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**DEVELOPMENT OF MUSCLE STRUCTURE AND FUNCTION
IN LOLIGINID SQUIDS**

BY

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DISSERTATION

Submitted to the University of New Hampshire
in Partial Fulfillment of
the Requirements for the Degree of

Doctor of Philosophy

in

Zoology

December, 2004

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DEDICATION

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ABSTRACT

DEVELOPMENT OF MUSCLE STRUCTURE AND FUNCTION
IN LOLIGINID SQUIDS

by

Gabriela Maria Martinez

University of New Hampshire, December, 2004

Squid embryos are able to contract their mantle early during the embryonic period. These contractions are initially sporadic but become progressively more coordinated over the embryonic period and by hatching squids are able to locomote using jet propulsion. The ability to jet propulse and respire emerges during the pre-hatching stages of development and it requires, in part, the differentiation and organization of the circular and radial muscles of the mantle. How and when the musculature of the mantle, funnel and fins develops and acquires functional ability has not been investigated in cephalopod embryos.

This dissertation examines the onset of contractile capabilities and subsequent maturation of the main locomotor structures in embryos of two species of loliginid squids with a focus on the mantle musculature. The functional implications of the differentiation and organization of the musculature is investigated using a combination of techniques including fluorescent labeling, light and transmission electron microscopy, high speed video and mathematical modeling

The results of these series of studies indicate that the development and emergence of functional competence of the mantle musculature in loliginid squids is a dynamic process. Differentiation and organization of the musculature of the main locomotor structures does not occur simultaneously and has a precise sequence with the mantle developing first, then funnel and the fins developing and organizing last. This sequence applies to both gross morphological and ultrastructural levels of development. Additionally, the two circular fiber types found in the adult mantle have different temporal and spatial patterns of development. The superficial mitochondria rich (SMR) fibers that drive respiratory contractions differentiate first at the inner and outer surfaces of the mantle. The central mitochondria poor (CMP) muscle fibers, which are active during fast and escape jetting, differentiate second and in the central region of the mantle.

The mantle of embryonic loliginid squids is able to produce contractions in the absence of a completely developed and organized musculature. Contractions are first observed and recorded in Arnold stage 25 embryos. These contractions are rare and sporadic. During subsequent stages, the mantle undergoes measurable ontogenetic kinematic changes as evidenced by an increase in the frequency and duration of the contractions. Moreover, mathematical modeling of these contractions indicates that the mantle in embryonic squids is capable of producing two distinct types of contractions. These different contraction types resemble the respiratory and locomotory contractions of the juveniles and adults. Furthermore, these functionally distinct contractions emerge at different times:

the respiratory-like contractions emerge first and locomotory like contractions second.

When these data are examined in conjunction with the morphological data they show that mantle morphology and mantle functional ability appear to be developing in parallel. Additionally, stage 27 emerges as a morphologically and functionally significant point in development. Stage 27 embryos have a robust, differentiated mantle whose morphological organization and functional repertoire begins to reflect that of the adults.

CHAPTER I

INTRODUCTION

An inherent relationship between musculature and function is found throughout the animal kingdom. Similarly, there is a relationship between development and function. During development, three features change over time: morphology (size and structure), function, and the relationship between the structures and their underlying functions. During the embryonic period, changes occur in what an organism is capable of doing with its existing structures. All embryos go through several functional transitions from random, uncontrolled twitching to coordinated movement. How and when those transitions occur is directly influenced by the extent of muscle and neural development (Bekoff, 1981; Drachman, 1963; Wu et al., 2001).

The development of different functional morphologies may not be as simple as the complete construction of a structure first, followed by a subsequent gain of function. It could well be that the process is more complex; structures and related functions may develop and mature in parallel rather than sequentially. This dissertation investigates the dynamic developmental relationship between form and function by looking at the differentiation and organization of the mantle musculature, as well as, its emerging functional abilities in embryonic loliginid squids.

Cephalopods, and squids in particular, are ideal organisms on which to investigate questions regarding the development of morphology and the development of function. Cephalopods must be able to swim immediately upon hatching, and are composed primarily of muscular tissue (Budelman, 1994). They are direct developers that do not undergo a typical radical molluscan metamorphosis and thus do not emerge as miniature replicas of the adults. Cephalopods must go through several morphological and functional ontogenetic transitions in the post hatching period as direct responses to changing Reynolds number and hydrodynamic environments (Preuss and Gilly, 2000; Preuss et al., 1997; Thompson and Kier, 2001a; Thompson and Kier, 2001b; Thompson and Kier, 2002). Moreover, the musculature of cephalopods is especially interesting because of the ability of a single structure to fulfill different functions, specifically the dual respiratory and locomotory functions of the mantle. All of these characteristics offer an excellent system in which to examine the development of form and function.

