff. 1989. Direct development in the sea urchin *Phyllacanthus parvispinus* (Cidaroidea): phylogenetic history and functional modification. *Biol. Bull.* **177**: 96-109.

Razinia, Z., E. J. J. Carroll, and S. B. Oppenheimer. 2007. Microplate assay for quantifying the developmental morphologies, effects of exogenous hyalin on sea urchin gastrulation. *Zygote* **15**: 1-6.

Schroeder, T. E. 1981. Development of a "primitive" sea urchin (*Eucidaris tribuloides*): irregularities in the hyaline layer, micromeres, and primary mesenchyme. *Biol. Bull.* **161:** 141-151.

Smith, A. B. 1984. *Echinoid Palaeobiology.* Allen & Unwin, London. 190 pp.

Smith, A. B., and N. T. J. Hollingworth. 1990. Tooth structure and phylogeny of the Upper Permian echinoid *Miocidaris keyserlingi. Proc. York. Geol. Soc.* **48**: 47-60.

Smith, A. B., D. Pisani, J. A. Mackenzie-Dodds, B. Stockley, B. L. Webster, and D. T. J. Littlewood. 2006. Testing the molecular clock: molecular and paleontological estimates of divergence times in the echinoidea (echinodermata). *Mol. Biol. Evol.* **23**: 1832-51.

Stephens, R. E., and R. E. Kane. 1970. The calcium-insoluble protein of the hyaline layer of the sea urchin egg. *J. Cell Biol.* **44**: 611-7.

Strathmann, M. F. 1987. *Reproduction and Development of Marine Invertebrates of the Northern Pacific Coast:Data and Methods for the Study of Eggs, Embryos and Larvae.* University of Washington Press, Seattle.

Wray, G. A. 1997. Echinoderms. Pp. 309-30 in *Embryology: Constructing the Organism*, S. F. Gilbert and A. M. Raunio, eds. Sinauer Associates, MA.

Wray, G. A., and D. R. McClay. 1988. The origin of spicule-forming cells in a 'primitive' sea urchin (*Eucidaris tribuloides*) which appears to lack primary mesenchyme cells. *Development* **103**: 305-15.

Chapter IV

Emlet, R. B. 1988. Larval form and metamorphosis of a 'primitive' sea urchin, *Eucidaris thouarsi* (Echinodermata: Echinoidea: Cidaroida), with implications for developmental and phylogenetic studies. *Biol. Bull.* **174:** 4-19.

Mortensen, T. H. 1937. Contributions to the study ofthe development and larval forms of echinoderms. III. *Kgl. Dan. Vedens. Selsk. Skr. Naturvid. Math. Ser. 9* **7**:14-20.

Mortensen, T. H. 1938. Contributions to the study ofthe development and larval forms of echinoderms. IV. *Kgl. Dan. Vedens. Selsk. Skr. Naturvid. Math. Ser. 9* **7**:13-26.

Parks, A. L., B. W. Bisgrove, G. A. Wray, and R. A. Raff. 1989. Direct development in the sea urchin *Phyllacanthus parvispinus* (Cidaroidea): phylogenetic history and functional modification. *Biol. Bull.* **177**: 96-109.

Prouho, H. 1887. Recherches sur le *Dorocidaris papillata* et quelques autres echinides de al Méditerranée. *Arch. Zool. Exp. Gén. Ser. 2.* **5**: 213-380.

Schroeder, T. E. 1981. Development of a "primitive" sea urchin (*Eucidaris tribuloides*): irregularities in the hyaline layer, micromeres, and primary mesenchyme. *Biol. Bull.* **161:** 141-151.

Smith, A. B., and C. W. Wright. 1989. British cretaceous echinoids, part 1. General introduction and cidaroida. *Monogr. Palaeont. Soc.* **141:** 1-101.

Smith, A. B., and D. T. J. Littlewood. 1997. Molecular and morphological evolution during the post-Paleozoic diversification of echinoids. Pp. 559-583 in *Molecular Evolution and Adaptive Radiation,* T. J. Givnish, and K. J. Systma, eds. Cambridge.

Smith, A. B., D. T. J. Littlewood and G. A. Gray. 1996. Comparing patterns of evolution: larval and adult life-history stages and small ribosomal RNA of post-Paleozoic echinoids. *Phil. Trans. R. Soc. Lond.* **349:** 11-18.

Wray, G. A., and D. R. McClay. 1988. The origin of spicule-forming cells in a 'primitive' sea urchin (*Eucidaris tribuloides*) which appears to lack primary mesenchyme cells. *Development* **103**: 305-15.

Young, C. M., and George, S. B. 2000. Larval development of the tropical deep-sea echinoid *Aspidodiadema jacobyi*: phylogenetic implications. *Biol. Bull.* **198:** 387-395.