MESENCHYME CELL DIFFERENTIATION IN INTERSPECIES HYBRID ECHINOID EMBRYOS

by

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Abstract

The sea urchins, *Strongylocentrotus purpuratus* and *Lytechinus pictus* diverged from a common ancestor 40-60 million years ago. Despite this divergence, reciprocal interspecific hybrids are possible under suitable laboratory conditions. This thesis has two main foci: morphological and molecular assessment of paternal and maternal characters in these hybrids. The morphology of the interspecies hybrids appears to be maternal until gastrulation. *S. purpuratus* embryos exhibit an oral-aboral axis earlier in gastrulation than *L. pictus* and both heterotypic crosses appear to be like *S. purpuratus* in this character. The number of skeletogenic primary mesenchyme cells (PMCs) in these embryos has been assessed using immunocytochemistry with the monoclonal antibody Ig8. New information regarding PMC numbers at the time of ingression is reported and is the same in both species and in hybrids. The morphology of the larval pigment cells of the two species and the hybrid embryos has been examined. The morphology of the larval skeletons has been examined and compared using polarized light microscopy. Three criteria were selected for assessing contributions of the parental species in the hybrid pluteus larvae: body rod curvature, body rod termini structures, and presence/absence of recurrent rods. Data suggest that in both heterotypic crosses, the *S. purpuratus* spicule structures predominate; however, some variability in spicule morphology was observed in embryos

derived from fertilizing *L. pictus* eggs with *S. purpuratus* sperm suggesting that the inheritance of these structures is complex.

Previous molecular analyses of the expression of two aboral ectoderm specific gene homologues, LpS1 and Spec1, in these reciprocal hybrid embryos have demonstrated a developmentally progressive restriction of expression of the *L. pictus* homologue in both hybrid crosses. The second focus of this thesis is a molecular characterization of the expression of two spicule matrix gene homologues, LSM34 and SM50, in these reciprocal hybrid embryos. RNA gel blot hybridization analysis has revealed that both these genes are expressed in both heterospecific crosses with no apparent down regulation. Whole mount *in situ* hybridization has localized both of these transcripts to a subpopulation of the PMCs in these heterospecific crosses suggesting that proper temporal and spatial regulation is maintained for both homologues.

For A. E. Dean
(1902-1949)

Who Would Have Understood

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Chapter I

Introduction

1.1 General Introduction

Sea urchins have long provided developmental biologists with a model system with which to examine embryonic development. Great importance has been placed on obtaining a clear understanding of mechanisms that control the gradual elaboration of structure and function that follows fertilization. Transformation of a single cell into a complex multicellular organism requires well regulated and interactive control mechanisms limited in both time and space. Development is initially orchestrated by maternally supplied information stored within the egg during oogenesis. As the maternal mRNAs and proteins are exhausted, transcription of the zygotic genome becomes necessary for continued development. Determining the nature of maternal factors and the mechanisms regulating zygotic gene expression will assist in gaining an understanding of both morphogenesis and cellular differentiation.

1.2 Fertilization

Sea urchin eggs range in diameter from 70-1,000 microns depending upon the species (Giudice, 1973; Hinegardner, 1975), though most indirect developing species have eggs less than 150 microns across. Seasonally gravid females shed meiotically mature ova that contain a moderate amount of yolk. At the time of release, eggs have an animal-vegetal axis of polarity which is maternally imposed during oogenesis. It is believed that there are cytoplasmic factors with polarized distributions along this axis, and that the rapid invariant

cleavages segregate these constituents into early blastomeres. The initiation of cellular specialization occurs in part as a result of the factors that are isolated within a particular blastomere, as wells as via cellular interactions.

The outermost layer of the egg is a jelly coat chiefly composed of mucopolysaccharides. In addition, this layer contains a small peptide chemo-attractant that diffuses from the egg and attracts sperm in a concentration dependent manner (Hansbrough and Garbers, 1981). This peptide is often, but not always, species specific. Experiments demonstrated that the sperm of the sea urchin *Arabacia punctulata* is not attracted to the eggs of *Strongylocentrotus drobachiensis* or vice versa. However, sperm from *S. purpuratus* is attracted to the jelly of *Lytechinus variegatus* but not to that of *A. punctulata* (Summers and Hylander, 1976).

Beneath the egg jelly lies a glycoprotein layer known as the vitelline envelope which is closely associated with the egg plasma membrane. The plasma membrane contains approximately 1.5×10^3 species specific glyoprotein sperm receptors that pass through the vitelline layer (reviewed by Giudice, 1986). These sperm receptors and the chemo-attractant in the egg jelly work synergistically with geographical distributions of sea urchin species, and timing of gamete release, to ensure that interspecific fertilizations are rare.

The sperm passes through the egg jelly and undergoes an acrosome reaction which releases bindin, a species specific egg binding protein. The bindin on the acrosome process attaches to the corresponding receptor on the

Figure 1:

Normal Sea Urchin Embryonic Development:-A Schematic

Representaion.

[A] fertilized egg [B] hatched blastula [C] mesenchyme blastula

[D] gastrulating embryo [E] prism stage larva [F] pluteus larva

Abbreviations : a, animal pole; v, vegetal pole; fe, fertilization envelope; hl, hyaline layer; sp, sperm; at, apical tuft cilia; bc, blastocoel; pmcs, primary mesenchyme cells; vp, vegetal plate; arc, archenteron; smcs, secondary mesenchyme cells; g, gut; m, mouth; an, anus; spic, spicule; aoe, aboral ectoderm; oe, oral ectoderm.

4b
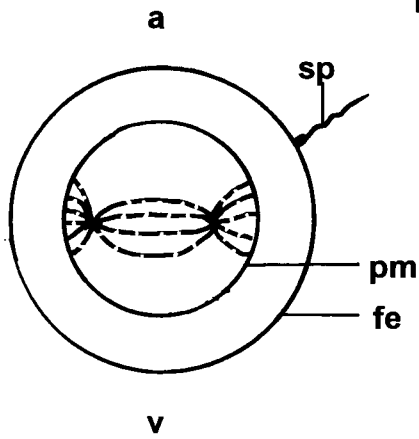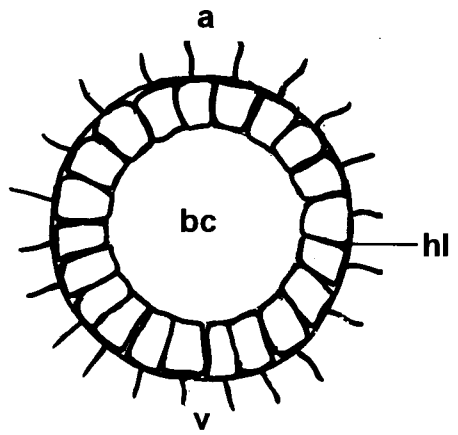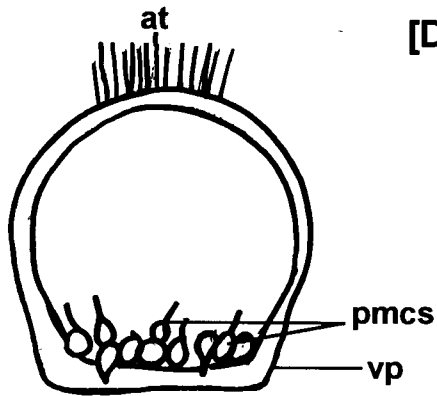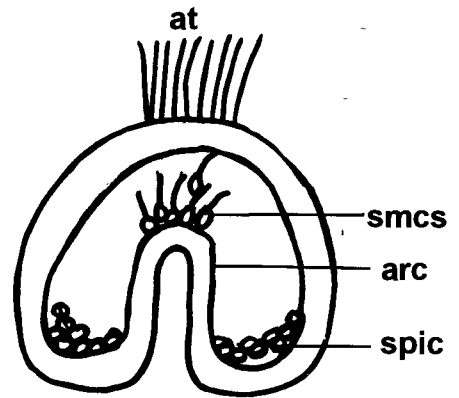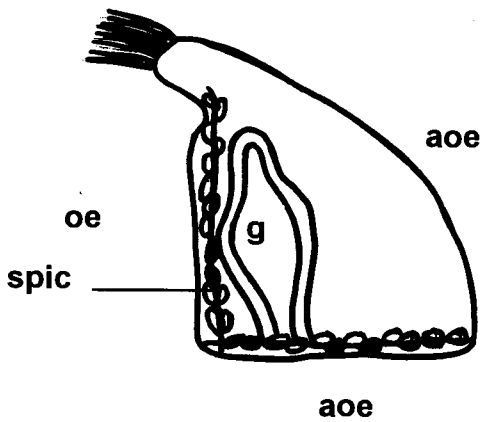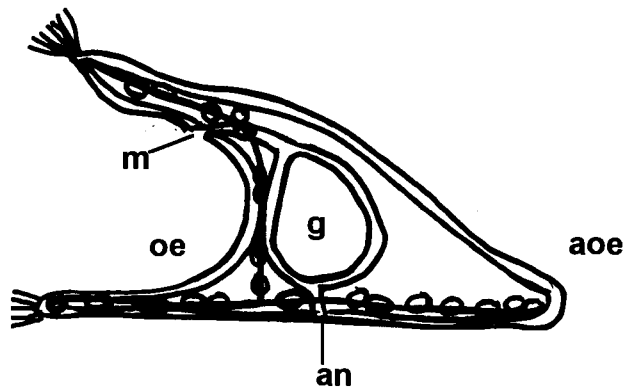
surface of the egg and the process penetrates the vitelline layer. The egg and sperm plasma membranes fuse, a fertilization cone forms, and the male pronucleus is drawn into the egg (reviewed by Giudice, 1986). The interaction between the sperm bindin and its receptor is thought to trigger a signal transduction pathway that initiates exocytosis of the egg cortical granules, which in turn, has several consequences. First, proteases cleave the remaining sperm receptors and the proteins that link the vitelline layer to the egg plasma membrane (Glabe and Vacquier, 1978). Second, mucopolysaccharides stored within the cortical granules are released into the perivitelline space. The ensuing osmotic gradient causes an influx of water into this space consequently lifting the vitelline layer away from the plasma membrane. Third, a peroxidase activity released during this reaction is responsible for hardening the fertilization envelope by crosslinking adjacent tyrosine residues (Mozingo and Chandler, 1991). This hardened envelope is elemental in the prevention of polyspermic fertilization (see Figure 1A). Finally, the cortical granules contain the components of the hyaline layer that coat the embryo and provide support during cleavage as well as preventing cellular dissociation (Hylander and Summers, 1982).

1.3 Cleavage

Indirect developing species of sea urchins exhibit almost invariant embryogenesis. Radial holoblastic cleavage begins approximately 1 hour post

fertilization. The first two divisions occur meridonally along the animal-vegetal axis perpendicular to one another. The third division occurs equatorially and separates the animal and vegetal regions. The fourth cleavages are the most striking. The blastomeres in the animal half divide meridonally producing eight mesomeres of equal size, and the vegetal cells divide in an unequal oblique, subequatorial manner producing four large macromeres and four small micromeres. The mesomeres will ultimately give rise to ectodermal structures in the larva, while the macromeres produce endoderm, mesoderm and some ectoderm. The micromeres mark the position of the vegetal pole and give rise to mesodermal derivatives. These micromeres then cleave unequally and produce four large micromeres and four small micromeres. The large micromeres proliferate and differentiate into a population of skeletogenic mesenchyme cells; and, the small micromeres cease dividing and give rise to the coelomic pouches during larval development.

At the 60 cell stage, the animal half of the embryo contains two tiers of cells referred to as an1 and an2. Below an2, in the vegetal half of the embryo, are two vegetal tiers, veg1 and veg2 (reviewed by Horstadius, 1973). The micromeres lie below veg2 at the extreme vegetal pole. By the 60 cell stage the S. purpuratus embryo consists of five distinct spatial territories (Cameron et al., 1987; Davidson, 1989; Cameron and Davidson, 1991). These are the oral ectoderm, the aboral ectoderm, the vegetal plate, the small micromeres, and the skeletogenic mesenchyme, descended from the large micromeres. Each

territory is clonally established by several founder cells. The discovery of territorial specific molecular markers has helped elucidate territorial specification and tissue specific differentiation. Cell marking experiments indicate that there is an almost invariant relationship between cell lineage and cell fate in the undisturbed embryo (Cameron *et al.*, 1987). *L. pictus* embryos probably form these territories in a similar fashion to *S. purpuratus.* Several *L. pictus* genes show the same patterns of expression as their *S. purpuratus* homologues, and can be used as markers to assess differentiation in cell isolation experiments and cell recombination experiments.

Sea urchin embryos exhibit regulative development. Classic isolation and recombination experiments performed by Horstadius (1935) have greatly contributed to our understandings of egg axis determination and interactions between layers of the 60 cell stage sea urchin embryo (reviewed by Horstadius, 1973). Blastomeres separated at the two and four cell stages develop into normal but smaller plutei. When animal tiers and vegetal tiers are separated, the animal tiers form ciliated ectoderm lacking vegetal structures (gut, skeleton). In contrast, the vegetal tiers form recognizable pluteus larvae with exaggerated endodermal structures. Horstadius proposed that the egg cytoplasm contains two opposing gradients of morphogenetic determinants, one concentrated at the animal pole and the other concentrated at the vegetal pole. His model suggests that normal development is dependent upon the ratio of animalizing to vegetalizing determinants; and, that the fate of a particular blastomere is

dependent upon the relative amounts of these diffusible morphogens that a blastomere receives during cleavage. This model has recently been reexamined. New information regarding cell signaling pathways suggests that the fate of a particular blastomere may be determined by intercellular communications implemented by ligand-receptor interactions (reviewed by Brandhorst and Klein, 1992). Positional information and inductive interactions are likely mediated in this way. The potential of a given blastomere is greater than its fate in the intact embryo. It is therefore reasonable to assume that cell interactions limit the developmental potential of a given blastomere. It has been proposed that cell fates are limited by a series of suppressive inductive interactions occurring between adjacent tiers of cells in the intact embryo. This model suggests that there are suppressive interactions occurring between tiers of cells in the 60 cell embryo, and that the potential of a given blastomere may be restricted by its neighbors (reviewed by Brandhorst and Klein, 1992).

Cell divisions continue until a hollow, fluid filled blastula comprised of approximately 256 cells is formed (see Figure 1B). The blastula then secretes a hatching enzyme, digests the fertilization envelope and begins to swim via the beating of epithelial cilia

1.4 Primary Mesenchyme Cell (PMC) Ingression and Skeletogenesis

Prior to the onset of gastrulation, a population of cells in the vegetal plate lose their affinities for each other and for the hyaline layer and gain affinity for

the basal lamina that lines the blastocoel (Fink and McClay, 1980; Fink and McClay, 1985). These primary mesenchyme cells, derived from the large micromeres, ingress into the blastocoel and send out filopodial extensions (see Figure 1C). These filopodia make stable contacts with the inside of the blastocoel and eventually the cells fuse to form an extensive syncytial ring just below the equator (Gibbons et al, 1969; Millonig, 1970). These filopodia penetrate the basal lamina and directly contact the overlying ectoderm (Spiegel and Spiegel, 1992). These are the skeletogenic cells and the number per embryo is a species specific characteristic. The basal lamina differs in composition both temporally and spatially in the region where the PMCs make stable contacts (Wessel and McClay, 1985; Galileo and Morrill, 1985) Fluorescently marked PMCs implanted in the animal pole of embryos migrate to the vegetal hemisphere and position themselves at the correct sites on the blastocoel walls (Ettensohn and McClay, 1986; Ettensohn and Malinda, 1993). This suggests that positional cues for PMC localization are not restricted to the vegetal hemisphere. Other cell types injected do not migrate in this manner (Ettensohn and McClay, 1986; McClay and Ettensohn, 1987).

The syncytial ring of PMCs becomes organized into two ventrolateral clusters that produce triradiate spicules of the skeleton (Okazaki, 1975) composed of $CaCO_3$, $MgCO_3$, and several secreted matrix glycoproteins (Decker and Lennarz, 1988; Wilt and Benson,1988). Cultured micromeres produce some skeletal structures autonomously when supplemented with horse serum or grown

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on an artificial extracellular matrix (Okazaki, 1975; Harkey and Whiteley, 1980; Carson et al 1985; Benson and Chuppa, 1990). Although the resulting skeletal spicules show some species specific characteristics, they are somewhat disordered and do not exhibit the normal triradiate structure. Therefore, various morphogenetic cues that specify the spicule shape must be in place in order for proper skeletogenesis to occur. In addition, the timing of spicule formation is delayed *in vitro* (Fink and McClay, 1985). When PMCs from an older embryo are transplanted into the blastocoel of a younger host, the timing of spicule formation follows that of the host. When PMCs are transplanted into the blastocoel of a host embryo prior to ingression, the donor PMCs do not synthesize spicules until the host PMCs have ingressed (Ettensohn and McClay, 1986). These experiments indicate that the timing of skeleton formation is not an intrinsic feature of the PMCs, and that proper timing occurs as a result of interactions with the rest of the embryo (Ettensohn and McClay, 1986).

Cultured micromeres produce spicules that are up to five times longer than those seen in the intact embryo (McClay *et al.*, 1992). In addition, half and quarter sized embryos produce skeletons that are appropriately reduced in size. When PMCs are transplanted from full size embryos into the blastocoels of half or quarter sized embryos, the spicules produced are also half or quarter sized (McClay *et al.*, 1992). Therefore, in addition to the timing of spiculogenesis, the size of the larval skeleton is regulated via interactions with the rest of the embryo (Fink and McClay, 1985; McClay *et al.*, 1992).

Reciprocal transplantation of PMCs between the species *Tripneustes ventricosus* and *Lytechinus variegatus,* from which the PMCs of the host have been removed, result in the formation of spicules that morphologically resemble the donor phenotype (Armstrong and McClay, 1994). *Tripneustes* skeletons have fenestrated (composed of three connected rods) anterolateral rods and the recurrent rods extend to the posterior and are joined to the body rods by the posterior connecting rods (see figure 9 on page 62 for a description of spicule nomenclature). *Lytechinus* skeletons are composed of single, simple rods and do not have posterior connecting rods. Primary mesenchyme cells within chimeric embryos containing both *L. variegatus* and *T. ventricosus* PMCs interact and produce mosaic skeletons with features of both species (Armstrong and McClay, 1994). The pattern of the spicules reflect the genotype of the local PMC type. All PMCs from a given donor embryo are equivalent in their potential in that they can produce the part of the skeleton that the positional information in the ectoderm specifies (Ettensohn, 1990). Chimeric embryos respond to cues from the ectoderm according to their genotypic specifications which results in skeletons with both fenestrated and simple rods (Armstrong and McClay, 1994). This result suggests that the signal information is conserved between these two species such that the PMCs from one species respond to the ectodermal signal from the other species. In hybrid embryos made between these two species, the spicules also follow both patterns randomly along the rods (Armstrong and McClay, personal communication, 1994). These experimental results

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demonstrate that although PMCs make the appropriately patterned spicules in the absence of the rest of the embryo, the cues responsible for the timing and the overall skeletal shape originate from the embryonic ectodermal epithelium (Ettensohn and McClay, 1986).

1.5 Gastrulation

Gastrulation results in the formation of the three primary germ layers from a spherical epithelium. Gastrulation can be broken down into three stages (reviewed by Burke, 1990). The first stage is primary invagination preceded by a flattening and thickening of the vegetal plate. Invagination is a mechanically autonomous event that does not require interaction with the rest of the embryo. Isolated vegetal plates invaginate to about two thirds of their normal lengths and the gut differentiates normally (Ettensohn et al., 1984). In exogastrulae in which the gut protrudes away from the vegetal pole, the gut extends in a normal fashion in the absence of any interactions with the extracellular matrix or ectoderm delimiting the blastocoel (Hardin et al., 1986; Nocente et al., 1991). Following invagination, the archenteron extends by convergent extension until it reaches two thirds of the way across the blastocoel. The final stage of extension appears to depend upon contact between the stomadeum (presumptive mouth) and the filopodial extensions from the secondary mesenchyme cells at the tip of the archenteron (Hardin, 1986). As in all deuterostomes, including vertebrates,

the blastopore at the vegetal pole becomes the anus. The archenteron

differentiates into the esophagus, stomach, and intestine.