Cephalopod Overview

Cephalopods are diverse and successful molluscs. There are approximately 700 species of cephalopods (Sweeney and Roper, 1998; Voss, 1977) and all but one are exclusively marine (*Lolliguncula brevis* lives in brackish water). They are found in all of the world's ocean main zones from the Arctic Basin to Antarctica (Roper et al., 1984). Cephalopods differ dramatically from their molluscan cousins in almost all aspects of their biology including their

embryology, ecology, behavior and locomotion. This class is characterized by a body plan that has retained the bilateral symmetry of its ancestors with an anterior portion that includes the head, funnel and the circumoral arm crown and a posterior portion that includes the mantle, mantle cavity and its organs (Beasley et al., 1998). Like other molluscs, all cephalopods have a radula and most have a shell remnant in the form of either an internal cuttlebone (cuttlefish) or a gladius (squids). Of all the extant cephalopods, only one genus, *Nautilus*, has retained an external shell (Beasley et al., 1998).

Cephalopods differ from other molluscs because they posses a sophisticated suite of behaviors, and a novel combination of methods for locomotion. These animals have successfully invaded all of the oceanic zones and this is reflected in their morphological adaptations. For example, some benthic octopods crawl using their arms (Beasley et al., 1998). Pelagic octopods, such as the Bolitinaids and Japatellids, use their webbed arms in a medusoid form of movement (Seibel et al., 1998). Cephalopods use jet propulsion to varying degrees as part of their locomotory repertoire. Squids are by far the most sophisticated cephalopod swimmers, and have evolved a highly proficient method of jet propulsion that allows them to swim with speed and agility that rivals fish(O'Dor and Webber, 1986; Wells and O'Dor, 1991). Squids can reach speeds of 10-15 knots (Packard, 1969; Young, 1975), swim continually for over two months, and can cover distances up to 2000 km (Gosline and DeMont, 1985; Packard, 1969). In fact, the locomotory system of squids

produces the fastest locomotion of any aquatic invertebrate (Moon and Hulbert, 1975; Packard, 1969).

Cephalopod Muscle Arrangement and Structure

Jet propulsion in squids is possible in part because of the arrangement and structure of the muscles in the squid mantle (Bone et al., 1981; Budelman et al., 1997; Gosline and Shadwick, 1983a; Gosline and Shadwick, 1983b; Gosline et al., 1983; Kier, 1988a). The mantle is also used for respiration, and its complex arrangement of muscle fibers allows it to function as a hydrostatic skeleton to provide movement and structural support. Hydrostatic skeletons are skeletal support systems that are normally comprised of two-dimensional or three-dimensional arrays of muscles surrounding fluid filled cavities (Kier, 1988a; Kier, 1992; Wainwright, 1982) that remain constant in volume. Hydrostatic skeletons create movement by shuttling fluid (which is incompressible at physiological pressures) in hydrostatic cavities from one part of the body to another (Kier, 1988a; Kier, 1992). In structures where fluid filled spaces are small or lacking, the muscles themselves serve as the incompressible fluid; changes in the dimensions of the muscular structures themselves create movement (Kier, 1988a; Kier, 1992; Kier and Smith, 1985; Smith and Kier, 1989).

The structure of the squid mantle is a complex, three-dimensional arrangement of muscle fibers between two stiff collagenous tunics that provide extra support (Bone et al., 1981; Budelman et al., 1997; Gosline and Shadwick, 1983b; Kier, 1982; Kier, 1985; Kier, 1987; Kier, 1988b). Within the mantle

muscles is a network of connective tissue fibers against which the muscle fibers can work. Squids possess different types of muscle fiber that are functionally and metabolically analogous to vertebrate red and white muscle (Bartol, 2001a; Bone et al., 1995b; Bone et al., 1981; Gosline and Shadwick, 1983b; Gosline et al., 1983; Mommsen et al., 1981). These different fibers are distributed differently within the mantle enable the mantle and provide the ability to carryout the dual functions of locomotion and respiration (Bone et al., 1994; Wells, 1988; Wells and Wells, 1982). The muscle fibers responsible for quiet ventilation and respiration are located at the inner and outer zones of the mantle and are characterized by a core of multiple mitochondria. The muscle fibers that power escape jetting are found in the central zone of the mantle and are mitochondria-poor (Bartol, 2001a; Bartol, 2001b; Bone et al., 1981; Gosline et al., 1983; Hochachka et al., 1975; Mommsen et al., 1981; O'Dor, 1982).