1.6 Secondary Mesenchyme Cells

During gastrulation, a second population of mesenchyme cells ingress

from the tip of the archenteron. These cells, derived from the veg2 tier of the 60

cell embryo, are the secondary mesenchyme cells whose timing of ingression is

species and cell type specific (Gibson and Burke, 1985; Wray and McClay,

1988). Following the primary invagination of the archenteron in S. purpuratus,

the chromogenic mesenchyme cells release from the tip and begin an active

period of migration within the blastocoel. These cells eventually invade the

ectoderm and differentiate into the pigment cells, whose functions are not known

at this time (Gibson and Burke, 1985; Gibson and Burke, 1987). As gastrulation

nears completion, the SMC derivatives at the tip of the archenteron extend

filopodia that contact and direct the archenteron to its attachment site on the roof

of the blastocoel: the stomadeum. It appears that these cells make stable

contacts with the basal lamina in the target region and exert a mechanical force

that pulls the archenteron to the target site marking the site of the future mouth

(Hardin, 1986; Crawford and Burke, 1994). In addition, a population of fibroblast

like cells known as the blastocoelar cells are released from the archenteron and

distribute themselves in the blastocoel where they form a network of cellular

processes that associate with various tissues (Tamboline and Burke, 1992).

Shortly before the completion of gastrulation the tip of the archenteron expands laterally in both directions to yield the coelomic pouches. It is debatable as to whether these cells are properly considered SMCs as they are not migratory and remain part of an epithelium despite the presence of active filopodia (Gustafson and Wolpert, 1963). Upon completion of gastrulation, 10-15 cells from each coelomic pouch migrate to the foregut. These are the myogenic mesenchyme cells that give rise to the circumesophageal musculature of the pluteus larva in which several muscle specific genes are expressed [Ishimoda-Takagi *et al.*, 1984; Cox *et al.*, 1986; Burke and Alvarez, 1988; Wessel et al, 1990; Fang, 1994 (Ph.D. Thesis Simon Fraser University , 1994)].

It has been shown that if the PMCs are eliminated from the blastocoel at the early gastrula stage, 65-75 of the SMCs convert to the PMC fate (Ettensohn and McClay, 1988). Under these conditions, the converted SMCs migrate to the correct position in the vegetal hemisphere, initiate transcription of PMC specific genes, and ultimately produce a normal skeleton. During gastrulation PMCs are seen to contact SMCs via their filopodia and it is has been speculated that the PMCs signal the SMCs thereby inhibiting the SMCs from adopting a PMC fate (Ettensohn, 1992). Such an inhibition mechanism is reminiscent of the lateral inhibition during vuval development in *Caenorhabditis elegans* (reviewed by Sternberg 1993). The population of SMCs that convert normally form pigment cells, which are reduced in number after conversion (Ettensohn and Ruffins, 1993).

1.7 Larval Formation and Metamorphosis

Embryonic development is completed with the formation of the bilaterally symmetrical pluteus larva which begins to feed. Until this stage there has been no net increase in mass of the embryo. The pluteus has a posteriorly tapering body and anteriorly projecting arms (see Figure 1F). The anterolateral and the postoral arms are the first to appear and these are supported by rods that originate from the triradiate spicules. A band of ciliated cells forms on the larval arms and the preoral lobe and is used for locomotion and in feeding (Strathman, 1987). The mature skeleton of the larva consist of two oral rods, two anal rods, two transverse rods, and two body rods. Some species have recurrent rods and in some species these rods are connected via posterior connecting rods (*Tripneustes*). During larval development the arms grow, and the addition of more arms is observed in some species. As the coelomic pouches grow posteriorly, the left pouch becomes enlarged and a hydropore connected to the outside environment is formed. An invagination begins on the left side of the embryo which, in conjunction with the left coelomic pouch forms the adult rudiment. This rudiment grows forming the juvenile echinoid in preparation for metamorphosis. The timing of this metamorphosis is directly related to food supplies and availability of substrate (Burke, 1983). During metamorphosis, the larval epithelium collapses, the juvenile rudiment everts, and the larval features are replaced with the radially symmetrical adult form which begins its benthic

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lifestyle. The events that control the process of metamorphosis are complicated and at this point poorly understood, though sensory responses are clearly involved (Burke, 1983).

1.8 Hybrid Embryos and Inheritance

The process of fertilization involves the fusion of the maternal and paternal haploid pronuclei within the egg. The contribution of genetic material from both parents is essentially equal, but the egg cytoplasm and the stored materials are overwhelmingly maternal in origin. Stored maternal mRNAs and proteins are sufficient to carry fertilized sea urchin eggs through the cleavage stages in the absence of nuclear transcription (Brachet, 1963; reviewed by Giudice, 1973). Although stored materials are known to be sufficient for early embryogenesis, zygotic transcription is initiated prior to the first cleavage (reviewed by Davidson, 1986). A great deal of interest lies in determining which processes are under maternal control and which processes are zygotically regulated during embryogenesis.

Historically, various types of interspecies hybrids have been used to assess maternal and paternal contributions during early development. Many species within a given genus can be cross fertilized *in vitro*, and result in viable embryos, larvae, and occasionally, adults. Classic studies involved both morphological analysis and timing of cleavages. Modern studies use sensitive

molecular techniques to investigate developmental gene regulation and gene interactions of two divergent genomes within a single organism.

1.9 Amphibian Hybrids

1.9.1 Rana Hybrids

In 1941 Moore *et al.* explored various morphogenetic processes in crosses between different species of the frog genus *Rana*. In viable hybrids, Moore observed that the rates of morphogenesis are virtually indistinguishable from that normally seen in the maternal species; however, morphogenetic events occurring during and following neural plate formation deviate somewhat from the rates characterized by the maternal species. These results suggest that zygotic gene expression does not predominate until the neurula stage. The subpopulation of embryos that are inviable arrest after the completion of blastulation, at the onset of gastrulation. This observation indicates that although stored maternal information is sufficient to direct early events, development past blastula formation requires zygotic gene activity.

1.9.2 Xenopus Hybrids

Crosses between species of *Xenopus* are possible and the most widely studied cross is between *Xenopus laevis* and *Xenopus borealis* (previously referred to as *Xenopus mulleri*). These reciprocal hybrid crosses are viable and produce mature adults. Studies revealed that these hybrid frogs preferentially

transcribe *X. laevis* ribosomal DNA regardless of the maternal species (Honjo and Reeder, 1973). The cells of these individuals contain one nucleolus suggesting that the presence of the *X. laevis* nucleolar organizer inactivates the *X. borealis* nucleolar organizer. At the swimming tadpole stage, a low level of *X. borealis* rRNA becomes apparent (within a population). Although the *X.laevis* rRNA predominates, some adult hybrid individuals express a significant amount of *X. borealis* rRNA. More recent studies demonstrate that this phenomenon is the result of two mechanisms working in combination. First, it has been reported that the *X. laevis* ribosomal genes have four times the number of enhancers associated with their expression than do *X. borealis* ribosomal genes. Second, there appears to be species specific transcription factors required for RNA polymerase I (reviewed by Reeder, 1985). Although both mechanisms work synergistically to contribute to the observed dominance, the enhancer imbalance mechanism is the major factor. This nucleolar dominance phenomenon is also reported for *Drosophila* hybrids, many plant hybrids, and for some mammalian somatic cell hybrids (reviewed by Reeder, 1985).

Studies pertaining to the expression of the histone H1 genes in hybrid frogs indicate that the paternal H1 transcript is detectable at the midblastula transition stage. This suggests that the zygotic genome is actively making a significant contribution to development prior to gastrulation and that paternal histone H1 gene is actively transcribed and retained in hybrid frogs (Cassidy and Blackler, 1978).

Two dimensional SDS gel electrophoretic comparisons of proteins synthesized by the ovaries of *X. laevis* and *X. borealis* individuals revealed variations in electrophoretic properties in approximately one third of all proteins. With two exceptions, hybrid frog larvae produce detectable levels of both parental proteins. This demonstrates that while allelic exclusion does occur for the rRNA genes, there is no overall dominance of one genome over another in *Xenopus* hybrids (De Robertis and Black, 1979).

1.10 Interspecific Hybrid Sea Urchin Embryo

1.10.1 Classical Studies

In 1893 Theodore Boveri initiated the study of interspecific hybrid sea urchin embryos in order to determine the extent of maternal and paternal contributions during early embryogenesis. He fertilized eggs and anucleate merogones of *Spherechinus granulatus* with sperm of another genus *Echinus microtuberculatus*. From these experiments Boveri reported that while diploid hybrids formed intermediate skeletons, fertilized merogones (hybrid androgenotes) developed skeletal morphology of the paternal species (for historical review see Giudice et al, 1973; Davidson *et al.* 1976). While this result proved controversial, it focused attention on the role of the nucleus in heredity and early development. Echinoid hybrids with notably different timing of development, and easily identifiable differences in morphological characteristics, have been used as a system to assess the initiation of zygotic gene activity

(reviewed by Davidson *et al.*, 1976; Giudice, 1973). The timing of cleavages in sea urchin embryos is relatively invariant at a given temperature and interspecies hybrids exhibit the maternal cell cycle length. In the hybrids made by fertilizing eggs of *Cidaris tribuloides* with sperm from *Lytechinus variegatus* the development of the embryos is maternal until PMC formation. The number and point of ingression of the PMCs prior to gastrulation is paternally specified and is therefore indicative of zygotic gene activity (Dreisch, 1898; Tennet, 1914; reviewed by Davidson 1976). These morphological characters are far less sensitive than modern molecular techniques which have been used to detect initiation of zygotic transcription prior to the first cleavage. Treatment of sea urchin eggs with the transcription inhibitor, actinomycin D results in chemical enucleation (Gross *et al*, 1964; Giudice *et al.*, 1968; reviewed by Brandhorst, 1985). In these experiments, parthogenetically activated enucleated eggs cleave several times and some form irregular blastulae. These abnormal blastulae may form as a result of incomplete transcriptional inhibition, however, these experiments indicate that the earliest events that occur after fertilization do not require zygotic transcription.

Interspecies echinoid hybrid embryos develop with varying degrees of success. Some crosses arrest prior to gastrulation, while others form healthy feeding pluteus larvae (reviewed by Davidson, 1976). Loss of chromosomal material may occur as a result of incompatible homolog pairing and loss of paternal DNA was reported for some crosses. Alternatively, incorrect or lack of

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paternal gene expression may occur due to incompatible ooplasmic factors

necessary for proper regulation (reviewed by Davidson, 1976; Nisson *et al.*,

1989)

The species *Strongylocentrotus purpuratus* and *Lytechinus pictus*

diverged from a common ancestor 40-60 million years ago (Smith 1988). It has

been estimated using hybridization kinetics that the proportion of shared single

copy DNA between these two species is 12-22 % (Angerer *et al.*, 1976; Harpold

and Craig, 1978; Hall *et al.*, 1980) Although these species are clearly divergent,

under appropriate laboratory conditions, reciprocal interspecific crosses can be

generated which develop at least to the feeding pluteus larval stage. These

crosses provide a system for studying the interactions of two divergent genomes

within a single organism.

1.10.2 Restricted Expression of Paternal Genes

Two dimensional electrophoresis has been used to analyze the abundant

polypeptides that are synthesized in three crosses of sea urchin hybrids: These

crosses are *S. purpuratus* eggs fertilized with *L. pictus* sperm, and *S. purpuratus*

and *S. drobachiensis* reciprocally crossed. Paternally derived proteins are

underrepresented in these hybrid embryos when compared to the homotypic

embryos. This underrepresentation occurs with no apparent loss of paternal

DNA (Tufaro and Brandhorst, 1982). Hybridization studies revealed a reduced

prevalence of *L. pictus* paternal mRNAs, particularly those that are normally abundant.

In 1987, Conlon *et al.* investigated the transcriptional activity of the paternal genes in the reciprocal hybrid crosses between *L. pictus* and *S. purpuratus*. The previously described underrepresentation of paternal transcripts was again observed in SxL embryos. These *L. pictus* mRNAs are zygotic products, so their underrepresentation is not as a result of their being of maternal origin. In the reciprocal cross, several of these *L. pictus* transcripts were also reduced indicating that the activity of these genes does not depend on maternal specific factors. Nuclear run-on experiments used to assess the rate of transcription of a *L. pictus* specific metallothionein gene in these hybrids indicate that the *L. pictus* form of this mRNA is actively transcribed but does not accumulate to normal levels. These data suggest that the accumulation of *L. pictus* metallothionein transcripts, and possibly other gene products, are post-transcriptionally restricted in these hybrids. This restriction may be occurring at the level of RNA processing, export, or stabilization.

1.10.3 Histone Gene Expression

The expression of the histone genes in the cross between *S. purpuratus* eggs and *L. pictus* sperm was investigated (Maxson and Egrie, 1980). As early as the two cell stage, both maternal and paternal histone mRNAs are present at detectable levels. This mRNA is recruited into polysomes by the 16 cell stage

and is present in similar levels. These experiments show that in the case of the repetitive histone genes, there is no exclusion of expression of *L. pictus* genes observed in this cross. In addition, this result indicates that the zygotic (and paternal) genome is actively being transcribed by the first cleavage in sea urchin embryos.

1.10.4 Actin Gene Expression:

The expression of the *S. purpuratus* cytoplasmic actin genes CyI, CyIIIa, and the muscle actin gene, show the appropriate temporal and spatial patterns of expression in reciprocal hybrid embryos. This indicates that the *trans* regulatory factors necessary for the proper expression are present in both crosses of hybrid embryos (Bullock *et al.*, 1988; Nisson *et al.*, 1989). The recently identified *L. pictus* actins have not yet been carefully investigated to assess their expression in hybrids (Fang et al, 1994); but, preliminary results indicate that the *L. pictus* orthologue of CyIIIa actin is underexpressed in hybrid embryos (Fang and Davenport, unpublished results). When a CyIIIa actin promoter-reporter gene fusion is injected into *S. purpuratus* it shows the proper temporal and spatial specificity of expression. When this construct is injected into *L. variegatus* (a species closely related to *L. pictus*) its expression is aberrantly regulated spatially. This observation indicates that products of the *S. purpuratus* genome must be present for normal expression of the CyIIa gene, and that these products are produced in hybrid embryos.

1.10.5 Restricted Expression of the LpS1 Gene

The Spec1 and 2 genes from *S. purpuratus* and the *L. pictus* homologues LpS1 α and β are aboral ectoderm specific calcium binding proteins present late in embryogenesis (Klein *et al.*, 1987; Xiang *et al.*, 1991). Although their functions are unclear at this time, protein sequence similarity, expression patterns, and immunocrossreactivity between the proteins indicate that the Spec and LpS1 genes are homologues. Despite distinct differences between the promoters of these homologues, LpS1 and Spec2a promoters produce the correct temporal and spatial patterns of expression when injected into *L .pictus* embryos (Gan *et al.*, 1990). RNase protection assays performed on hybrid RNA show that while Spec1 mRNA accumulation is almost normal, the amount of LpS1 mRNA is drastically reduced, most notably at the pluteus stage (Brandhorst *et al.*, 1991). This reduction is not a result of loss or obvious rearrangement of the LpS1 gene. Nuclear run-on analyses of transcription indicate that the reduction in LpS1 mRNA is transcriptionally mediated. Serial sections hybridized *in situ* show that both genes are expressed exclusively in the aboral ectoderm (Nisson *et al.*, 1992). These data imply that regulatory factors required for the tissue specific accumulations of LpS1 and Spec1 mRNAs must be present in hybrids. LpS1 mRNA is present in only 2 % of all hybrid plutei (both LxS and SxL crosses), while Spec1 is detected in almost all hybrid plutei. In LxS embryos, approximately 1 % of all plutei show strictly LpS1 expression in

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the aboral ectoderm at normal levels. In another 1% of LxS embryos, patches of cells solely expressing LpS1 were interspersed with patches of cells expressing normal levels of Spec1 exclusively. These patches do not always correspond to the lineage of a single founder cell. They often represent less than an entire founder cell lineage, or cells from lineages of different founder cells (reviewed by Brandhorst and Klein, 1992). In SxL plutei, 2 % of the embryos express LpS1 exclusively in the aboral ectoderm while the majority only contain Spec1 mRNA in the aboral ectoderm. Interestingly, in SxL gastrula stage embryos, while 2 % of the embryos show exclusive expression of LpS1 and 50 % of the embryos express Spec1 in lieu if LpS1, the remaining hybrids were coexpressing both homologues in the same cells. This demonstrates the developmentally progressive restriction of LpS1 transcription in cells that express Spec1. LpS1 reduction is observed in both heterotypic crosses which suggests that this is not a maternal or paternal dominance. Perhaps this phenomenon occurs as a result of a dominance of the S. purpuratus genome over the L. pictus genome, and that the LpS1 homologue is inactivated . Another possible explanation is that LpS1 and Spec1 compete for a common transcription factor and that Spec1 has a higher affinity for this factor resulting in reduced LpS1 accumulation (Brandhorst et al., 1991). This does not explain why some cells only accumulate LpS1 transcripts unless in these rare cases the Spec1 gene is lost from or modified in a small number of embryos during development (Brandhorst et al.,

1991). This result prompts the question as to whether or not this phenomenon is observed for other pairs of homologous genes in these hybrid embryos.

1.10.6 msp130

An *S. purpuratus* 130 kD primary mesenchyme cell surface protein gene has been cloned and sequenced (Harkey *et al.*, 1985). This protein has been reported to be involved in the uptake of Ca^{2+} necessary for the deposition of $CaCO_3$ in sea urchin larval spicules (Leaf *et al.*, 1987). A monoclonal antibody, 1223, has been raised against this polypeptide and is seen to inhibit spicule elongation in cultured PMCs treated with this antiserum (reviewed by Decker and Lennarz, 1987). The developmental expression of this protein has been determined using this monoclonal antibody in both *S. purpuratus* and *L. pictus*. There is a markedly different expression pattern in each of these species. In *S. purpuratus*, the epitope is detectable in the egg and at low levels in all cells until the gastrula stage. At the time of skeletogenesis this epitope appears to be restricted of the PMCs. In contrast, *L. pictus* embryos do not have detectable levels of this epitope until the formation of PMCs, after which the antiserum only reacts significantly with the PMC surfaces. In the reciprocal hybrid crosses between these two species, the pattern of expression of this protein is indistinguishable from the maternal species (Decker and Lennarz, 1987).

1.11 Spicule Matrix Protein Genes LSM34 and SM50

The larval skeleton of the sea urchin *S. purpuratus* is composed of 20:1 $CaCO_3:MgCO_3$ and at least ten different matrix proteins (Benson *et al.*, 1986). The major component is the 47 kD SM50 protein that has been cloned, sequenced, and characterized by Sucov *et al.* (1987). An *L. pictus* skeletal protein, LSM34, has been subsequently cloned and sequenced and found to encode a 34 kD polypeptide (Livingston *et al.*, 1991). Both LSM34 and SM50 are secreted, basic and nonglycosylated, and show an overall similarity of approximately 74% at the amino acid level. These polypeptides are deemed homologous based upon immunocrossreactivity, amino acid sequence similarity, and identical expression patterns of their genes. In both cases, the mRNAs transcribed from these genes are accumulated to detectable levels around the time of PMC ingression, and are spatially restricted to the PMCs. Although both polypeptides have similar organizational properties, signal sequences, proline rich regions, and repeat regions; there is considerable divergence between the two species. The number of repeats in SM50 is 29 while there are only 14 in LSM34. The majority of the LSM34 repeats have a glutamine in the third position while the SM50 repeats have an arginine in this position. There are 10 prolines conserved between the two species, however each cDNA encodes additional prolines not observed in the other. The proline rich region and the repeats appear to be important to the tertiary structure of the polypeptides. The structure suggests that these proteins are elongated and have a β spiral domain

at the carboxy terminus. The basic nature of these proteins may be necessary for interactions with acidic components of the matrix. These data suggest that LSM34 and SM50 share a common ancestral gene comprised of fewer repeats; and, that subsequent divergence is responsible for the present dichotomy..

1.12 Thesis Objectives

This thesis has two main foci. First, a comparative morphological study of the embryological development of the sea urchin species *L. pictus*, *S. purpuratus* and their reciprocal hybrid crosses is reported. Second, the temporal and spatial expression patterns of the two spiculin homologues, LSM34 and SM50 in reciprocal interspecific hybrid crosses between *L. pictus* and *S. purpuratus* are assessed.

Chapter II

Materials and Methods

2.1 Sea Urchin Cultures

Sea urchin embryos were cultured for experimental purposes under the conditions outlined below. These conditions were selected because they conferred a high degree of normal and synchronous development. All centrifugation steps were performed quickly at room temperature in a Sorvall GLC-1 clinical centrifuge. Plasticware used for culture purposes was washed without detergent and rinsed in double distilled H_2O (ddH_2O). The quality of eggs and sperm obtained from individuals was assessed using light microscopy and a small test fertilization was performed for homospecific crosses.

2.1.1 *Lytechinus pictus* Cultures

Lytechinus pictus (*L. pictus*) individuals were obtained from Marinus, California and held year round at 12-14ºC. Gravid urchins are available year round when maintained under optimal conditions consisting of good water quality, minimal temperature fluctuations, and with regular cleaning and feeding. Sex was determined by gonopore size or by gentle electrostimulatation causing minimal gamete release. Large amounts of gametes were obtained via an intracoelomic injection of 0.10 ml of 0.55 M KCl. Eggs were collected in sea water which had been filtered through 45 μm Millipore nitrocellulose filters (MFSW) at 16ºC. Eggs were then washed through four-ply cheesecloth to remove debris. Immediately prior to fertilization, eggs were washed and settled 3-4 times in excess MFSW. Dry sperm was collected on ice using a wide bore

pipette to prevent damage. A 100 ml suspension of eggs was fertilized by adding 1.0 ml of 0.1 % sperm freshly diluted in MFSW. Fertilization typically occurred within 2 minutes following insemination, and the degree of fertilization was assessed by scoring for the presence of fertilization envelopes. Fertilized eggs were then washed, settled, and the less dense excess sperm removed. This step was repeated three times. Cultures were gently stirred at 60 RPM, at 16°C, at densities not exceeding 1000 embryos per ml. For improved appearance, later stage embryos used for morphological studies were cultured at densities of 50 to 100 embryos per ml. Upon hatching, swimming embryos were separated from unfertilized eggs by filtering through 44 μm Nitex mesh. Embryos were resuspended in fresh MFSW. Late stage cultures were supplemented with 50 μg/ml penicillin and 50 μg/ml streptomycin to prevent bacterial contamination.

2.1.2 *Strongylocentrotus purpuratus* Cultures

Strongylocentrotus purpuratus (S. purpuratus) individuals were collected intertidally on the west coast of Vancouver Island and were held at 6-10°C year round under conditions detailed above. Individuals were sexed by electrostimulation and gametes were obtained by a intracoelomic injection of 1.0-3.0 ml of 0.55 M KCl. Preparation of eggs, fertilization methods, and post-fertilization methods were the same as for *L. pictus* with the exception that all

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steps were carried out on ice. Cultures were incubated at 10-12°C under the conditions and densities described for *L. pictus*.

2.2 Hybrid Sea Urchin Embryo Cultures

Interspecific hybrid crosses between the species *L. pictus* and *S. purpuratus* were accomplished using various methods for disrupting species specific barriers. The success of fertilization and normal development varied among batches of eggs, but conditions were optimized in order to maximize fertilization and ensure healthy synchronous development.

2.2.1 *L. pictus* x *S. purpuratus* (LxS) Cultures

Due to the relatively small numbers of eggs that can be obtained from a single *L. pictus* female, eggs from 3-5 individuals were combined and prepared as described for *L. pictus* cultures. Settled eggs were transferred to a 50 ml conical tube and the species specific sperm receptors located on the egg vitelline layer were digested in 250 µg/ml trypsin. A stock of trypsin, stored frozen at - 20°C was reserved for these purposes to reduce variability due to differences in enzyme activity between batches. Treated eggs were incubated at 16°C for 5 minutes, diluted two-fold in MFSW and centrifuged at 1,000 RPM to remove trypsin. This wash step was repeated three times. Eggs were fertilized by adding 100 µl dry *S. purpuratus* sperm to a 50 ml egg suspension and incubating for 30 minutes at 14 -16°C on a rocker. Excess sperm was removed

as detailed for *L. pictus* cultures. The degree of fertilization was apparent at first cleavage. Embryos were cultured at 14-16°C under conditions and densities described for *L. pictus* cultures.

2.2.2 *S. purpuratus* x *L. pictus* (SxL) Cultures

S. purpuratus eggs were collected and prepared as described for *S. purpuratus* cultures. Settled eggs were resuspended in 100 ml MFSW and digested in 125 µg/ml trypsin for 30 seconds. Treated eggs were diluted two-fold and centrifuged at approximately 500 RPM for 2-5 minutes. Eggs were washed with MFSW three times to remove trypsin and transferred to a 50 ml Falcon tube. Eggs were fertilized by adding 100 µl of dry *L. pictus* sperm and incubating on ice for 30 minutes with occasional mixing. Washes and culture conditions were as described for *S. purpuratus* cultures.

Under these conditions, the hybrid crosses develop at a rate similar to the maternal homotypic cross until gastrulation. In some cultures, hybrid embryos tend to have slightly delayed gastrulation. Temperature and culture densities have been observed to impact the rate of development significantly.

2.3 Transformation of Bacteria with Plasmid DNA

All plasmids in this work are characterized by ampicillan resistant selection. Competent DH5∝ cells were prepared and transformed with 25-50 ng of recombinant plasmid DNA as detailed in the protocol by Sambrooke *et al*

(1989). Single colonies were selected, streaked on agar-LB (Luria-Bertani

Media) plates containing 100 µg/ml ampicillin for selection. Liquid media (also

containing 100 µg/ml ampicillin) was inoculated with single colonies and

bacterial cultures grown for 14-18 hours in a 37°C shaking incubator.

2.4 Isolation of Plasmid DNA

Plasmid DNA was purified from bacterial cells using either the alkaline

lysis method (Sambrooke *et al*. 1989) or by using the Magic™ Minipreps DNA

purification system (Promega).

2.5 Restriction Enzyme Digests

Restriction enzymes purchased from either BRL, Pharmacia or from New

England Biolabs were used in accordance with the manufacturers' specifications

to digest the DNA. Typical digestions were carried out at 37°C for 2 hours with

an enzyme concentration of 1-3 units per µg of recombinant plasmid.

2.6 Agarose Gel Electrophoresis

DNA was separated according to size using electrophoresis in 0.7-1.0 %

(w/v) agarose gel in 1 X TAE (40 mM Tris acetate: 10 mM

ethylenediaminetetraacetic acid (EDTA)) containing 1 µg/ml ethidium bromide.

Gels were run at 1-5 V/cm and visualized using a 300 nm U.V. transilluminator.

Photographs were taken using either Kodak Plus X pan film or by the UVP Image Store 5000 Video documentation system.

2.7 Preparation of Purified Insert DNA

In order to label DNA for use as hybridization probes, insert DNA was first isolated from plasmid DNA by one of the methods outlined below. In all cases, digested DNA was electrophoresed on 0.7 % (w/v) agarose in either 1 X TAE or 1 X TBE (90 mM Tris Borate: 10 mM EDTA) containing 1 µg/ml ethidium bromide to separate insert from vector. Insert bands were cut from gel using a 360 nm U.V. transilluminator to prevent nicking of the DNA. The DNA was eluted from gel by one of the three methods detailed below.

2.7.1 Method 1

Low melting point gel slices were warmed to 55-60°C and extracted once with TE (10 mM TrisHCl pH 8.0; 1 mM EDTA) saturated phenol; once with 50 % TE saturated phenol; 50 % 24:1 chloroform:isoamyl alcohol; and once with 24:1 chloroform:isoamyl alcohol. DNA was then precipitated in 0.3 M sodium acetate, pH 5.5, and absolute ethanol and recovered by centrifugation.

2.7.2 Method 2

Insert DNA was purified from low melting point agarose using the Promega Magic™ PCR Preps DNA purification system as detailed in the

instruction manual provided. Briefly, the gel slice was melted, combined with 1 ml of the purification resin, and vortexed 20 seconds. Samples were then passed through the column provided and the column was washed once in 80 % (v/v) isopropanol. The DNA sample was eluted by adding 50 µl 1 x TE, pH 8.0, to the column and collected by centrifugation.

2.7.3 Method 3

The agarose was removed from DNA sample by loading the gel slice onto a pipette containing a silanized glass wool plug and centrifuging for 30 minutes at 6,000 RPM (Hermle Z 320 K centrifuge). This DNA preparation could be used directly for oligolabelling reactions or concentrated by ethanol precipitation.

2.8 RNA Isolations

To destroy ribonuclease contamination, all solutions used for RNA work were pretreated with 0.1 % (v/v) diethylpyrocarbonate (DEPC) for a minimum of 3 hours prior to autoclaving. In order to further reduce possible RNA degradation by RNase contamination, disposable plastic tubes and pipettes were used. Total RNA was isolated from sea urchin embryos using the method of Chomczynski and Sacchi (1987) as outlined below. Some modifications were made in order to adapt the protocol for sea urchin embryos.

Embryos were collected by centrifugation in 50 ml conical tubes at 1,000 RPM (Sorvall GLC-1 clinical centrifuge) for 2-5 minutes and washed once in ice

cold Calcium Magnesium Free Sea Water [CMFSW (31g/L NaCl; 0.8 g/L KCl;

0.2 g/L NaHCO$_3$; 1.6 g/L Na$_2$SO$_4$)]. Sea water was decanted and pellets were

placed immediately on ice. 1.0 ml of homogenization buffer [(4 M guanidinium

thiocyanate: 25 mM sodium citrate : 0.5% sarcosyl) containing 0.36 ml

ß-mercaptoethanol per 50 ml] was added per 100 mg tissue. Samples were

homogenized in a Sorvall Omnimixer for 60-90 seconds at maximum speed and

the homogenate was transferred to a 50 ml polypropylene tube. The following

solutions were added in order: 0.1 volume of ice cold 2 M sodium acetate, pH

4.0, 1.0 volume of water saturated phenol; and 0.2 ml of 24:1 chloroform:isoamyl

alcohol. The sample was mixed gently after the addition of each solution. After

the addition of chloroform, the sample was shaken for 10 seconds and incubated

on ice for 15-30 minutes. Samples were transferred to 6 ml polypropylene tubes

and centrifuged at 10,000 RPM in a Beckman JA-20 rotor for 20 minutes at 4°C

using 15 ml Corex tubes as carriers. The acidic conditions of the extraction

segregated the sheared genomic DNA to the interphase and great care was

taken to prevent contamination. The upper aqueous layer was transferred to a

fresh tube and the RNA precipitated in an equal volume of isopropanol for one

hour at - 80°C. RNA was pelleted in a JA-20 rotor at 10,000 RPM for 20 minutes

at 4°C. Supernatant was discarded and air dried pellets were redissolved in 250

µl homogenization buffer per 100 mg original tissue. Samples were transferred

to 1.5 ml microfuge tubes and the RNA precipitated in 2 volumes of absolute

ethanol overnight at - 20°C. RNA was collected by centrifugation at 14,000 RPM

for 15 minutes at 4°C (Hermle Z 320 K Desktop microfuge), pellets were washed in 70 % ethanol, and dried briefly in a vacuum spin drier. Pellets were dissolved in 250 μl of DEPC-treated ddH$_2$O per 100 mg original tissue and precipitated with 0.5 M LiCl and 2 volumes ice cold ethanol for 30 minutes at - 80°C. RNA was collected by centrifugation, washed in 70 % ethanol , dried briefly in a vacuum spin drier, and resuspended in 25-50 μl DEPC-treated ddH$_2$O. Concentration and purity of samples was assessed via spectrophotometric analysis at 260nm and 280 nm. OD$_{260/280}$ ratios ranged from 1.9 to 2.1 indicating minimal protein contamination.

2.9 Fractionation of RNA by Denaturing Gel Electrophoresis

5.0 μg samples of RNA were dried in a vacuum spin drier, resuspended in 50 % (v/v) formamide, 2.0 % (v/v) formaldehyde, 1 X MOPS buffer (v/v), and heat-denatured at 55 - 60°C for 15 minutes. RNA samples were fractionated on 0.7-1.3 % agarose containing 2 % (v/v) deionized formaldehyde (pH > 6.0) in 1 X MOPs buffer at 3-4 V/cm. Buffer was circulated between the two chambers with a peristaltic pump to prevent the development of a pH gradient.

2.10 RNA Gel Blot Transfers

Gels were photographed, then washed three times in excess ddH$_2$O for 10 minutes. RNA was transferred from the gel onto Genescreen™ via the unidirectional capillary method of Southern (1975) as described by Sambrooke

et al. (1989) using 25 mM phosphate buffer, pH 6.8, as a transfer buffer. Transfers were normally allowed to proceed for 14-18 hours. The RNA was fixed onto the membrane via UV crosslinking in a on autocrosslink, and baked in an 80°C vacuum oven for a minimum of 2 hours to liberate any residual formaldehyde.

2.11 Preparation of ^{32}P-labeled DNA Probes

Isolated insert DNA was labeled by random priming method using the NEBlot kit (New England Biolabs) according to the instructions provided. 25-50 ng of template DNA was dissolved in 33 µl nuclease free ddH$_2$O and heat denatured (100°C) for 5 minutes, then quick cooled on ice. The sample was spun for 10 seconds in a microfuge and combined with 5 µl 10 x labeling mix containing random octadexoyribonucletides, 6 µl dNTP mix, 5 µL $\propto$-^{32}P-dCTP (3,000 Ci/mmol), and 5 units of DNA polymerase Klenow fragment. The labeling reaction was allowed to proceed for 60 minutes at 37°C, and unincorporated nucleotides were removed by Sephadex G-50 column chromatography.

2.12 Northern Blot Hybridizations

Membranes were prehybridized for a minimum of 4 hours in 5 X SSPE [20 X SSPE: 3 m NaCl, 0.2 M NaH$_2$PO$_4$, 0.02 M EDTA Na$_2$, pH 7.4], 5 X Denhardt's [0.1 % (w/v) bovine serum albumin (Sigma, Fraction V), 0.1 % (w/v) Ficoll 400 (Pharmacia), 0.1 % (w/v) polyvinyl pyrrolidone], and 0.3 % (v/v) sodium doedecyl

sulfate (SDS). Denatured ^{32}P labeled probe(s) were added to fresh hybridization solution (1,000,000 cpm/ml) and hybridizations were carried out at 65°C for 14-18 hours. To remove excess non-specifically bound probe, two or three post hybridization washes were carried out using an excess of 1 X SSPE; 0.4 % SDS for 20 minutes each at 65°C. Autoradiographs were produced using either Kodak X-OMAT AR film or Kodak X-OMAT XK-1 film at - 80°C with intensifying screens.

2.13 Whole Mount *In situ* Hybridizations

Whole mount *in situ* hybridizations using digoxygenin labeled riboprobes were performed as outlined in the protocols developed for *Drosophila* (Tautz and Pfeifle,1989) and *Xenopus* embryos (Hemmati-Brivanlou et al. ,1990) and adapted for sea urchins by Harkey and Whiteley (1992) and LePage and Gache (1992). Other modifications for specific probes and for interspecific hybrid embryos were worked out by myself in collaboration with Peter Andolfatto and are described below.

2.13.1 Synthesis of Digoxygenin Labeled Riboprobes

Freshly prepared recombinant plasmid DNA was digested with a restriction enzyme that cut at a site downstream from the insert in the polylinker. A 5' or 3' overhang was preferable because the efficiency of RNA synthesis is improved and reverse priming is eliminated. Linearized plasmids were

fractionated on an agarose gel to test for completion of digestion. Protein was removed from samples by a single phenol extraction, followed by a 25:24:1 phenol:chloroform:isoamyl alcohol extraction and a 24:1 chloroform:isoamyl alcohol extraction. Transcription templates were precipitated in ethanol and recovered by centrifugation. Vacuum dried pellets were resuspended in sterile ddH2O and the concentrations determined by viewing relative intensities of ethidium bromide stained bands on an agarose gel.

Transcription reactions were set up by sequentially adding the following to a microfuge tube on ice: 1 μg linearized template DNA in 4μl nuclease free ddH$_2$O; 2 μl each of 10mM solutions of ATP, GTP, CTP; 1.3 μl 10 mM UTP; 0.7 μl 1nM digoxygenin-11-UTP; 4 μl 5 X transcription buffer; 2 μl 0.1 M dithiothreitol (DTT); 40 units RNase inhibitor, and 50 units either T7, T3, or SP6 RNA polymerase. Reactions were incubated at 37ºC for T7/T3 and 40ºC for SP6 for 2-4 hours. Template DNA was removed by a digestion with 1 unit of RQ DNase I for 15 minutes at 37ºC. Unincorporated ribonucleotides were removed by Sephadex G-50 column chromatography and probe concentrations were determined by dot blot procedures as described for the Genius™ system (Boehringer-Mannheim).

2.13.2 Fixation and Storage of Embryos

Sea urchin embryos were collected on 44 micron Nitex to concentrate and washed with MFSW to remove debris. Samples were fixed in 3-5 volumes

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phosphate buffered saline (PBS) containing 2.5 % (v/v) glutaraldehyde for two hours on ice, followed by two 15 minute washes in 10-15 volumes PBS. Embryos were gradually dehydrated in 10 % , 35 %, 50 %, and 70 % ethanol. Fixed samples were transferred to - 20°C where they could be stored in good condition indefinitely.

2.13.3 Dehydration, Delipidation, Rehydration, Proteinase K Digestion, and Postfixation

To prepare for hybridization, embryos were dehydrated in single washes of 80%, 90%, and 2 x 100% ethanol. Subsequent incubation in 100% toluene for 15 - 20 minutes at room temperature removed lipids and improved accessibility to the hybridization probe. Samples were rehydrated by washes in 100%, 90%, 80%, 70 %, 50%, 35%, 10% ethanol, and 2 x in PBST (0.2 M phosphate buffer, pH 7.4; 0.15 M NaCl; and 0.1 % Tween20). Embryos were then digested for varying lengths of time and concentrations of proteinase K, dependent on species of embryo and the developmental stage. Briefly, S. purpuratus embryos were digested in 5 µg/ml proteinase K for 5 - 10 minutes (10 minutes for gastrulae and 5 minutes for plutei), L. pictus embryos 40 - 80 µg/ml proteinase K for 10 minutes (80 µg/ml for gastrulae and 40 µg/ml for plutei), LxS embryos in 20 - 40 µg/ml for 8 - 10 minutes (40 µg/ml for gastrulae and 20 µg/ml for plutei), and SxL embryos in 5 µg/ml for 2-5 minutes (5 minutes for gastrulae and 2 minutes for plutei). Afterwards, embryos were washed twice in PBST and

postfixed in 4 % paraformaldehyde in 50 % PBST for 30 minutes at room temperature.

2.13.4 Hybridization

After fixation, embryos were washed 5 times in excess PBST. Hybridization solution (50% formamide; 5 x SSC; 500 µg/ml yeast tRNA, 50 µg/ml heparin, 0.1% Tween20) was introduced gradually to avoid damage and embryos were prehybridized in 100% hybridization solution for 60-90 minutes at 52°C for *S. purpuratus* and SxL individuals, and at 55°C for *L. pictus* and LxS samples. Initially, probes that were greater than 500 bp were reduced in size by alkaline hydrolysis in 40 mM sodium bicarbonate; 60 mM sodium carbonate for 20-40 minutes depending on initial length. Full length probes of less than 1.5 kb were also successfully used in the absence of hydrolysis with no apparent detriment. Hybridizations were carried out at appropriate temperatures overnight; however, in some cases hybridizations of as little as four hours performed well and reduced damage to embryos.

2.13.5 Posthybridization Washes

Following hybridization the embryos were washed for 10 minutes each in 100% hybridization solution, 50% hybridization solution: 50% PBST, and then twice in PBST all at hybridization temperature. Embryos were then washed once in 1 x SSC, 0.1% Tween20 and three times in 0.1 x SSC:0.1% Tween20 at

hybridization temperate for 20 minutes each. Excess, non specifically bound single stranded probe was removed by a 30 minute digestion in PBST containing 20 µg/ml RNase A and 100 units/ml RNase T1 at 37°C, followed by 2 washes in PBST. This RNase digestion eliminated spurious binding of probe to pigment cells.