Cephalopod Respiration and Locomotion

In respiration, the mantle is mechanically responsible for the continual circulation of water in and out of the mantle (Bone et al., 1994; Wells, 1988; Wells and Wells, 1982), allowing water to pass across the gills where oxygen is extracted. During respiration, water is sucked into the mantle cavity by the expansion of the mantle (Packard, 1972). Water is expelled by contraction of the circular muscles and it is pushed through the funnel (Budelman et al., 1997). Respiration and locomotion are incompatible with one another because the sudden hyperinflation and exhalation required to produce a jet does not allow

enough time for oxygen extraction (Wells, 1988). Oxygen measurements in octopods, cuttlefish and *Nautilus*, have shown that oxygen levels drop as soon as jetting behavior begins (Wells, 1988). Respiration movements are mostly restricted to the anterior portion of the mantle, in the gill region (Packard and Trueman, 1974). Respiratory movements are also seen in the posterior half of the mantle; these movements are smaller, have a different frequency and thus are out of phase from the movements in the anterior region (Packard, 1972).

Functionally, respiration requires the mantle muscles to execute two different patterns of activity based on the type of respiration, either passive (at rest, ventilation) or active (preparing for a jet escape) (Bone et al., 1994). As previously mentioned, the ability to perform the two phases of respiration rests in the ultrastructural and biochemical differences of the radial and circular muscles (Gosline et al., 1983; MacGillivray et al., 1999; Mommsen et al., 1981). The circular and radial muscles are active at different points of the cycle and neither type of muscle is active during the refilling phase (Gosline et al., 1983). The radial muscles are involved in the inhalation phase of the respiration cycle and circular muscles are active during the exhalation phase (Gosline et al., 1983).

Jet propulsion has three distinct phases: inhalation, expulsion and refilling. Jet propulsion is an expensive mode of locomotion because it requires continual cycles of acceleration and deceleration (Gosline and DeMont, 1985; Wells, 1988). During inhalation the radial muscles contract, increasing the diameter of the mantle opening, sucking water into the mantle cavity, and filling it to its maximum capacity (Gosline et al., 1983). Expulsion is powered by the

contraction of the circular muscles decreasing the diameter of the mantle forcing water through the funnel resulting in the jetting action. The jet phase is immediately followed by the refilling of the mantle through the elastic recoil of the intramuscular connective tissue system (Gosline and Shadwick, 1983a; Gosline and Shadwick, 1983b; Kier, 1988a; Ward and Wainwright, 1972). Neither the radial or circular muscles appear to participate in the refilling of the mantle cavity (Gosline et al., 1983).

Cephalopod Development

Growth in cephalopods has traditionally been thought of in terms of overall body size and age, not necessarily as an increase in muscle mass (Forsythe and Van Heukelom, 1987). Recently however, growth has been studied as an increase in total muscle fiber growth and recruitment (Martinez and Moltschaniwskyj, 1999; Moltschaniwskyj, 1994). Several studies have determined that squids and cuttlefish grow continuously throughout their lifetime (Martinez, 2001; Martinez and Moltschaniwskyj, 1999; Pecl and Moltschaniwskyj, 1997). Moreover, this continual growth is due to a combination of hyperplasia and hypertrophy occurring at different rates at different stages during an animal's life span (Martinez and Moltschaniwskyj, 1999; Moltschaniwskyj, 1994; Pecl and Moltschaniwskyj, 1997). This continual addition of muscle fibers explains how cephalopods can have such rapid growth rates and can reach such extremely large sizes in a short period of time (Jackson and Choat, 1992; Moltschaniwskyj, 1994; Pecl and Moltschaniwskyj, 1997).

Unlike other molluscs, cephalopods have direct development (Arnold, 1965; Fields, 1965; Naef, 1928). They undergo meroblastic, radial cleavage that most closely resembles teleost development (Packard, 1972) as opposed to the developmental modes commonly seen in molluscs. Most cephalopod eggs are large - millimeters as opposed to micrometers - and telolecithal, with the embryo proper growing at the animal pole on top of a very rich yolk. They have no larval phase and thus no metamorphosis, yet they do not hatch out exactly as miniature adults (Boletzky, 1979; Nesis, 1979; Young and Harman, 1988). Cephalopods do undergo some notable, if not dramatic, changes in their overall body shape and some structures (such as the tentacles) in response to their changing hydrodynamic environment and other functional demands (feeding) (Shea, in press; Vidal, 1994).