2.13.6 Detection

Embryos were incubated in blocking solution (PBST containing 2% normal sheep serum) for 30 minutes at room temperature. Subsequently, they were suspended in blocking solution containing a 1:1000 dilution of anti-digoxygenin Fab fragments conjugated to alkaline phosphatase (Boehringer-Mannheim Biochemicals) and incubated at room temperature for 1 hour. Samples were washed twice in PBST, twice in 100 mM Tris(pH 9.5); 100 mM NaCl; 0.1% Tween20, and twice in alkaline phosphatase buffer (100 mM Tris (pH 9.5); 100 mM NaCl; 50 mM $MgCl_2$; 1 mM levamisole; 0.1% Tween20). Embryos were then resuspended in alkaline phosphatase buffer containing 3.5 µl/ml BCIP (50 mg/ml 5-Bromo-4-chloro-3-indolyl phosphate in 100% dimethylformamide (DMF)) and 3.375 µl/ml NBT (100 mg/ml Nitro blue tetrazolium salt in 70% DMF) and incubated in the dark until staining was complete. Staining began after 30-60 minutes and usually took 2-4 hours to complete. Stained embryos were washed twice in 100 mM Tris (pH 9.5) : 100 mM NaCl: 0.1% Tween20, three times in PBST, once in 25% glycerol in PBST,

and stored in 50% glycerol in PBST at 4°C indefinitely. Photographs were taken using an Olympus AHBS3 microscope using either Nomarski or bright field optics. Kodak 160 ISO tungsten film was used.

2.14 Immunocytochemical Staining

Primary mesenchyme cells (PMC) were visualized by the use of the *Lytechinus variegatus* PMC specific monoclonal antibody, Ig8, kindly provided by D. McClay (McClay et al. 1983). This antisera crossreacts with the *L. pictus* and *S. purpuratus* PMC surface antigen; however, there is some minor nonspecific background staining in *S. purpuratus* embryos. *L. pictus* and *S. purpuratus* embryos and their reciprocal interspecifc hybrids were immunostained and PMCs were visualized using epi-fluorescence microscopy. Flattening the embryos aided in the resolution of the PMCs, and images were recorded using Northern Exposure digital image software.

2.14.1 Fixation and Storage of Embryos

Embryos were collected at the early gastrula stage on 44 micron Nitex and fixed in 4 % (w/v) paraformadehyde dissolved in MFSW for 1 hour at room temperature. Fixed samples were washed three times in excess MFSW and stored at 4°C. Embryos could be kept for several months when supplemented with 2-5 mM sodium azide to prevent growth of bacteria.

2.14.2 Immunocytochemistry

Fixed embryos were washed three times in phosphate buffered saline and made permeable to the antibodies via a 15-30 minute treatment in PBS containing 0.1% (v/v) Triton X100 at room temperature. Embryos were then blocked in PBS containing 0.1% Triton X100 and 2% (v/v) bovine serum albumin (or fetal calf serum) for 30 minutes at room temperature, followed by three washes in PBS containing Triton X100. Primary antibody (Ig8) was used full strength and embryos were incubated at 4°C overnight. 12-18 hours later, the embryos were washed three times in PBS then blocked for 30 minutes in PBS containing 2% (v/v) normal rabbit serum. After three washes in PBS, rabbit anti-mouse-FITC was added at dilutions ranging from 1:100 to 1:500 and incubated for 2 hours at room temperature or overnight at 4°C. Embryos were then washed 3-4 times in PBS and mounted for viewing.

2.15 Preparation and Visualization of Larval Skeletons

L. pictus, S. purpuratus, and their reciprocal hybrid larvae were gently concentrated using 44 micron Nitex and were digested overnight in 10% Clorox in MFSW. Preparations were rinsed in MFSW and viewed using polarizing light microscopy. Northern exposure image capture software was used to document data viewed through an Olympus AHBS3 microscope.

Chapter III

Results

3.1 Optimization of Interspecies Hybrid Crosses

The research undertaken for this thesis relied upon generation of sufficient numbers of reciprocal interspecific hybrid embryos between the species *Lytechinus pictus* and *Strongylocentrotus purpuratus*. In order to achieve this, species specific barriers that are in place to prevent cross fertilization must be overcome. Previous protocols fell short in their ability to consistently yield a high rate of fertilization required for these studies. Conditions were optimized to maximize interspecies fertilization rates and improve their later development.

For best results, eggs collected should be washed thoroughly to remove coelomic fluid and fertilized within one hour of shedding. Older batches tend to have poorer fertilization rates and eggs not thoroughly washed have impeded fertilization that is corrected with more extensive washes. In addition, animals freshly collected generally contain better quality gametes than animals held in closed seawater facilities for extended periods. Specific conditions for gamete collection, fertilization, and culture growth are detailed in Materials and Methods

3.1.1 *Lytechinus pictus* ♀ x *Strongylocentrotus purpuratus* ♂ Crosses

The small size of *L. pictus* individuals limits the number of gametes that can be obtained from a single spawning. For this reason high rates of fertilization of *L pictus* eggs are desirable. All manipulations are performed in 14-16°C MFSW. To prepare eggs of this species for cross fertilization by

S.purpuratus sperm, they are pretreated with MFSW containing 25 μg/ml trypsin for 5 minutes with gentle mixing. It should be noted that batches of trypsin vary in activity and therefore must be optimized prior to use. Overdigestion of *L. pictus* eggs results in aggregation of the eggs and abnormal development. After digestion, the egg suspension is immediately diluted 2-fold and gently centrifuged to collect eggs. The supernatant is aspirated off and eggs are washed three times with MFSW. Fertilization of *L. pictus* eggs with *S.purpuratus* sperm without prior trypsin treatment has a very low frequency of success, even at very high concentrations of sperm.

3.1.2 *Strongylocentrotus purpuratus ♀ x Lytechinus pictus ♂* Crosses

Eggs of *S.purpuratus* can sometimes be efficiently fertilized in a large excess of *L. pictus* sperm. However, this method was found to be largely unreliable and was avoided during the course of this study. *S.purpuratus* eggs are much more sensitive to protease treatment than *L. pictus* eggs. Overdigested eggs tend to aggregate resulting in aberrant development. Therefore, both the concentration and time of protease digestion have been adjusted from previously used protocols. In short, eggs are digested in 100 ml of ice cold MFSW containing 12.5 μg/ml trypsin. The suspension is swirled for 30 seconds, diluted 2 fold, and gently collected by centrifugation 3 times through ice cold MFSW.

3.2 Comparative Morphology of *L. pictus*, *S.purpuratus*, and their reciprocal interspecific embryos.

3.2.1 Mesenchyme Blastulae

Figure 2, on page 51, shows the mesenchyme blastulae of *L. pictus*, *S.purpuratus*, and reciprocal interspecies hybrids. In all cases the primary mesenchyme cells are ingressing into the blastocoel at the vegetal pole. The epithelial layer surrounding the blastocoel appears thinner in the larger *L. pictus* embryos than in *S.purpuratus*, however statistical data show no significant differences. In both heterospecific crosses, the size and overall morphology of the hybrid blastulae closely resemble the maternal species.

Table 1:

Ectoderm thickness in *L. pictus*, *S. purpuratus*, LxS, and SxL Blastulae

Cross	Mean Ectoderm Thickness (microns)	One Standard Deviation
L. pictus	12.3	1.2
S. purpuratus	13.2	1.7
LxS	13.8	1.8
SxL	11.9	1.5

n=15

Figure 2:

Mesenchyme Blastula Stage Embryos

[A] *L. pictus* mesenchyme blastula magnified 365 times [B] *S. purpuratus*

mesenchyme blastula magnified 300 times [C] *L. pictus* (♀) x *S. purpuratus* (♂)

mesenchyme blastula magnified 355 times [D] *S. purpuratus* (♀) x *L. pictus* (♂)

mesenchyme blastula magnified 360 times.

50 microns

51b
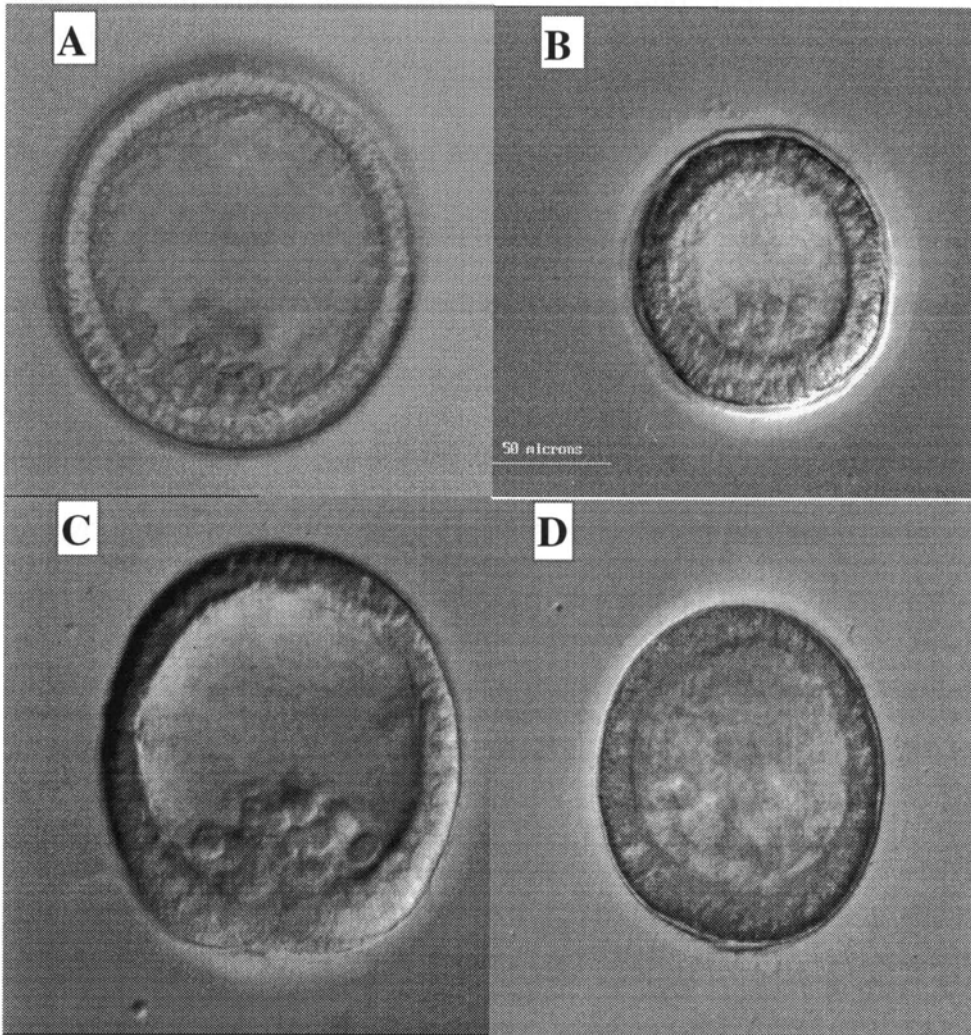
3.2.2 Gastrulation

Figure 3 shows early gastrulae of *L. pictus, S. purpuratus*, and reciprocal hybrids. The size and overall morphology of the hybrid embryos is again maternal. Figure 4 shows embryos in which the process of gastrulation is nearing completion. The *L .pictus* archenteron extends across the blastocoel along the animal vegetal axis in a radially symmetrical fashion, while in the *S. purpuratus* embryo the extending archenteron bends toward the oral side, establishing the plane of bilateral symmetry (right-left axis). In *L. pictus* embryos, the target site for attachment of the archenteron (the stomadeum) is closer to the animal pole than in *S. purpuratus*, and an obvious oral-aboral axis and bilateral symmetry appear chronologically later in this species. Both LxS and SxL hybrid embryos show an early extension of the archenteron toward the oral side, suggesting a possible dominance of the *S. purpuratus* condition for the control of these processes.

3.2.3 Pluteus Larvae

Figure 5 shows the pluteus larvae of *L. pictus, S. purpuratus*, and reciprocal hybrids. The overall morphology of the hybrid plutei is quite similar to those of the maternal species. The *L. pictus* and LxS plutei have characteristically longer arms than the *S. purpuratus* and SxL plutei of a similar stage. Both heterospecific crosses result in the formation of relatively healthy larvae which swim actively and are able to feed. However, to the best of our

Figure 3:

Early Gastrula Stage Embryos

[A] *L. pictus* early gastrula magnified 320 times **[B]** *S. purpuratus*

early gastrula magnified 315 times **[C]** *L. pictus* (♀) x *S. purpuratus* (♂) early

gastrula magnified 320 times **[D]** *S. purpuratus* (♀) x *L. pictus* (♂) early gastrula

magnified 315 times.
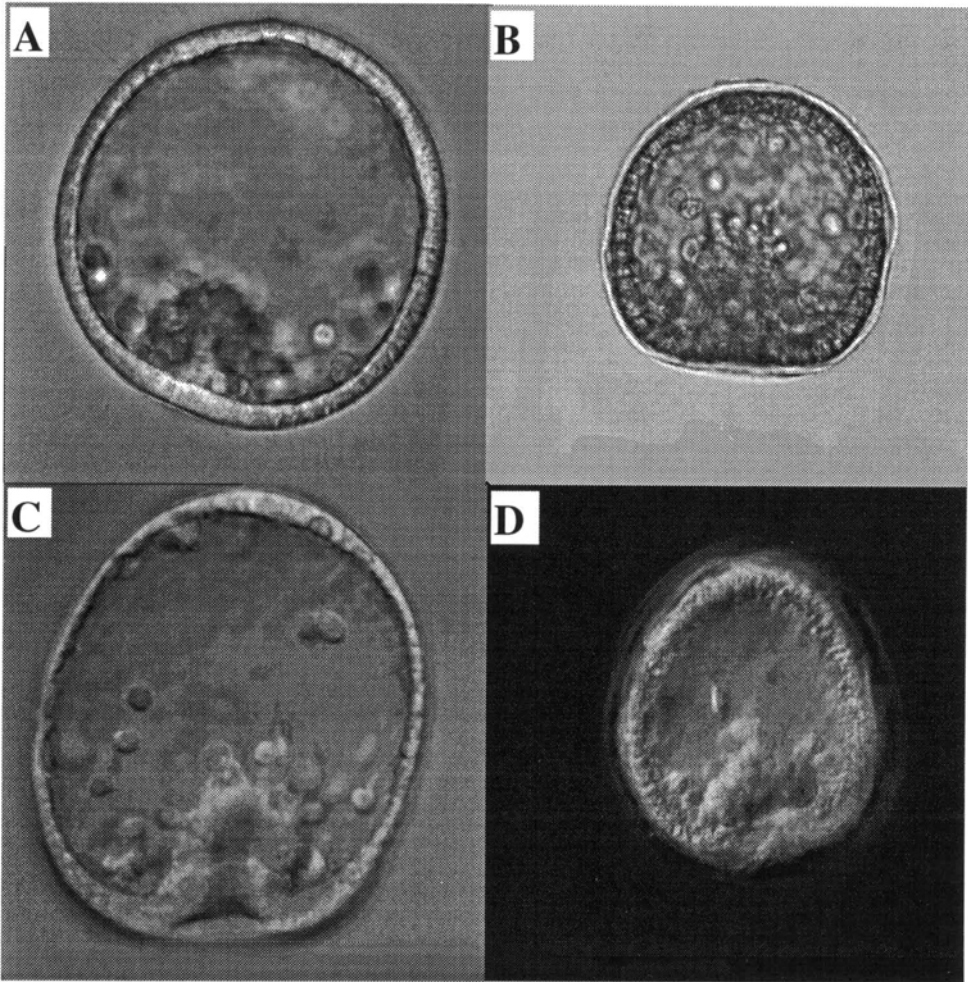
Figure 4:

Late Gastrula Stage Embryos

[A] *L. pictus* late gastrula magnified 315 times **[B]** *S. purpuratus*

late gastrula magnified 325 times **[C]** *L. pictus* (♀) x *S. purpuratus* (♂) late

gastrula magnified 315 times **[D]** *S. purpuratus* (♀) x *L. pictus* (♂) late gastrula

magnified 340 times.
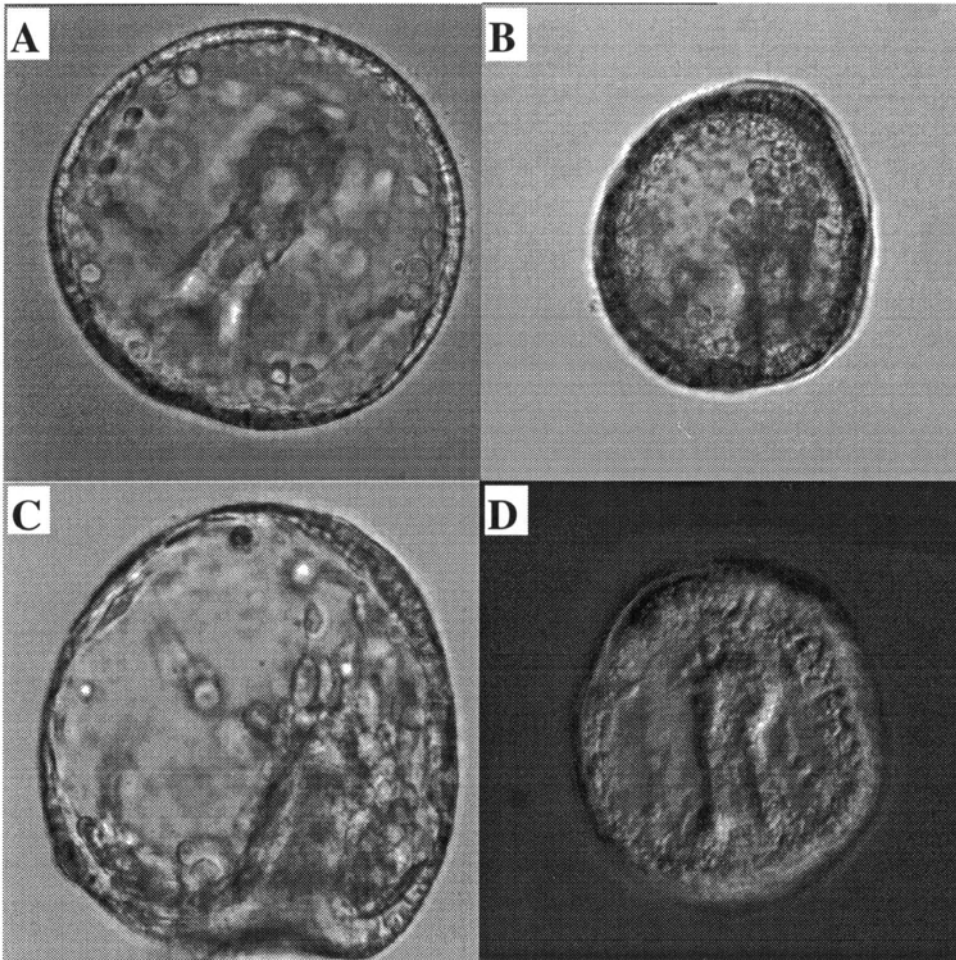
Figure 5:

Early Pluteus Larvae

[A] *L. pictus* pluteus larva magnified 130 times **[B]** *S. purpuratus*

pluteus larva magnified 125 times **[C]** *L. pictus* (♀) x *S. purpuratus* (♂)pluteus

larva magnified 130 times **[D]** *S. purpuratus* (♀) x *L. pictus* (♂) pluteus larva

magnified 140 times.
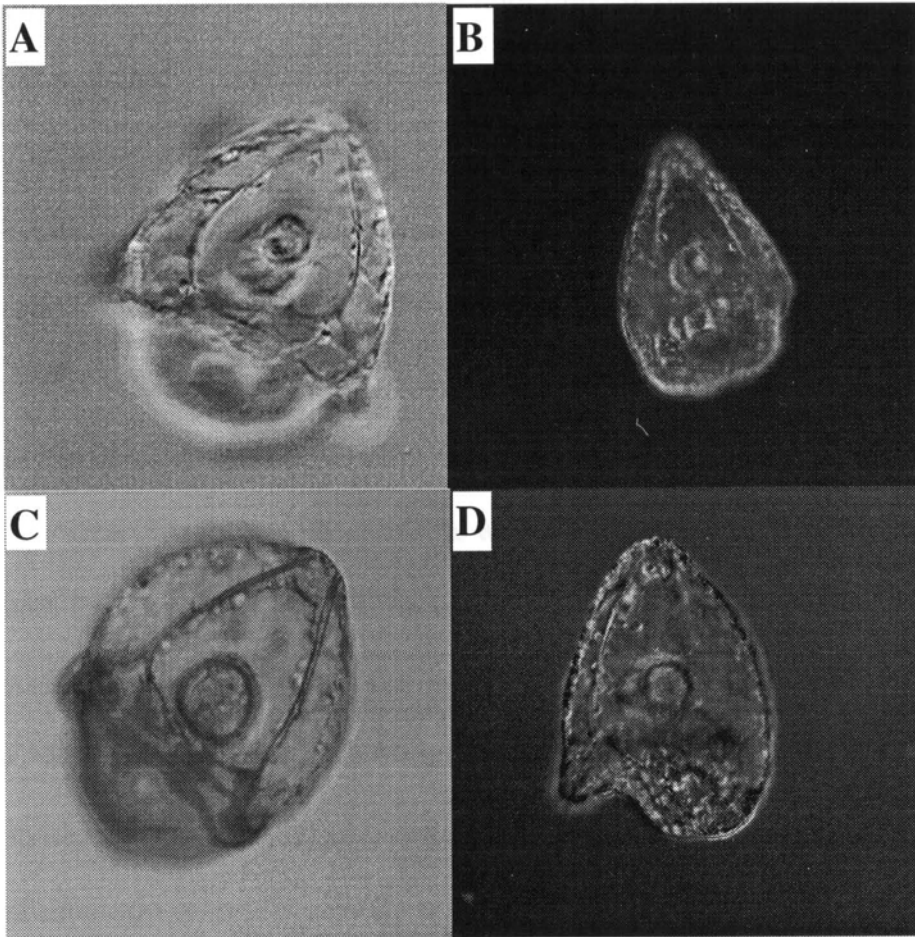
knowledge, none of these hybrid embryos have been reared through metamorphosis.

3.3 Determination Of Primary Mesenchyme Cell Numbers

The number and timing of ingressing primary mesenchyme cells differs among sea urchin species. These characteristics have been examined in a number of hybrid crosses revealing a maternal control. We thus examined this PMC ingression in *L. pictus* and *S. purpuratus* and hybrid embryos. Embryos were stained with the monoclonal antibody Ig8 (McClay et al. 1983) and viewed by indirect immunofluorescence microscopy. This antiserum was raised against *L. variegatus* PMC antigen and is thought to recognize the carbohydrate moiety of a cell surface glycoprotein and reacts with PMCs immediately after ingression but not with PMCs that are still in the vegetal plate. Mab Ig8 crossreacts with a similar surface molecule present on PMCs of *S. purpuratus* , but with reduced intensity. The images in Figures 6 and 7 show the results of the immunocytochemical staining of *L pictus, S. purpuratus*, LxS and SxL early to mid-gastrula stage embryos. PMCs are seen to be organized into a ring of 16 cells in both homotypic species and in the LxS cross (see Figures 6A and C, and 7A on pages 57 and 58 respectively). These data suggest 16 PMCs ingress; this observation contrasts with previous reports (reviewed in Davidson, 1986). This ring of syncytial cells migrates toward the equator and increases to 64 cell bodies. Two subsequent cleavages presumably result in the maximum of 64

Figure 6:

Visualization of Primary Mesenchyme Cells Stained with Mab Ig8 by Indirect Immunofluorescence

[A] and [B] *L. pictus* early gastrula stage preparations; [C] and [D] *S. purpuratus* early gastrula stage preparations. [A] magnified 285 times [B] magnified 345 times [C] magnified 305 times [D] magnified 320 times.
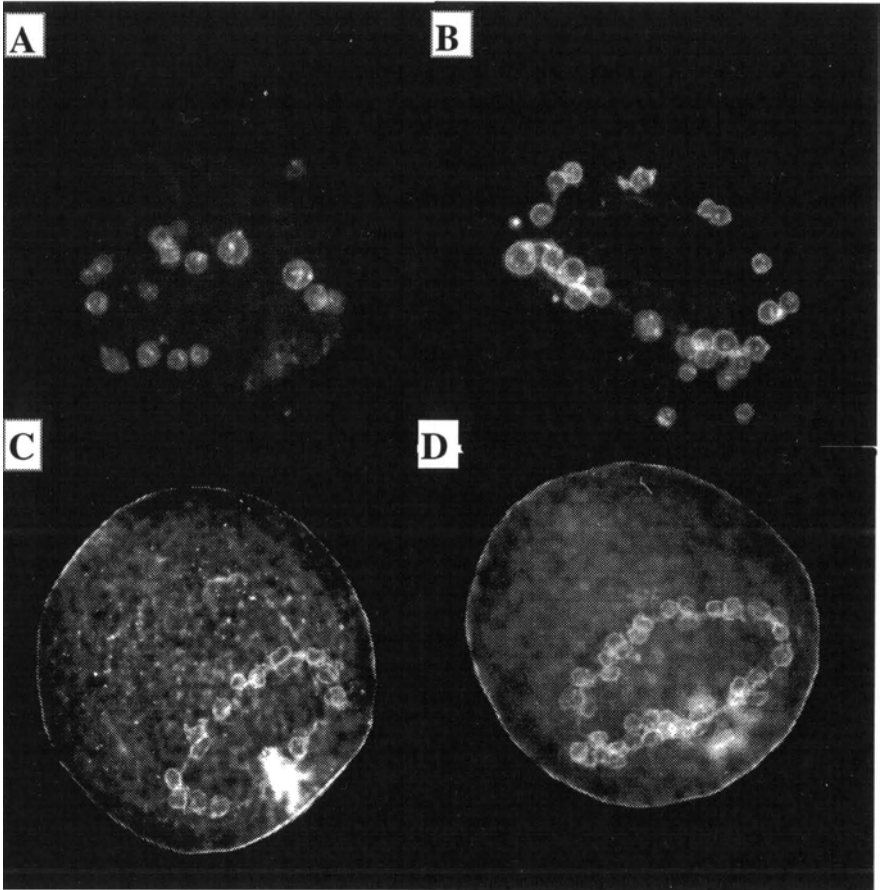
Figure 7:

Visualization of Primary Mesenchyme Cells Stained with Mab Ig8 by Indirect Immunofluorescence

[A] and [B] *L. pictus* (♀) x *S. purpuratus* (♂) early gastrula stage preparations;

[C] and [D] *S. purpuratus* (♀) x *L. pictus* (♂)early gastrula stage preparations.

[A] magnified 315 times [B] magnified 300 times [C] magnified 305 times [D] magnified 290 times.

58b
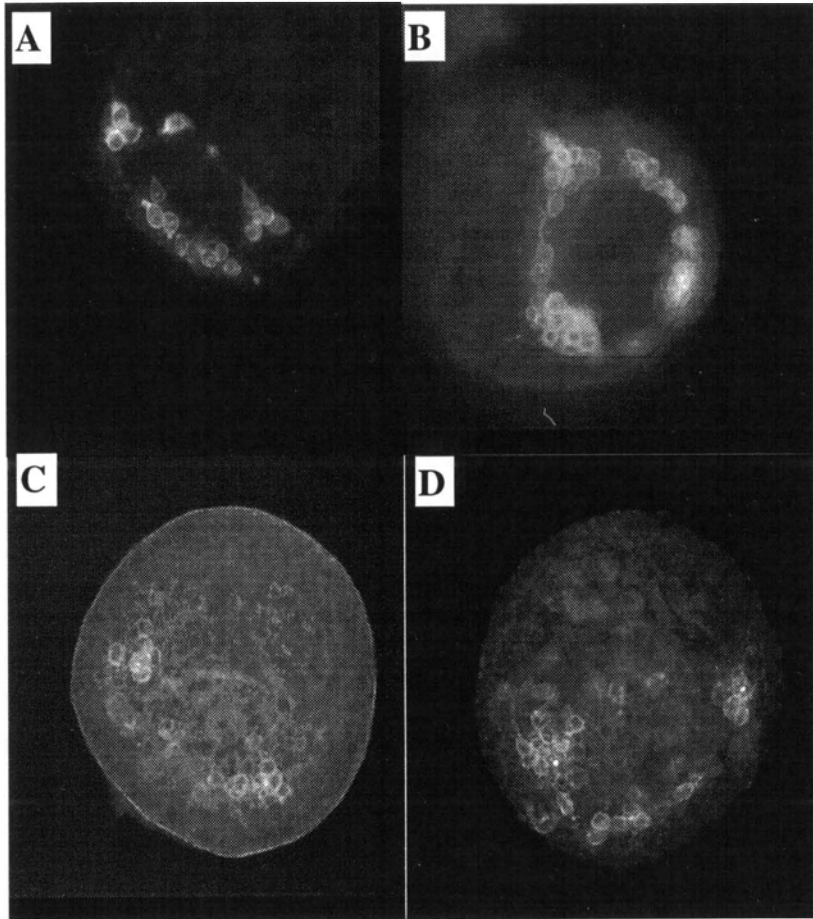
PMCs observed and in agreement with lineage information reported by Cameron and Davidson, (1987) for *S. purpuratus*. Cell counts show no significant differences in PMC number between *L. pictus* and *S. purpuratus*. Both homotypic embryos have an average maximum around 64 PMCs. In LxS and SxL embryos immunocytochemically stained with Ig8, similar numbers were observed. Many embryos of all four crosses were observed with 32 PMCs. Some embryos exhibited numbers of PMCs intermediate to 16 and 32, or 32 and 64. In these embryos, there is a distinct size differential observed between various PMCs within a single ring (see Figure 6B on page 57). It is likely that this observation can be attributed to some asynchrony in cleavage of PMCs. Immunocytochemical staining of the hybrid embryos clearly shows the proper organization of the PMCs. They form syncytial rings that later form the ventrolateral clusters that are the site of initiation of synthesis of the triradiate spicules of the skeleton.

It appears that Mab Ig8 reacts with the LxS PMCs as intensely as its does with the *L. pictus* PMCs. In addition, the weaker crossreactivity of Ig8 observed in the *S. purpuratus* PMCs is also observed in the heterotypic SxL PMCs. This suggests that the Ig8 antigen may be maternally specified. Another observation is that the hyaline layer of the *S. purpuratus* and SxL embryos appears to nonspecifically bind either Ig8 or the secondary antibody in a manner not observed in the *L. pictus* or LxS embryos. This nonspecific reactivity is more apparent at the vegetal plate. These observations suggest that the hyaline

composition of the layer may differ in these two species and that there may be a

territorial difference in the hyaline layer of *S. purpuratus* embryos. This

difference appears to be maternally controlled in hybrid embryos.

3.4 Pigment Cell Morphology

3.4.1 Pigment cells in *L. pictus* and *S. purpuratus*

The digital nomarski images shown in Figure 8 on page 61 depict the

comparative morphology of the pigment cells as observed on the surface of the

ectoderm of *L. pictus, S. purpuratus*, LxS and SxL pluteus larvae. These

derivatives of the secondary mesenchyme cells are clearly different in the two

species. The morphology of *S. purpuratus* pigment cells varies considerably

from that of *L. pictus* pigment cells. *S. purpuratus* pigment cells are generally

larger and have a stellate structure, commonly observed as having three or four

processes. The pigment granules in this species are concentrated in the

processes, away from the cell nuclei. *L. pictus* pigment cells have a small

spherical morphology and contain age dependent levels of echinochrome

granules. As plutei of this species mature, the pigment cells may in some cases

become less spherical and take on a filamentous morphology, however, they are

not consistently found to have the three or four projections consistently observed

in *S. purpuratus* larvae. In both species pigment cells are seen to be

concentrated at the apex of the larva and at the tips of the arms.

Figure 8:

Pigment Cell Morphology

[A] *L. pictus* early pluteus larva [B] *S. purpuratus* early pluteus larva

[C] *L. pictus* (♀) x *S. purpuratus* (♂) early pluteus larva [D] *S. purpuratus* (♀) x

L. pictus (♂) early pluteus larva.

61b
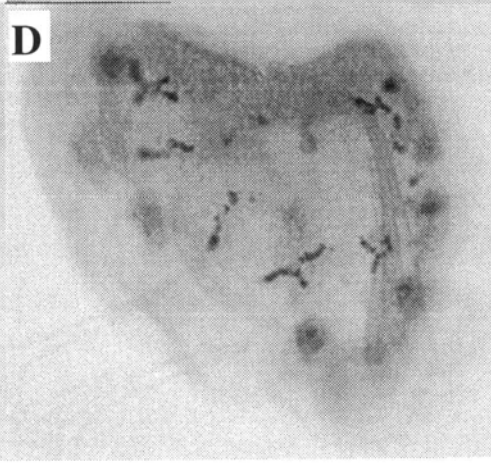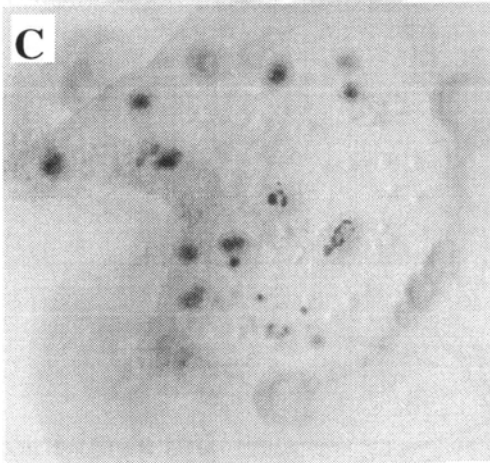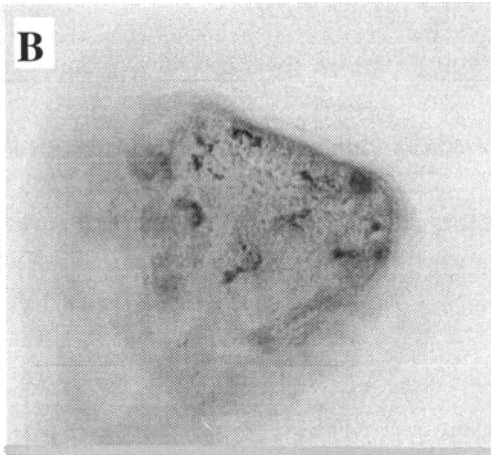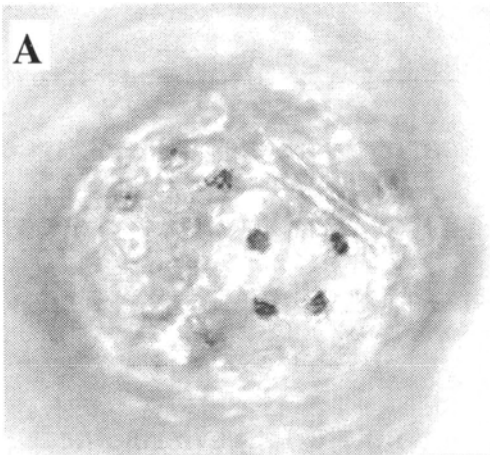
3.4.2 Pigment cells in Hybrid Embryos

In LxS embryos, the pigment cells resemble those of *L. pictus*. Most are spherical and are not observed to become large and stellate with increasing age. The morphology of these cells in the SxL individuals is indistinguishable from that of *S. purpuratus*, although early pigment cells of this cross in conjunction with those seen in the *S. purpuratus* larvae are somewhat more round immediately after invasion into the ectoderm. Despite variability observed between cultures and individuals within a particular hybrid culture, pigment cell morphology appears to be consistently maternal in origin.

3.5 Comparative morphology of Spicules
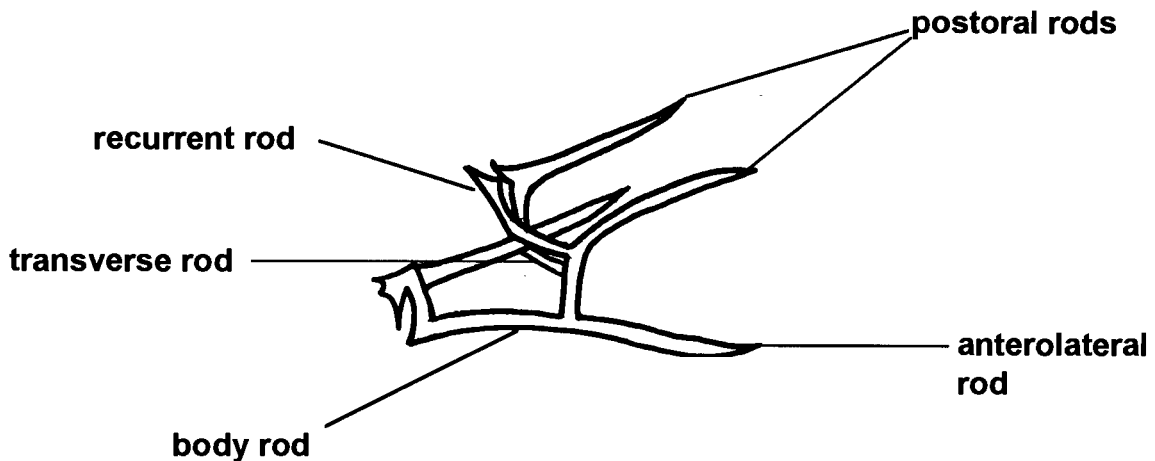
Figure 9:

Morphology of a Sea Urchin Larval Skeleton

Figure 10:

Spicule Morphology at the Pluteus Larval Stage

[A] *L. pictus* paired spicules magnified 325 times **[B]** *S. purpuratus* paired

spicules magnified 325 times **[C]** *L. pictus* apex structures magnified 325 times

[D] *S. purpuratus* apex structures magnified 325 times.

Abbreviations: **rr,** recurrent rod; **br,** body rod; **brt,** body rod terminus.

63b
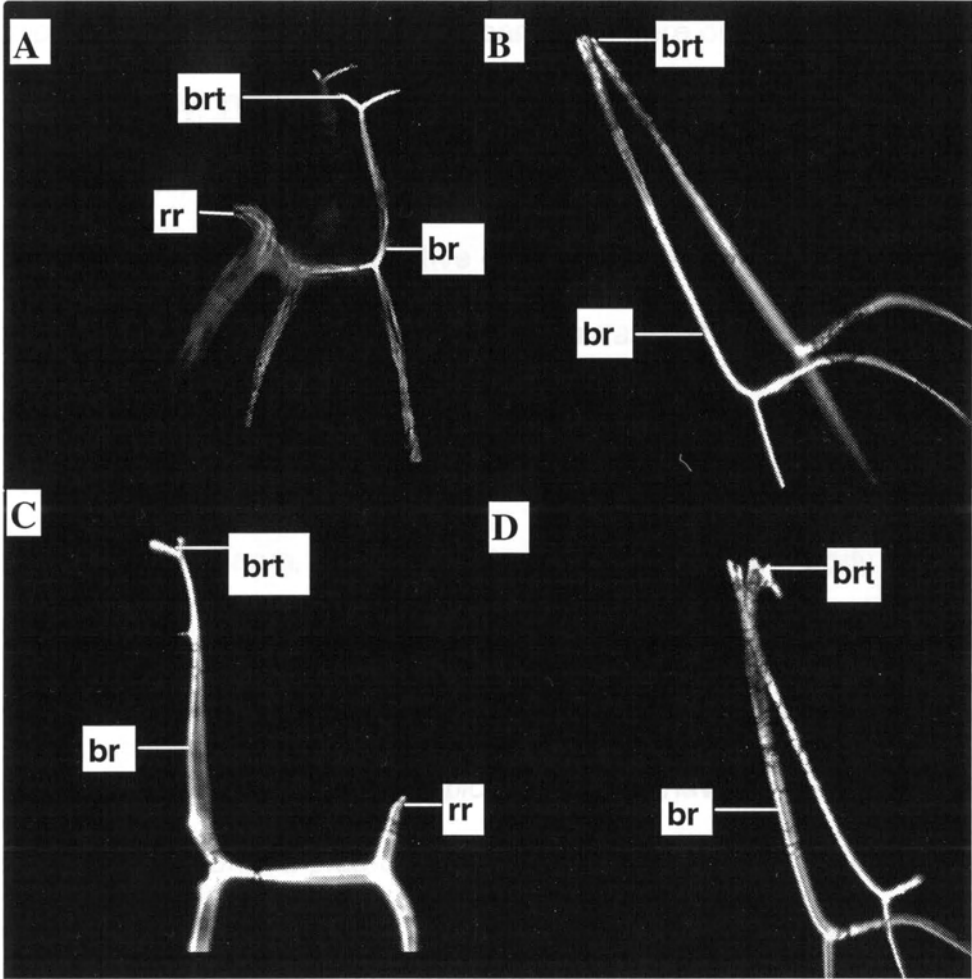
3.5.1 Spicule Morphology of *L. pictus* and *S. purpuratus*

Figure 9 on page 62 is a schematic drawing of a sea urchin skeleton composed of two spicules. Each rod is pointed out and labeled for use as a reference to the reader. Figure 10 on page 63 depicts spicule preparations of *L. pictus*, *S. purpuratus*. These preparations were observed using polarized light microscopy. The structural characteristics of the larval rods are simple and there are no consistent differences in the spacing of the barbs along these rods. There are however three distinctive differences which render *L. pictus* spicules easily distinguishable from those of *S. purpuratus* at this stage. First, *S purpuratus* body rods are straight and elongate while those of *S. purpuratus* are slightly curved. Second, the terminal ends of the body rods, corresponding to the apex of the larva, also differ. In *L. pictus* plutei, the body rods terminate into branched structures running perpendicular to the rod axis. *S. purpuratus* body rods have thickened termini elaborated into forked structures which run parallel to one another. Third, *L. pictus* spicules have a recurrent rod which is absent in *S. purpuratus* pluteus larvae of the same stage. These three differences are the criteria used for assessing the maternal and paternal inheritance of skeleton morphology in hybrid pluteus larvae.