Cephalopod embryology has been well described in classical studies by Naef (1928) and Arnold (1965) and it continues to be studied in more species. Loliginid squids make up the bulk of the embryological literature and has become the "textbook" representative of cephalopod embryology (Wells, 1988). Both Naef (1928) and Arnold (1965) created staging tables based on explicit morphological characteristics and landmark events in order to standardize cephalopod embryology. These two staging schemes have been widely used and subsequently modified to accommodate the differences in development of the increasing number of species described (Blackburn et al., 1998; Shigeno et al., 2001a; Watanabe et al., 1996).

Recently, there has been an increase in the number of developmental morphological descriptions of the organogenic period of many different species of cephalopods (Blackburn et al., 1998; Shigeno et al., 2001a; Shigeno et al., 2001b; Shigeno and Yamamoto, 2002; Warnke, 1999; Watanabe et al., 1996). These descriptions tend to focus on the overall development of the animal or more specifically, the nervous system (Shigeno et al., 2001b; Shigeno and Yamamoto, 2002; Warnke, 1999; Watanabe et al., 1996). These and more recent studies of other cephalopod species have paid little attention to the development of muscles, except for brief references to the growth of the mantle and the fusion of the funnel folds into a tube. Muscle differentiation and growth is inferred but not described. Similarly, early embryonic movements displayed by cephalopod embryos are referred to in passing, with no elaboration or mention of possible functional significance (Arnold, 1965; Fields, 1965; Naef, 1928). Furthermore, little is known about respiration and other early embryonic movements. Aside from observations by Naef (1928) and oxygen consumption studies of egg masses (Parra et al., 2000), almost nothing is known about embryonic respiration in squids. Embryonic respiration is thought to occur by diffusion of oxygen through the outer epidermal epithelial layer (Cronin and Seymour, 2000; DeWachter et al., 1988; Ruppert et al., 2004) until the mantle is able to contract enough to effectively move water around within the chorion that encapsulates the embryo. Presumably, once the mantle has developed enough to contract and the gills can extract oxygen, then respiration is similar to that of adults.

The muscular system of cephalopods offers an excellent opportunity to examine the development of form and function because it has been well studied in adults and juveniles, and thus offers a good comparative base. The available ontogenetic information about cephalopod musculature is based on studies that have been conducted on post-hatching juveniles and adults (Table I-1) (Bone et al., 1981; Chen et al., 1996; Hoyle, 1964; Kier, 1988a; Preuss et al., 1997; Shea, in press; Thompson and Kier, 2001a; Thompson and Kier, 2001b; Thompson and Kier, 2002). Fundamental information such as the embryonic origins of muscle, muscle cell differentiation and muscle function are virtually unknown. Studies by Preuss (1997,2000), and Thompson (2001a, 2001b, 2002) have integrated development, morphology and function of the mantle musculature and connective tissue in paralarval squid. (Gilly et al., 1991; Preuss and Gilly, 2000; Preuss et al., 1997) have effectively shown the functional and morphological maturation of the circular mantle muscles in hatchling and paralarval *Loligo opalescens* by integrating structural, histological and functional data. Similarly, Thompson and Kier. (2001a, 2002) described the substantial morphological and functional changes that occur in the intermuscular collagen system in hatchling and paralarval *Sepioteuthis lessoniana*. The changes in these collagenous networks have significant effects on the kinematics and mechanics of jet propulsion in young squids that in turn affects not only their ecology, but also their behavior. Ontogenetic changes in somatic and muscle growth in juvenile and adult sepiolids and sepiids have been studied (Martinez and Moltschaniwskyj, 1999; Pecl and Moltschaniwskyj, 1997). Gilly et al. (1991) have

described the development of neural control of escape responses in embryos and hatchlings and inadvertently also mentions changes in muscles and their functional capacities.

Development of Function

Historically, the field of functional morphology has not been considered within a developmental context. Most functional morphologists conduct their studies on adult morphologies and adult functions (Savazzi, 1999). In general, there is more biomechanical and kinematic data for vertebrate systems than Fish swimming studies encompass everything from different modes of locomotion across a diverse group of fishes to startle/escape responses to the contribution of fins to locomotion (Beddow et al., 1995; Domenici and Blake, 1997; Drucker and Jensen, 1996; Hale, 1999; Hove et al., 2001; Jayne and Lauder, 1994; Johnston, 1980; Johnston, 1991; Westneat et al., 1998). Different types of musculature in fish have also been identified and their role in fish locomotion as well as their physiology and growth has been well documented (Bone, 1966; Bone, 1978; Devoto et al., 1996; Jayne and Lauder, 1994; Veggetti et al., 1990; Weatherley and Gill, 1987; Weatherley et al., 1988; Zimmerman and Lowery, 1999).