3.5.2. Spicule Morphology in Hybrid Embryos

3.5.2.1 *S. purpuratus* x *L. pictus*

The morphology of skeletons prepared from SxL hybrid pluteus larvae almost exclusively resemble those of *S. purpuratus* larvae. In particular, the body rods are straight and have thickened, forked body rod termini. Most lack recurrent rods, although on some rare occasions, a spicule having a recurrent rod is observed. 50 spicules have been observed and the structural morphology is summarized in Table 2 below.

Table 2:

Summary of SxL Spicule Morphology

L. pictus-like	*S. purpuratus*-like	Intermediate
0	48	2

n=50

3.5.2.2 *L. pictus* x *S. purpuratus*

The spicule morphology observed in LxS hybrid plutei is more complex. Of the 50 LxS pluteus larvae spicules examined, approximately 70% lack recurrent rods, have straight body rods and display forked body rod termini consistent with *S. purpuratus* morphology. Approximately 15% of the spicules

closely resemble the L. pictus spicules. They have the recurrent rod, curved

body rods, and the characteristic branched apex. The remaining hybrid spicules

in the sample observed, have intermediate structures between L. pictus and S.

purpuratus characteristics. The results of the spicules examined are tabulated in

Table 3 below.

Table 3:

Summary of LxS Spicule Morphology

L. pictus-like	S. purpuratus-like	Intermediate
7	35	8

n=50

3.6 Temporal Expression of LSM34 in L. pictus Embryos

LSM34 and SM50 are Spicule matrix protein gene homologues from

L. pictus and S. purpuratus respectively. Their temporal expression patterns

have been determined in the homospecific embryos and both heterotypic

crosses of embryos by RNA gel blot hybridizations.

Figure 11A on page 68 depicts a RNA gel blot of L. pictus total RNA

prepared from mesenchyme blastula stage, gastrula stage, prism stage, and

pluteus stage embryos hybridized with an LSM34 DNA probe. The LSM34

transcript was weakly detected at the mesenchyme blastula stage, increased at

the gastrula stage and continues to be expressed through to the pluteus stage.

The corresponding ethidium bromide stained gel of this gel blot is depicted in Figure 12A on page 69.

3.7 Temporal Expression of SM50 in *S. purpuratus* Embryos

Figure 11B on page 68 shows an RNA gel blot of *S. purpuratus* total RNA prepared from mesenchyme blastula stage, gastrula stage, prism stage, and pluteus stage. This gel blot was hybridized with an SM50 DNA probe. The SM50 transcript is detected at all stages on this blot. The corresponding ethidium bromide stained gel is shown in Figure 12B on page 69.

3.8 Temporal Expression of LSM34 in *L. pictus* x *S. purpuratus* Hybrid Embryos

Figure 13A on page 70 shows an RNA gel blot of LxS total RNA prepared from hatched blastula, mesenchyme blastula, gastrula, prism and pluteus stages hybridized with an LSM34 DNA probe. Lane 1 shows *L. pictus* gastrula stage total RNA and lane 2 shows total RNA from gastrula stage *S. purpuratus*. The LSM34 probe hybridizes with the *L. pictus* RNA but not with the *S. purpuratus* RNA. No detectable LSM34 transcript was seen at the hatched blastula or mesenchyme blastula stages. LSM34 becomes detectable at the gastrula stage and continues to be expressed through until the pluteus stage. The greater intensity of LSM34 RNA in the *L. pictus* embryos may be due to there being one

Figure 11:

RNA Gel Blot Hybridizations

[A] Total *L. pictus* RNA gel blot hybridized with LSM34 ^{32}P-labeled DNA [B]

Total *S. purpuratus* RNA gel blot hybridized with ^{32}P-labeled SM50 DNA

Abbreviations: **mb,** mesenchyme blastula stage RNA; **g,** gastrula stage RNA; **pr,** prism stage RNA; **pl,** pluteus larva RNA.

[A] mb g pr pl

[B] mb g pr pl
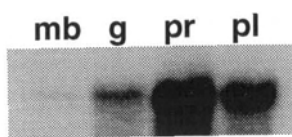
68b
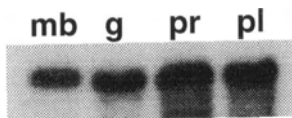
Figure 12:

Ethidium Bromide Stained Denaturing RNA Gels

[A] *L. pictus* total RNA gel of blot shown in Figure 11A. **[B]** *S. purpuratus* total RNA gel of blot shown in Figure 11B.

Abbreviations: **mb,** mesenchyme blastula stage total RNA; **g,** gastrula stage total RNA; **pr,** prism stage total RNA; **pl,** pluteus larva RNA.

[A]

mb g pr pl

[B]

mb g pr pl
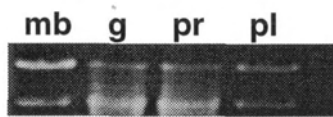
Figure 13:

RNA Gel Blot Hybridizations of LxS Total RNA

[A] Total LxS RNA gel blot hybridized with LSM34 ^{32}P-labeled DNA [B] Total

LxS RNA gel blot hybridized with ^{32}P-labeled SM50 DNA [C] Total LxS RNA

hybridized with ^{32}P-labeled LSM34 and SM50 DNA.

Abbreviations: **hb,** hatched blastula stage RNA; **mb,** mesenchyme blastula

stage RNA; **g,** gastrula stage RNA; **pr,** prism stage RNA; **epl,** early pluteus larva

RNA; **pl,** pluteus larva RNA; **lp,** *L. pictus* gastrula stage total RNA; **sp,**

S. purpuratus gastrula stage total RNA.

[A] lp sp   hb mb g   pr epl pl

[B]   lp sp   hb mb g pr epl pl

[C]   lp sp   hb mb g pr epl pl

70b
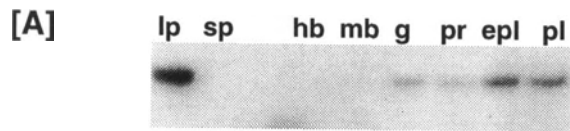
Figure 14:

Ethidium Bromide Stained Denaturing RNA Gels

[A] LxS total RNA gel of blot shown in Figure 13A [B] LxS total RNA gel of blot shown in Figure 13B [C] LxS total RNA gel of blot shown in Figure 13C.

Abbreviations: **hb,** hatched blastula stage total RNA; **mb,** mesenchyme blastula stage RNA; **g** gastrula stage total RNA; **pr,** prism stage total RNA **epl,** early pluteus larvae total RNA; **pl,** pluteus larva RNA.

[A] lp sp   hb mb g pr epl pl

[B] lp sp   hb mb g pr epl pl

[C] lp   sp   hb mb g pr epl pl

71b
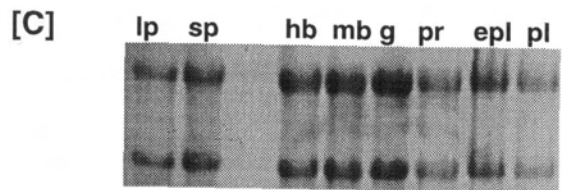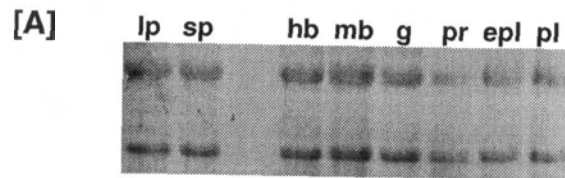
copy of the gene, or may represent a reduced activity of the gene in hybrids.
The ethidium bromide stained gel of this blot is shown in Figure 14A on page 71.

3.9 Temporal Expression of SM50 in *L. pictus* x *S. purpuratus* Hybrid Embryos

The RNA gel blot in Figure 13B on page 70 shows the expression of SM50 in LxS hybrid embryos. SM50 hybridized to the *S. purpuratus* RNA but not to the *L. pictus* RNA. Lanes 1 and 2 show *L. pictus* gastrula total RNA and *S. purpuratus* gastrula stage total RNA, respectively. No detectable transcript was present in the hatched blastula stage, although SM50 RNA was clearly present in the mesenchyme blastula stage hybrids and increased through to the pluteus stage. The corresponding ethidium bromide stained gel is shown in Figure 14B on page 71.

3.10 Comparative Expression of LSM34 and SM50 in *L. pictus* x *S. purpuratus* Total RNA.

The RNA gel blot in Figure 13C on page 70 shows LxS total RNA probed with both LSM34 and SM50 radiolabelled DNA. The difference in size of these transcripts allows for a comparison of their expression using the same RNA sample. Lane 1 shows total RNA from *L. pictus* gastrula stage embryos and lane 2 shows *S. purpuratus* RNA from the same stage. The SM50 transcript initially appeared at low abundance at the mesenchyme blastula stage hybrids, earlier

72

than the LSM34 transcript. The LSM34 probe was of slightly higher specific

activity and therefore produces a stronger signal than the SM50 probe. Clearly,

both transcripts are present in the LxS hybrid gastrula, prism and pluteus stages.

Figure 14C on page 71 depicts the ethidium bromide stained gel corresponding

to this blot.

3.11 Temporal Expression of LSM34 in *S. purpuratus* x *L pictus* Hybrid Embryos

Figure 15A on page 74 shows RNA gel blot analysis of SxL total RNA

hybridized with a LSM34 DNA. Lane 1 and 2 are again control lanes of *L. pictus*

and *S. purpuratus* gastrula stage total RNA respectively. Again the probe

hybridized to the *L. pictus* RNA but not to that of *S. purpuratus*. No LSM34 RNA

was detected in the hatched blastula or mesenchyme blastula stages of the SxL

embryos. The transcript accumulates later, in the SxL gastrula and pluteus

stages. The corresponding ethidium bromide stained gel for this blot is shown in

Figure 16A on page 75.

Figure 15:

RNA Gel Blot Hybridizations of SxL Total RNA

[A] Total SxL RNA gel blot hybridized with LSM34 ^{32}P-labeled DNA [B] Total

SxL RNA gel blot hybridized with ^{32}P-labeled SM50 DNA [C] Total SxL RNA

hybridized with ^{32}P-labeled LSM34 and SM50 DNA.

Abbreviations: **hb,** hatched blastula stage RNA; **mb,** mesenchyme blastula

stage RNA; **eg,** early gastrula stage RNA; **lg,** late gastrula stage RNA; **pl,**
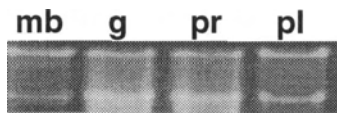
pluteus larva RNA; **lp,** *L. pictus* gastrula stage total RNA; **sp,** *S. purpuratus*
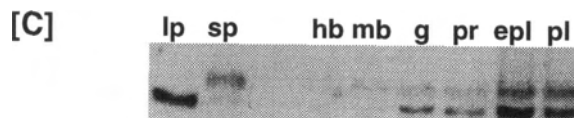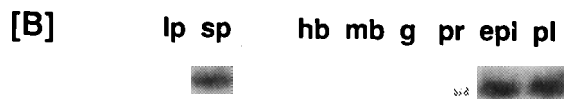
gastrula stage total RNA.

[A]   lp sp hb mb eg lg pl

[B]   lp sp hb mb eg lg pl

[C]   lp sp hb mb eg lg pl

74b
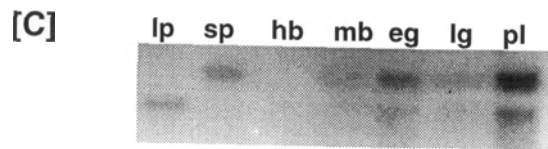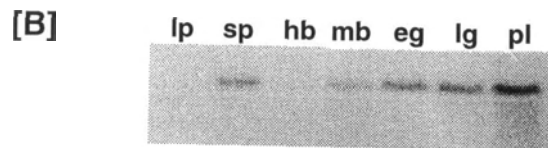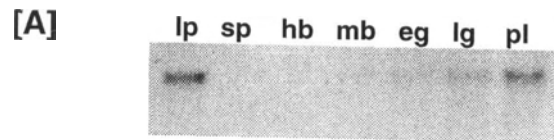
Figure 16:

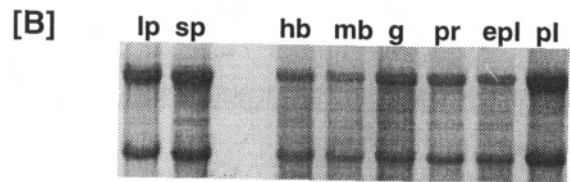**Ethidium Bromide Stained Denaturing RNA Gels**

[A] SxL total RNA gel of blot shown in Figure 15A **[B]** SxL total RNA gel of blot shown in Figure 15B **[C]** SxL total RNA gel of blot shown in Figure 15C.

Abbreviations: **hb,** hatched blastula stage total RNA; **mb,** mesenchyme blastula stage RNA; **eg,** early gastrula stage total RNA; **lg,** late gastrula stage total RNA; **pl,** pluteus larva RNA.

[A]   lp sp hb mb eg lg pl

[B]   lp sp hb mb eg lg pl

[C]   lp sp hb mb eg lg pl
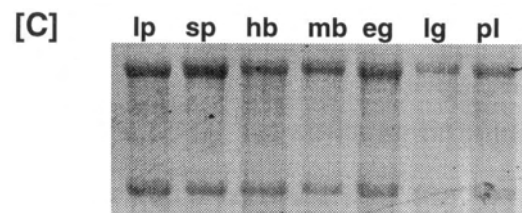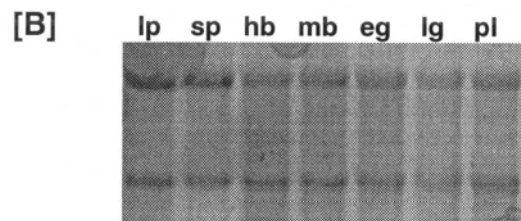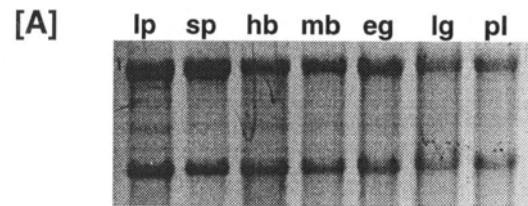
3.12 Temporal Expression of SM50 in *S. purpuratus* x L .pictus Hybrid Embryos

Figure 15B on page 74 shows an RNA gel blot of SxL total RNA probed with SM50 radiolabelled DNA. No cross hybridization was observed to RNA in the *L. pictus* lane. The SM50 transcript is clearly present in the mesenchyme blastula stage hybrids and increased through to the pluteus stage. No SM50 RNA is detectable in the hatched blastula stage hybrids. The ethidium stained gel of this blot is depicted in Figure 16B on page 75.

3.13 Comparative Expression of LSM34 and SM50 in *S. purpuratus* x *L. pictus* Total RNA

The RNA gel blot depicted in Figure 15C on page 74 shows SxL hybrid total RNA probed with radioactively labeled LSM34 and SM50 DNA. The SM50 probe was of a higher specific activity than the LSM34 probe resulting in a stronger signal for this transcript. The SM50 transcript was first detected earlier than the LSM34 transcript, at the mesenchyme blastula stage. The LSM34 transcript was initially detected at the early gastrula stage. Lane 6 shows late gastrula stage RNA and appears to be underloaded in comparison to the other samples. This is corroborated by the ethidium bromide stained gel of this blot depicted in Figure 16C on page 75. This blot indicates that both LSM34 and SM50 transcripts are present early gastrula, late gastrula, and pluteus stage hybrid embryos.

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3.14 *In situ* Hybridization of *L. pictus* Gastrula Stage Embryos with LSM34 Antisense RNA

The embryo shown in Figure 17A on page 78 is of an *L. pictus* late gastrula stage embryo hybridized *in situ* with an digoxygenin labeled antisense LSM34 RNA probe synthesized *in vitro*. The alkaline phosphatase reaction using the subbstrates NBT and BCIP produce the purple/blue colour shown. The darkly stained cells are the PMCS and cell counts reveal that only a subset of these cells have detectable levels of LSM34 transcript. The mean number of LSM34 expressing PMCs was 35.24 with a standard deviation of 8.10. The range of this sample was 17-54. This data is summarized in Table 4 below. PMC counts using Ig8 (discussed in section 3.3 on page 56) indicate that L. pictus embryos at this stage have 64 PMCs, and this in turn implies that LSM34 expression is restricted to a fraction of the total PMC population.

3.15 *In situ* Hybridization of *L. pictus* (♀) x *S.purpuratus* (♂) Gastrula Stage Hybrid Embryos with LSM34 Antisense RNA.

The LxS hybrid late gastrula depicted in Figure 17B on page 78 was hybridized *in situ* with an LSM34 antisense riboprobe labeled with digoxygenin. This transcript is localized to the PMCs. 50 embryos were viewed and scored for the number of PMCs that stained positive for LSM34. This data is summarized in Table 4 below. The mean number of PMCs that stained positive

Figure 17:

Whole Mount *In Situ* Hybridizations

[A] *L. pictus* late gastrula hybridized with LSM34 antisense RNA [B] SxL hybrid late gastrula hybridized with LSM34 antisense RNA **[C]** LxS hybrid late gastrula hybridized with LSM34 antisense RNA **[D]** *S.purpuratus* late gastrula hybridized with SM50 antisense RNA. **[E]** SxL hybrid late gastrula hybridized with SM50 antisense RNA **[F]** LxS hybrid late gastrula hybridized with SM50 antisense RNA.

Figures **[A]**, **[B]**, **[C]**, **[D]**, and **[F]** are oriented animal pole toward the top of the page. **[E]** is viewed aboral side up.
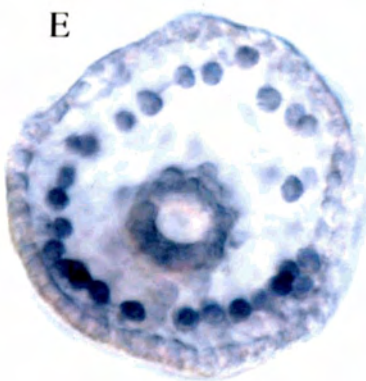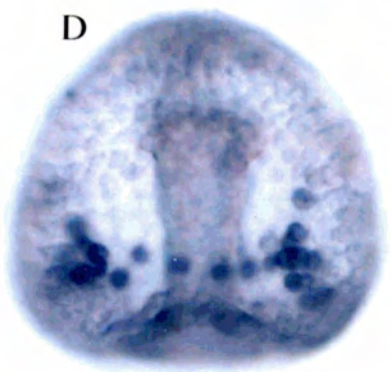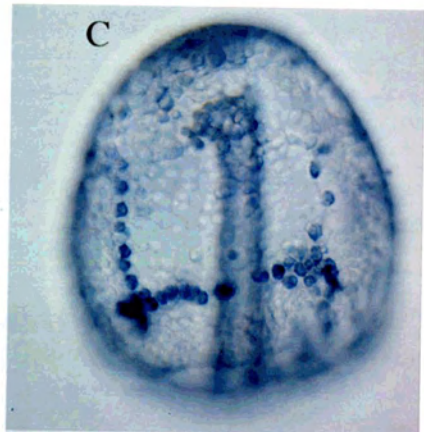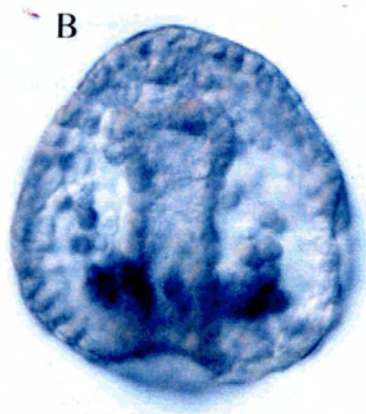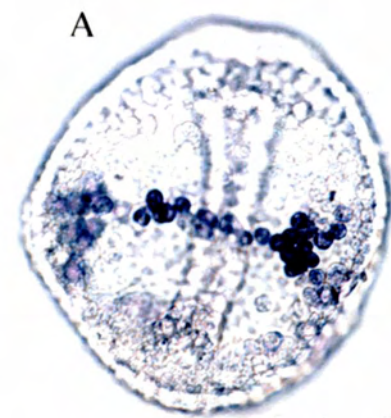
for LSM34 in these hybrids was 32.18 with a standard deviation of 5.73. The range of this sample was 17-44.

3.16 *In situ* Hybridization of *S. purpuratus* (♀) x *L. pictus* (♂) Gastrula Stage Hybrid Embryos with Antisense LSM34 RNA

The late gastrula stage SxL hybrid embryo in Figure 17C on page 78 was hybridized *in situ* with an LSM34 riboprobe labeled with digoxygenin. The proper tissue specific expression is maintained for LSM34 in this cross. Cell counts of PMCs staining positive for this transcript were performed on 50 embryos from this sample. The mean number of PMCs that are expressing LSM34 at detectable levels was 30.10 with a standard deviation of 7.03. The range observed for this sample was 16-43.

Table 4:

Summary of PMC counts Stained with LSM34 via Whole Mount *in situ* Hybridization.

Cross	Mean # PMCs Positive for LSM34	One Standard Deviation
L. pictus	35.24	8.10
LxS	32.18	5.73
SxL	30.10	7.03

n=50

3.17 *In situ* Hybridization of *S.purpuratus* Gatrula Stage Embryos with SM50 Antisense RNA

The S. pupuratus late gastrula embryo shown in figure 17D on page 78 is the result of an *in situ* hybridization experiment using digoxygenin labeled antisense SM50 RNA. The stained cells observed are PMCs and cell counts were performed on a sample of 50 embryos. The number of PMCs that stain positive for SM50 were scored. (see Table 5 below) The average number of PMCs that appear to be expressing SM50 in *S.purpuratus* embryos is 36.56 with a standard deviation of 4.37. The range of this sample is 28-47. These data in conjunction with the fact that *S.purpuratus* appear to have 64 PMCs imply that only a subpopulation of these cells express SM50.

3.18 *Insitu* Hybridization of *L. pictus* (♀)x *S.purpuratus* (♂) Hybrid Embryos with SM50 Antisense RNA

The embryo depicted in Figure 17E on page 78 shows a LxS hybrid gastrula stage embryo that has been probed *in situ* with a digoxygenin labeled SM50 riboprobe. This photomicrograph shows proper spatial expression of this gene in the PMCs. Cell counts of PMCs that stain positive for the SM50 transcript are shown in Table 5 below. A population of 50 embryos were viewed and the SM50 expressing PMCs were counted. The average number of PMCs

seen to express SM50 is 31.80 with a standard deviation of 10.58. The observed range in this sample is 12-54.

3.19 *In situ* Hybridization of *S.purpuratus* (♀) x *L. pictus* (♂) Hybrid Embryos with SM50 Antisense RNA

The gastrula stage embryo in Figure 17F on page 78 shows the expression of SM50 in *S. purpuratus x L. pictus* embryos. The expression is observed to be consistently limited to the PMCs suggesting proper spatial regulation of this gene in this hybrid cross. Cell counts of 50 individuals yield an average of 30.74 PMCs that stain positive for SM50 expression. The standard deviation of this sample is 8.17 and the range is 18-52.

Table 5:

Summary of PMC counts Stained with SM50 via Whole mount *in situ* Hybridization.

Cross	Mean # PMCs Positive for SM50	One Standard Deviation
S. purpuratus	36.46	4.37
LxS	31.80	10.59
SxL	30.74	8.17

n=50

Chapter IV

Discussion

4.1 General Morphology

The morphological studies detailed in this thesis are somewhat inconsistent with previous reports indicating that morphological features observed in interspecies hybrid sea urchin embryos are predominantly of maternal origin (reviewed by Davidson, 1976). Although some gross morphological characteristics do appear to be maternally specified, there are clear exceptions to this rule in the reciprocal hybrid embryos examined.

L. pictus and *S. purpuratus* have notably different eggs. Primarily, *L. pictus* eggs are larger and less pigmented than *S. purpuratus* eggs. Because there is no net change in mass from fertilization until initiation of feeding in the pluteus larva, the size of the embryo directly reflects the size of the egg. Therefore, in both heterotypic crosses, the embryos and early larvae are very similar in size to those of the maternal species. In addition, the degree of pigmentation prior to pigment cell formation of the embryos is a product of the egg and is therefore, a maternally derived feature. At the mesenchyme blastula stage, prior to invagination of the archenteron, the overall morphology of the heterospecific embryos appears strictly maternal. The smaller size of the *S. purpuratus* eggs gives the impression that the ectoderm that surrounds the blastocoel is thicker in this species than the ectoderm observed in *L. pictus* blastulae. My measurements indicate that there is no significant statistical difference between the thickness of the ectoderm between these two species at the same stage, but the blastocoel of *S. purpuratus* is smaller (see Table 1 on

page 50). Although the size, clarity, and overall general appearance of the heterospecific embryos do resemble the maternal species, there are some deviations from this generalization.

Archenteron extension differs between the two species of sea urchins used in these studies (see Figure 4A & B on page 54 in Results). In the *S. purpuratus* embryos, the gut bends toward the oral side earlier in development than in the *L. pictus* gastrulae. Interestingly, in hybrid embryos of both crosses between these two species, the extending gut bends toward the oral side soon after invagination, as in *S. purpuratus* (see Figure 4c & D on page 54 in Results). This result suggests the events that control this process are controlled by the zygotic genome, and that the *S. purpuratus* genome dominates the *L. pictus* genome in this regard. It is unknown whether this bending of the gut is autonomous to the endodermal cells, or if it occurs in response to signals from the ectoderm. If this process is mediated by ectodermal cues that provide oral-aboral axis information, it is interesting to speculate that perhaps both the *L. pictus* and *S. purpuratus* signals are present but that the endodermal cells are only competent to respond to the *S. purpuratus* information. Alternatively, perhaps the signal is the same in these two species and that the dominance effect observed in the hybrids occurs as a result of the signal being generated earlier in hybrids than in *L. pictus* embryos. This signal could be released in two separate waves in hybrids but the endoderm having responded to the first signal may be no longer competent to respond to the later signal. If this bending

occurs as an intrinsic feature of the endodermal cells, the *S. purpuratus* controls may predominate in hybrids. This possibility is supported by the literature that reports an overall underrepresentation of *L. pictus* gene products in SxL embryos (Tufaro and Brandhorst, 1984; Conlon and Brandhorst, 1987). The molecule(s) that control the timing of archenteron bending may be represented by one or several of the underexpressed transcripts previously reported. The apparent *S. purpuratus* dominance may occur due to the lack of the *L. pictus* contribution of zygotic transcripts.

4.2 Primary Mesenchyme Cell Numbers and Ingression

Previous literature has considered the inheritance of the number and timing of ingressing PMCs in hybrid embryos (for review see Davidson, 1976). The number of PMCs in hybrid embryos generally corresponds to that of one parent or another. These cells are the descendants of the large daughters of the micromeres that come about by an unequal fifth cleavage. The large micromeres subsequently divide either three or four times to produce 32 or 64 PMCs depending upon the species. These numbers of PMCs remain constant through embryonic development to the early larval stage (Ettensohn, 1992). I have investigated the number of PMCs in *L. pictus* and *S. purpuratus* embryos to establish whether a difference exists in PMC numbers between the two species. The literature indicates that *Lytechinus variegatus* embryos have 64 PMCs (Armstrong and McClay, 1994), and to the best of our knowledge, there is no

information regarding the number of PMCs in the closely related species,

L. pictus. The information available for S. purpuratus regarding the number of

PMCs is contradictory. Harkey et al. (1992) have reported that this species has

32 PMCs , while lineage studies indicate that although there are 32 PMCs at the

time of ingression, this number increases to about 60 before the pluteus stage

(Cameron and Davidson, 1987). In addition, Ettensohn (1990) reported that

L. variegatus have a widely variable number of PMCs. The authors report a

mean of 68 PMCs with a standard deviation of 7, and a range of 50-79. Based

on the literature, I considered it possible that the number of ingressing PMCs

would be a useful species-specific character. I have used the monoclonal

antibody, Ig8, kindly provided by David McClay's lab, in order to examine the

number of PMCs in L pictus embryos. I have found that L. pictus embryos also

contain up to 64 PMCs, and no significant differences were seen in the

reciprocal hybrid embryos which were also seen to have up to number of 64

PMCs. McClay has reported (1992) that in Lytechinus variegatus, 64 PMCs

ingress into the blastocoel from the vegetal pole and that these cells do not

divide further. In addition, previous studies report that the number of PMCs at

the time of ingression in S. purpuratus is 32 (Davidson, 1986, Cameron and

Davidson, 1987). I have found that the number of PMCs present at the time of

ingression is 16 in both L. pictus and S. purpuratus. 16 PMCs arranged in a ring

in the vegetal hemisphere are clearly visible in Figures 6A & C and 7A on pages

57 and 58 in Results. MAb Ig8 recognizes a cell surface antigen that is only

detectable in PMCs that have delaminated from the vegetal plate and reside within the blastocoel (McClay, 1983). This information rules out the possibility that the 16 cells that are in a ring are still within the vegetal plate. Although it is possible that the cells that stain positive for Ig8 are not the only PMCs localized to this ring of cells, no unlabeled PMCs were visualized using DIC microscopy. In addition, numbers of PMCs in the blastocoel that lie between the numbers 16 and 32 and 32 and 64 were observed. This indicates that there are two mitotic events that occur subsequent to ingression. It has been reported that mitotic figures have been observed in the PMCs after ingression implying post-ingression divisions occur (Nislow and Morrill, 1988). In contrast, Ettensohn and McClay (1988) suggest that the consistent number of PMCs observed in embryos argue against cell divisions after ingression. The detection of mitotic figures supports the observed numbers of PMCs that lie between 16 and 32, and, 32 and 64. In addition, there is an observable size difference between PMCs in embryos with PMC numbers between 16 and 32, and 32 and 64, suggesting that the larger PMCs have not yet divided (see Figure 6B on page 57 in Results).

Although the possibility that the number of PMCs may vary to some degree between embryos cannot be ruled out by the observations reported , the overt invariant nature of developing sea urchin embryos, detailed in the available literature, argue against this. PMCs also bind wheat germ agglutinin (WGA) and other lectins. The properties of these cells that cause WGA to bind

to their surfaces is present in the micromeres of the 16 cell stage embryo (Ettensohn and McClay, 1988). FITC-labeled WGA can be injected into the blastocoels of mesenchyme blastula stage embryos where it preferentially binds PMCs that can be viewed using fluorescent microscopy (Ettensohn and McClay, 1988). This method provides an alternate method for viewing and counting PMCs which could be used as a basis for comparison with the results reported here. This could be used to rule out any possibility that not all PMCs express the MAb Ig8 antigen shortly after ingression. Alternatively, single embryos could be injected with FITC-WGA and stained with Ig8 using a rhodamine labeled secondary antibody. This would allow PMC cell counts to be performed using both methods and compared within the same embryo.

4.3 Pigment Cell Morphology

The chromogeneic secondary mesenchyme cells ingress into the blastocoel during gastrulation, migrate, and eventually invade the ectoderm. This population of SMCs gives rise to the pigment cells of the larva whose function remains unclear at present (Gibson and Burke, 1987). Historical biochemical studies report the inheritance of the pigments that are localized to these cells in various hybrid sea urchin embryos. In crosses between L. variegatus (♀) and A. punctulata (♂), the larval pigmentation is maternally specified (Young, 1958; reviewed by Czihak, 1975). In the reciprocal crosses between S. purpuratus and S. droebachiensis, the timing of echinochrome

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synthesis is maternal. In the *S. purpuratus* (♀) x *S. droebachiensis* (♂) cross, the rate of accumulation is intermediate between the two species, while in the reciprocal cross the rate of accumulation is maternal (Chaffee and Mazia, 1963; reviewed by Czihak, 1975). The interpretation of these experiments is that there must be a difference in the amounts of pigment enzyme precursors, or that there are other limiting factors involved in pigment formation in the egg cytoplasm from these two species.

A more informative pair of reciprocal hybrid crosses for studying pigment inheritance are those made between *S. purpuratus* and *S. droebachiensis*. This is due to these two species having distinctively different pigments. Two *S. purpuratus* napthaquinone pigments, A and B are absent in the *S. droebachiensis* larvae. *S. droebachiensis* larvae have by two pigments referred to as X and Y, neither of which are detected in *S. purpuratus* larvae. Both pigments A and B have absorbance spectra indistinguishable from the adult echinochromes. In contrast, X and Y pigments spectra differ significantly from the adult echinochromes. Experiments performed by Griffiths (1965; reviewed by Czihak, 1975) involving reciprocal hybrid crosses between these two species have revealed that both crosses produce the *S. purpuratus* A and B pigments but not the *S. droebachiensis* pigments X and Y. It is thought that the absence of detectable levels of X and Y occurs as a result of these pigments being biosynthetic intermediates in the formation of the adult pigments and that they are converted in the hybrids to A and B. The timing of pigment synthesis is

S. purpuratus-like in both heterospecific crosses, but, while the rate of pigment synthesis is comparable in both hybrid crosses, it is significantly lower than the rate specified by the *S. purpuratus* genome (Griffiths, 1965). These data suggest that the cytoplasm supplied during oogenesis is not influencing the timing or rate of pigment synthesis or the type of pigments produced in these hybrids embryos.

In the hybrid crosses made between *Dendraster excentricus* (♀) and *S. droebachienesis* (♂), and *L. variegatus* (♀) and *Tripneustes* (♂), no detectable pigments are synthesized in the hybrid larvae (Whiteley and Whiteley, 1972; Badman and Brookbank, 1970; reviewed by Czihak, 1975). The interpretation is that the pigment biosynthetic pathways of the parental species somehow interact in these hybrids to produce colourless hybrid products.

The morphology of pigment cells in *L. pictus* and *S. purpuratus* is strikingly different see Figure 9A & B on page 61 in Results). *L. pictus* larvae generally have round or elliptical pigment cells, while *S. purpuratus* plutei have stellate pigment cells having three or four processes. In the reciprocal crosses made between these two species, the overall morphology of these pigment cells resemble the maternal species at the early pluteus larval stage (see Figure (c & D on page 61 in Results). Although these data suggest maternal inheritance of the descendants of the chromogenic mesenchyme, no conclusions can be drawn regarding the pigments that are synthesized in these heterospecific crosses. A monoclonal antibody has been raised against the *S. purpuratus* pigment cells

and is species specific (Burke, 1995 personal communication). It would be of interest to use this antiserum to determine whether or not the antigen recognized is also maternally supplied in the reciprocal hybrid plutei. In addition, the isolation and characterization of the main pigments from species and their reciprocal hybrids would be useful in determination of the heritable nature of these echinochromes.

4.4 Spicule Morphology

Previous experiments by Okazaki (1975), Harkey and Whiteley (1980), Carson et al. (1985), and Benson and Chuppa (1990) have shown that the PMCs synthesize spicules in the absence of cells from the rest of the embryo. Although these cells have been reported to be somewhat autonomous, certain factors are required for initiation and continuation of spiculogenesis. When cultured micromeres isolated at 16 cell stage are supplemented with horse serum, or grown on an artificial extracellular matrix, the synthesis of skeletal components is initiated, although the timing is delayed. This indicates that cell-cell interactions are not required for activation of the spicule matrix protein genes, or other genes required to promote synthesis of the larval skeleton. Attempts have been made to purify and characterize the component(s) of horse serum that are necessary for the activation of the genes involved in spicule synthesis *in vitro*, but as of yet no factor(s) have been identified.

The structure of the skeletons produced is greatly improved when isolated

PMCs combined with basal lamina are allowed to reaggregate with ectodermal

cells (Harkey and Whiteley, 1980). Furthermore, the filopodial processes of the

PMCs have been observed to penetrate the basal lamina and directly contact

the overlying ectoderm (Spiegel and Spiegel, 1992). It is unclear at this time as

to whether the PMCs respond directly to the ectoderm cells, or, to a component

of the extracellular matrix. Although there is no direct evidence to suggest that a

component of the extracellular matrix is involved in this process, it is possible

that a putative peptide or growth factor may exist that has not been recovered

from isolated extracellular matrix with the techniques available.

There is a great deal of literature that supports the hypothesis that the

ectoderm is involved in the regulation of proper skeleton formation in sea urchin

larvae. It has been concluded that the information that specifies the structure of

the rods produced (simple versus fenestrated) is intrinsic to the PMCs. In

contrast, the timing of spiculogenesis and the size of the skeletons produced are

shown to be orchestrated by the remainder of the embryo (Armstrong and

McClay, 1994). In *L. variegatus* plutei that have had the ectoderm at the tip of

the arms ablated, spicule outgrowth ceases (Ettensohn and Malinda,1993). This

indicates that the presence of this ectoderm is required for spicule growth in this

region, and that it is possible that it provides a factor that is recognized by the

PMCs that potentially activates specific gene transcription necessary for growth

of the spicules at the tip. It has been demonstrated that all PMCs within an

embryo are equivalent and that any PMC can synthesize any portion of the skeleton (Armstrong and McClay, 1994). It is possible that the information specified by the ectoderm instructs the PMCs to form the appropriate part of the spicule by providing positional information which might be conveyed via a ligand-receptor mediated signal transduction mechanism. The aforementioned observations are compelling evidence in support of a model that suggests that the signals from the ectoderm are essential for proper spicule growth. In addition, these experiments clearly indicate that the described autonomy of the PMCs is limited.

At this point, the putative signal from the ectoderm has not been identified, nor has its mode of action been elucidated. It is very likely that the complexity of this process requires a multifaceted signaling system. The fact that any given PMC can respond to information specified elsewhere in the embryo suggests that these PMCs all have equal potentials, and that the localized production of the appropriate skeletal component by a particular PMC occurs as a result of different localized positional cues. Alternatively, it is possible that this signal is graded and that the PMCs respond differently to varying levels of this signal.

The ablation studies discussed above demonstrate that the ectoderm is indeed producing a factor necessary for spicule out growth. In addition, the fact that PMCs can be induced to produce spicules in culture via the addition of horse serum suggests that a component of this serum may mimic the effects of

the ectodermal factor. This factor may be a ligand for a PMC-specific receptor that activates a cascade of events that result in the activation of nuclear genes responsible for some aspect of spicule production. In addition, because the PMC filopodial processes are seen to directly contact the ectoderm, they may have receptors localized at the tips of these extensions that recognize a specific membrane protein localized on the surface of specific ectoderm cells. Armstrong and McClay (1994) suggest that in addition to ectoderm-PMC signaling, the PMCs likely communicate among themselves. The authors postulate that because these cells form an intricate and interactive network, then it follows that these cells have direct effect on their neighbors.

The morphology of *L. pictus* and *S. purpuratus* spicules differs sufficiently such that reciprocal interspecies hybrid plutei spicules have been examined herein and compared to the homospecific structures. The spicule rods of *L. pictus* and *S. purpuratus* are both composed of simple single rods having barb-like projections along these rods at similar intervals in both species. Although there is considerable similarity between the spicules of both species, there are some distinct differences. Three criteria have been selected from these differences and have been used to assess the inheritance of spicule morphology in the reciprocal heterospecific pluteus larvae. First, the structure of the terminus of the body rods differ significantly. *L. pictus* have branched termini while *S. purpuratus* have thickened, forked termini. Second, the *L. pictus* spicules have a recurrent rod and the *S. purpuratus* spicules of the same stage

are missing this structure. Finally, the body rod in the L. pictus skeleton is curved while the same rod in S. purpuratus is nearly straight.

In the SxL hybrid cross, the S. purpuratus spicule characteristics predominate. In the reciprocal cross of LxS, this S. purpuratus predominance is also true, but there is a great deal more variability. A significant number of spicules from this cross (approximately 15%) have spicules that are L. pictus like. In addition, approximately 15% of the assayed spicules have structures that are characteristic of both parental species. The degree of contribution of structures from either species varies between spicules. The process of digesting the organic material in a dilute bleach solution results in the spicules becoming unpaired. This is unfortunate in that it is difficult to comment as to whether the two spicules from a single hybrid pluteus are similar, or, if there is some variability of these structures within a single organism. It has been shown that the rate of spicule synthesis is almost indistinguishable between the two spicules in a single embryo (Ettensohn and Malinda, 1993). This is likely due to well regulated communication between the two ventrolateral clusters. It has been proposed that the rate of spicule growth is independent of the number of PMCs involved (Ettensohn and Malinda, 1993). It is possible that the rates of spicule synthesis differs between the two species. The rate of spicule formation in the heterospecific embryos could be investigated to determine whether both rods form at similar rates. This could provide insight into the inheritance of the factors that govern these rates in these hybrid embryos.

Unpublished research by McClay and associates indicates that hybrid

embryos between the species *Tripneustes ventricosus* and *Lytechinus*

variegatus have rods that show alternative species specific patterns distributed

randomly along the length of the rods. This was relatively simple to assess due

to the fact that *L. variegatus* have simple rods and *T. ventricosus* have

fenestrated anterolateral rods. The similarity between the rod structures of

L. pictus and *S. purpuratus* makes determination of parental contributions along

individual spicules difficult. This was not possible to determine from these

studies as to whether the spicules in the hybrid embryos between *L. pictus* and

S. purpuratus also exhibit this alternating pattern. However, the criteria used in

this study indicate that there is predominance of *S purpuratus* spicule structures

in both crosses of hybrids pluteus larvae.

In PMC transplantation experiments between *T. ventricosus* and

L. variegatus, the structure of the rods is specified by the species of PMC. This

information is intrinsic to the PMCs and genetically specified. *Tripneustes*

skeletons have fenestrated anterolateral rods and the recurrent rods extend to

the posterior and are joined to the body rods by the posterior connecting rods.

In contrast, *Lytechinus* skeletons are composed of simple rods and do not have

posterior connecting rods. Of the 31 *L. variegatus* embryos whose PMCs had

been replaced with *Tripneustes* PMCs, all but one produced spicules

characterized by fenestrated anterolateral rods(Armstrong and McClay, 1994).

11 of these chimeras produced posterior connecting rods In the reciprocal

transplantation experiment, all 8 *Tripneustes* embryos containing *L. variegatus*

PMCs produced simple anterolateral rods. In addition, 7 of these embryos

lacked the posterior connecting rods. Although there is some variability in the

structures produced in these chimeric embryos, these data support the

genotypic specification of the rod structure by the PMCs. In addition, some rods

are synthesized in hosts that do not normally produce the structure. This

suggests that the information specified by the ectoderm is conserved between

these two species. For example, *L. variegatus* ectoderm must still generate the

signal necessary to produce the posterior connecting rods that the *Tripneustes*

PMCs are specified to synthesize. In contrast, although the *Tripneustes*

ectoderm produced the information to specify these rods, the majority of the

L. variegatus PMCs do not produce this structure in the chimeric embryos.

The variation in the structures formed by these chimeras is not

commented on by the authors. In one of the eight chimeras generated by

combining *L. variegatus* PMCs with PMC-less *Tripneustes* embryos, these

posterior connecting rods were produced. Although there is no direct evidence

for this, perhaps the *L. variegatus* genome contains the genes necessary to

respond to the information from the ectoderm that specifies this particular rod

structure normally absent in embryos of this species.

There are many genes involved in the process of skeletogenesis in the

sea urchin embryo, some which have been isolated and characterized. At least

ten matrix proteins are associated with spicules in *S. purpuratus* larvae. In

addition, a modulator protein, msp130 thought to be involved in calcium transport, has been cloned and characterized (Harkey et al., 1985). The expression of these genes must be stringently regulated to ensure appropriate accumulation of the products necessary during spicule formation. This temporal and spatial expression must be governed by factors whose synthesis and accumulation must also be tightly controlled. Because the formation of the larval skeleton is similar in *L . pictus* and *S. purpuratus*, it is likely that mechanisms controlling these functions are also similar. The apparent dominance of the *S. purpuratus* skeletal features over those of *L. pictus* in both crosses of hybrid plutei may be due to a given regulator factor or set of factors having a higher affinity for *S. purpuratus*-specific gene regulatory sequences. This does not however, explain the significant but variable contribution made by the *L. pictus* genome in the LxS cross.

If the signal supplied by the overlying ectoderm works via a receptor-mediated signal transduction pathway that subsequently alters the gene expression of the stimulated PMCs, it is likely that a similar signaling pathway operates in both *L. pictus* and *S. purpuratus*. This is supported by the previously discussed transplantation experiments that demonstrate the ability of PMCs to respond to the ectodermal signal of another species (Armstrong and McClay, 1994). Although there is some conservation of this signal and the PMC response, there may be some divergence such that PMCs of different species will respond in different degrees depending upon the source or concentration of

the signal. In cells of hybrid embryos there should be a mixture of maternal and paternal species-specific transcription factors . However, it is possible that both types of factors will have a higher affinity for the regulatory sequences of one species. This could result in the preferential expression of genes from one species in both heterospecific crosses as is observed. Assuming that all PMCs are equivalent in a given embryo, and that each PMC in hybrid embryos contains the genetic information from both species necessary for response to the ectoderm and skeletogenesis, then it is possible that the regulatory factors preferentially activate the *S. purpuratus* genes. The *L. pictus* contribution seen in the LxS cross may be a result of a maternally supplied factor. Perhaps there is a ooplasmic factor involved in gene regulation such that the *L. pictus* genes are expressed sufficiently to have an observable effect in a subset of PMCs in the LxS cross. Alternatively, the *L. pictus* spicule characteristics synthesized in this cross may occur as a result of loss, inactivation, or rearrangement of the *S. purpuratus* gene that encoded this putative regulatory factor in this subset of PMCs.

4.5 Expression Of LSM34 and SM50 In Reciprocal Hybrid Embryos

Previous work by Tufaro and Brandhorst (1982) has described a reduced synthesis of paternal proteins in three heterotypic crosses of sea urchin embryos. Conlon *et al.* (1987) further investigated the expression of species-specific genes in the reciprocal crosses between *L. pictus* and *S. purpuratus* and

confirmed an underrepresentation of paternal transcripts in the SxL cross. In addition, they reported an underrepresentaion of the same *L. pictus* specific transcripts in the LxS cross. These transcripts are of zygotic rather than maternal origin in *L. pictus*. These observations indicate that the reduced expression is not occurring as a result of the *L. pictus* genes being maternally rather than zygotically expressed. Further investigations have shown that the reduced levels of several *L. pictus* transcripts, in particular metallothionein, are not correlated to transcriptional inactivity of these genes, suggesting that the reduced accumulation of these mRNAs is post transcriptionally restricted in the hybrid embryos (Conlon et al., 1987). This may be occurring at the level of mRNA processing, export, or stability.

Gene-specific analyses have been conducted to determine if reduced accumulation of other RNAs is observed. There is no exclusion of expression of histone genes in SxL hybrid embryos (Maxson and Egrie, 1980). *S. purpuratus* actin gene expression is properly regulated both temporally and spatially in both heterospecific crosses, indicating that the regulatory factors necessary for proper expression must be present in both crosses of hybrid embryos (Bullock et al., 1988; Nisson et al., 1989). Although the *L. pictus* homologues of these actin genes have recently been cloned and characterized, (Fang and Brandhorst, 1994) their expression has not yet been carefully characterized in the heterospecific crosses. Preliminary data indicate that the aboral specific

L. pictus C2 actin gene, the putative orthologue of the aboral ectoderm specific S. purpuratus CyIIIa actin gene, is underexpressed in these hybrid embryos.

The *L. pictus* LpS1 gene and the *S. purpuratus* Spec1 gene are aboral ectoderm-specific calcium-binding proteins whose functions have not been elucidated. These genes have been characterized as homologues (Nisson et al., 1992), and their expression in interspecies hybrids has been investigated. In both hybrid crosses, Spec1 is expressed at virtually normal levels while the LpS1 transcript is underrepresented, most notably at the pluteus stage. This observed reduction in LpS1 transcript level is a developmentally progressive restriction of expression, and LpS1 is detectable in only approximately 2% of the aboral ectoderm cells of plutei of both hybrid crosses. These cells expressing LpS1 in hybrid embryos are in some cases derived from more than one founder cell, and in other cases represent less than the lineage of a single founder cell. At the gastrula stage, 50% of the SxL embryos express Spec1 exclusively, 2% express LpS1 exclusively, and the remaining individuals are seen to coexpress both genes in the same cells. Various models have been proposed to account for this observation. First, this phenomenon may occur as a result of a dominance of the *S. purpuratus* genome over the *L. pictus* genome, and that the LpS1 gene is some how inactivated in most cells. Another possible explanation is that these two genes compete for a common transcription factor and that Spec1 has a higher affinity for this factor resulting in lower LpS1 accumulation. This model is plausible if the Spec1 gene is lost in a small number of cells during

hybrid embryo development. However, the promoters of the LpS1 and Spec genes are quite dissimilar.

The work in this thesis further contributes to the body of knowledge regarding gene expression in hybrid embryos generated between *L. pictus* and *S. purpuratus*. The LSM34 and SM50 genes are only expressed in primary mesenchyme cells and encode spicule matrix proteins involved in skeletogenesis. These genes are reported to be homologues (Livingston et al, 1991), and in the homospecific crosses, these genes are initially turned on at the mesenchyme blastula stage and continue to be expressed through to the pluteus stage during active skeleton formation. We have investigated their patterns of expression in reciprocal interspecies hybrids. RNA gel blot analysis shows that both transcripts are present in later stages of embryogenesis, during larval skeleton formation. There appears to be no down regulation of either of these genes in either hybrid cross. In some blots, the level of each gene appears to roughly half (or less) in intensity when compared to its level in the homospecific embryo at the same stage, although no quantitative analysis has been conducted. This reduction is expected as the hybrids contain only one copy of each gene. The SM50 transcript appears to accumulate slightly earlier than the LSM34 transcript in both crosses of hybrids. These data are preliminary and due to relatively low abundance of these messages in the total population of RNA in embryonic cells, further experiments are required to quantify these RNA levels. If there is indeed a temporal difference in the expression of these two

homolgues, there are several possible explanations. First, the two genes may be regulated by different transcription factors suggesting that these factors are also both expressed in these hybrid embryos. Although each transcript is present at the mesenchyme blastula stage in the homospecific crosses, perhaps the SM50 is initiated slightly earlier in *S. purpuratus* than LSM34 is in *L. pictus*. The observed difference in the accumulation of LSM34 and SM50 in both hybrid crosses may be as a result of a slightly later temporal activation of the LSM34 gene that is more evident when the gene is present in a single copy. Perhaps the one copy of this LSM34 gene is being transcribed but that the accumulation is slower and therefore more difficult to detect on a RNA gel blot at this stage. Secondly, the same regulatory factor(s) may be involved, but the *S. purpuratus* gene promoter may initially bind this factor more efficiently. The later appearance of the *L. pictus* transcript may result from an increase in the level of activity of a limiting factor.

The apparent coexpression of these genes in both crosses of hybrids is interesting in contrast with the results reported for LpS1 and Spec1, the metallothionein genes, and the preliminary data for *L. pictus* C2 actin. Although there is an underrepresentation of some *L. pictus* transcripts in these heterospecifc crosses, this is an example in which this is not the case.

Insitu data detailed in this thesis shows that both mRNAs are present in the appropriate cells suggesting that the proper regulation of spatial expression is maintained in both crosses of hybrids. This may be as a result of shared

spatial regulatory factors that are conserved between the two species; or it may imply that both sets of factors for proper spatial expression are active in these hybrid embryos..

Cell count experiments indicate that there are similar numbers of PMCs expressing SM50 in *S. purpuratus* gastrulae and in both crosses of hybrid gastruale. In addition, LSM34 is also expressed in a similar number of PMCs in *L. pictus* and SxL embryos. Although this is not absolute, these data indicate that there are a population of PMCs that are actively engaged in spicule formation, and a population of PMCs that are not. The similarities in LSM34 positive PMCs in L. pictus and SxL gastrulae; and SM50 positive PMCs between *S. purpuratus* and the reciprocal hybrid embryos observed, in conjunction with the information provided by the RNA gel blot data that shows both transcripts are present, suggests that at least a portion of the PMCs are coexpressing both transcripts. To further test this, a method for detecting two transcripts in situ would be required.

Attempts to produce an effective double labelling protocol for detecting two different transcripts within a single embryo, have met with limited success. In our hands, the use of fluorescein-UTP and anti-fluorescein conjugates has not provided the sensitivity required for the detection of mRNA *in situ*. In addition, other substrates for alkaline phosphatase that produce different colour detection have been met with limited success.

In this thesis, it is reported that the expression of spicule matrix protein genes is limited to a subpopulation of PMCs. This is supported by work done by Harkey and Whiteley. In addition, it has been reported that msp130 mRNA is accumulated to higher levels in PMCs that are localized to areas of skeleton growth (Ettensohn and Malinda, 1993). msp130 is a putative modulator of calcium transport, and the increased expression in areas of active spicule synthesis is not suprising given that calcium is a necessary component of the spicules (Harkey et al., 1992). Recent experiments published by Ettensohn and Malinda (1993) have shown by measuring [^{45}Ca] uptake, that the lengthwise growth of the larval spicules occurs at the tips and not by intercalary growth. Intercalary growth appears to be restricted to radial growth that increases the girth of the rods. In addition, it has been reported that in the feeding *Lytechinus variegatus* pluteus larva, the number of PMCs is approximately double the number reported for pre feeding larvae. 105-110 cells are reported to be present five days after fertilization in this species (Ettensohn at Malinda, 1993). This may be due a requirement of new populations of PMCs for arm addition and elaboration of the skeleton that occurs after feeding and growth are initiated.

Several elegant transplantation experiments clearly demonstrate that the skeleton size is regulated by the ectoderm, and is independant of the number of PMCs present and actively engaged in spicule synthesis (Armstrong and McClay ; Ettensohn and Malinda, 1993). Half and quarter size embryos generated by cell separation after the fist or second cleavages produce spicules that are also

half and quarter size respectively. PMCs from normal size embryos have been transplanted into the blastocoel of these half and quarter size embryos with the same results. Spicules synthesized by PMCs grown in culture are up to five times longer than normally found in the intact embryo. These two results combined indicate that the size restrictions are not intrinsic to the PMCs and that interactions with the rest of the embryo place constraints upon the growing skeletons and dictate the final size attained. Ettensohn and Malinda have proposed that there are two factors involved in the regulation of skeleton size. first is the rate of rod growth, and second is an internal PMCs "clock" that results in the restriction of time that a given PMC can partake in rod synthesis. The molecular clock hypothesis is supported by experiments in which mesenchyme blastula stage PMCs are transplanted to the tips of growing rods in pluteus larva. It has been shown that the number of PMCs at the tip of a rod does not affect the rate of synthesis of spicules. The microinjection of the young PMCs obtained soon after ingression to the tips of the pluteus larval arms results in an increase in rod length. This suggests that although the number of PMCs located at the tip of the growing rod has no affect on the rate of synthesis, the developmental stage of these cells affects the amount of further synthesis that will occur (Ettensohn and Malinda, 1993). This observation suggests that either there is a preprogrammed time that a PMCs can engage in spicule formation, or that there is a restricted amount of biosynthesis that any given PMC is capable of during development.

The reported observation that only a portion of the total PMC population expresses the spicule matrix protein genes may be explained in several different ways. First, it is possible that there is a divsion of labour between groups of these cells. Some cells may synthesize large quantities of matrix proteins, while other cells may express calcium modulator protein genes in abundance. This is unlikely because I have observed that most PMCs in the ventrolateral clusters in the gastrula stage embryos, express spicule matrix protein genes. Because these clusters are the sites of active spicule formation, this information argues that PMCs actively synthesizing spicules are likely expressing more than just a single spicule matrix gene. This could be further tested by using msp130 as a probe for whole mount in situ hybridizations, and by comparing the populations of PMCs that express spicule matrix genes and calcium modulator genes.

Another possibility is that a subpopulation of PMCs are reserved for later structural additions. This subpopulation may be the group that proliferates to increase the PMC number in feeding larvae. This model is supported by Ettensohn's molecular clock hypothesis. It is possible that if a PMC has a limited contribution to make during skeleton formation, that some will contribute early and some later. It is interesting to speculate as to the function of the PMCs that do not appear to partake in early skeletogenesis. The possibility that these cells are necessary for later additions to the skeleton preceding metamorphosis could be tested by removing PMCs trom the embryos such that the minimal number remained necessary for initial skelton formation without having SMC conversion.

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These embryos could then be reared through the feeding pluteus larval stages to assess whether all subsequent skeletal structures are formed normally. These experiments may also reveal any other possible functions of these cells.

The strict correlation between cell lineage and cell fate in the sea urchin embryo suggests that the PMCs that are involved in early skeleton formation (prior to feeding pluteus stage) may be derived from the same lineage. It is possible that the PMCs give rise to subpopulations of cells forming several lineages, and that each has a specific functional significance during skeletogenesis. Although all PMCs are equivalent in their potentials, perhaps there are positional assignments given to PMCs during cell divisions such that PMCs migrate to predetermined positions in the blastocoel according to their lineage. These discrete populations of PMCs may be clonally derived from one or several founder cells. It is also possible that the PMCs involved in the initial stage of skeletal from engaging in this process until later in development. This subpopulation of cells may then proliferate to produce the additional PMCs observed in older plutei (Ettensohn ans Malinda, 1993).

Conclusions and Directions for Future Research

In this thesis I have described the comparative morphology of *L. pictus*, *S. purpuratus*, and their reciprocal hybrid embryos. Although the overall morphology of the hybrid embryos is very similar to the maternal species, there are some characters that deviate from this. The process of gastrulation differs in these two species. *S. purpuratus* embryos exhibit a bilateral symmetry imposed by the position of the archenteron earlier than do the *L. pictus* embryos. It appears that both heterospecific crosses gastrulate in a fashion similar to the *S. purpuratus* embryos indicating a putative *S. purpuratus* dominance for the processes involved. In addition, this result indicates that the zygotic genome is responsible, at least in part, for the control of this process. This phenomenon requires further investigation in order to gain an overall understanding of gastrulation in sea urchin embryos.

The morphology of the pigment cells differs drastically between *L. pictus* and *S. purpuratus*. These differences were used to assess the inheritance of these secondary mesenchyme derivatives in the reciprocal hybrid larvae. This character appears to be maternally specified at the early pluteus stage. However, a more complete ontogenetic analysis is required to assess the further development of these cells. In order to do this, the larvae would require feeding. Feeding and rearing these hybrid larvae through metamorphosis would be of interest for numerous reasons. Preliminary results indicate that these hybrids

have the functional anatomy for feeding, although I have not raised them to metamorphosis. If these larvae were able to complete metamorphosis then this would allow for investigations into the interactions of these genomes in adults.

The morphology of the larval skeletons was examined herein. Three criteria were selected based on the ability to distinguish the species of origin of the particular characteristic. Inheritance of these characteristics was assessed in the hybrid larvae and an overall dominance of the *S. purpuratus* spicule morphology was observed in both crosses. The intrinsic pattern of spicule synthesis observed in culture and in other hybrid embryos made between species with significantly different rod structures makes the determination of maternal and paternal contributions relatively simple. The structure of the rods in *L. pictus* and *S. purpuratus* are very similar and are indistinguishable with the methods used herein. This makes it difficult to ascertain the contribution of a single PMC in a given hybrid embryo. It would be useful to use scanning electron microscopy to examine the spicules more closely with the aim to find some discriminating feature that differs between these two species. Measurements of thickness of various rods at specific locations may reveal differences that are tractable in larvae of these hybrid crosses.

The molecular characterization of the spicule matrix protein genes LSM34 and SM50 in these reciprocal hybrid embryos has revealed that both genes are expressed in both heterospecific crosses. The regulation of these genes differs temporally, a characteristic that is also observed in embryos of both

heterospecific crosses. *In situ* hybridizations with LSM34 and SM50 probes have allowed LSM34 and SM50 positive cells to be counted. In all cases, the counts reveal that only a subset of the total PMC population is engaged in the synthesis of these specific transcripts at a given time, It is of interest to determine whether this subset of cells is constant or if there is a limited amount of time that any given PMC can partake in spicule production. In addition, it is clear that these two transcripts are properly localized to the PMCs in the heterospecific embryos. There is a literature that demonstrates an underrepresentation of *L. pictus* transcripts in both crosses made between the two species. The data reported in this thesis for the LSM34 and SM50 gene homologues differs in that they are coexpressed. Future endeavors should involve the characterization of the expression of other pairs of homologous genes in these species. It would be interesting to examine other spicule matrix gene homologues in the hybrid embryos to determine if the coordinate expression seen for LSM34 and SM50 also occurs for other genes encoding spicule matrix proteins. It is possible that the coordinate expression/regulation of spicule matrix protein genes of both genomes within these hybrid embryos will be maintained throughout all aspects of spiculogenesis.

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