Recently there has been a slight shift in the types of kinematic and biomechanical studies being conducted on fish. There is an increasing body of literature regarding changes in locomotion and musculature in a wide variety of larval and juvenile fish (Batty, 1981; Batty, 1984; Hale, 1999; Johnston, 1994; Wakeling et al., 1999). This work ranges from changes in muscle fiber types to

changes in function through ontogeny (Patruno et al., 1998; Rowlerson et al., 1995; Sanger and Stoiber, 2001; Stoiber et al., 1998; Stoiber et al., 2002; Stoiber et al., 1999; Veggetti et al., 1990). Many studies however, focus either on changes in muscle morphology and structure or changes in swimming movements (Galloway et al., 1999; Osse, 1990; Webb and Weihs, 1986; Zimmerman and Lowery, 1999). Few delve into how changes in both morphology and function relate to one another.

One of the few studies examining the relationship between form and function during development was conducted on larval herring (Batty, 1984). Batty examined the changes in swimming kinematics and found that swimming style changed with growth and development. As the caudal and dorsal fins formed, the swimming style changed dramatically from what was first seen in early post-yolk sac larvae. Batty found that the distribution of red and white muscle fibers also changed, from a single layer on the outside of the myotomes to their adult distribution at the midline of the flank near the skin. This transition did not occur until after the gills had become fully functional (Batty, 1984).

Stoiber et al. (1999) took a different approach to investigating the development of morphology and function. Instead of looking at changes in kinematics they looked at changes in muscle cell morphology, muscle fiber growth and ultrastructure over the course of larval and juvenile ontogeny. Stoiber's work looks at the formation of fish muscle and correlates it with significant developmental functional landmarks such as hatching, onset of free swimming, and exogenous feeding (Stoiber et al., 1999). Both Batty and Stoiber

come to the same conclusions however, that changes in form and function of fish musculature are a direct result of the different functional demands experienced in changing Reynolds number environments (Batty, 1984; Stoiber et al., 1999).

Studies of functional morphology are generally conducted on juveniles or adults of the organism of interest, and seldom are form and function investigated together in an embryological context. Studies that have examined the dynamic between form, function, and development have shown that changes in morphology can have direct consequences on the proper development and function of several systems. For example, in chicken embryos, early embryonic movements are essential for the proper formation and development of a locomotor system (Wu et al., 2001). In fish embryos, studies have shown that progression through significant life stage transitions are directly linked to the extent of muscle formation and differentiation (Balon, 1985; Stoiber et al., 1998; Stoiber et al., 2002; Stoiber et al., 1999)

Functional morphological studies in post-hatched and juveniles of a number of invertebrate and vertebrate species have established that even in the post-embryonic period significant morphological changes continue to occur that in turn affect the ecology of the organism (Batty, 1984; Chen et al., 1996; Hunt and Seibel, 2000; Seibel et al., 1998; Shea, in press; Thompson and Kier, 2001b; Thompson and Kier, 2002).

Dissertation Objectives

Cephalopods use the same structure, the mantle, for locomotion and respiration. The biomechanics and kinematics of adult cephalopod jet propulsion, as well as, the physiology and ultrastructure of the musculature involved has been well studied. In contrast, there have been a limited number of similar investigations in juveniles and embryos. Fundamental questions pertaining to the ontogeny of the primary locomotor structures and subsequent functional capabilities remain to be investigated. The objectives of this study were (1) to describe the gross morphological and ultrastructural development of the mantle musculature in two species of loliginid squids; (2) to correlate muscular development and organization with the development of functional ability.

In addition to direct observations of developing embryos, I used fluorescent labeling to view emerging muscle development, transmission electron microscopy to analyze ultrastructural development, high-speed video and mathematical modeling to examine and quantify functional development. Though all of these techniques have been employed individually in functional morphology studies in a variety of different organisms, they have not been used together in a comprehensive attempt to address fundamental questions of cephalopod muscle differentiation and function. These data illustrate that functional ability can be measured both quantitatively and qualitatively prior to hatching and that biomechanical and kinematic experiments should no longer be limited by stage. Moreover, these studies reveal that form and function develop in parallel. There

are differences in the developmental timing of individual structures such that one structure may be further developed and therefore more functional prior to another, a process which is also species specific (Naef, 1928) (Balch et al., 1985; Blackburn et al., 1998).

Table I-1. Compilation of previous work on cephalopod musculature.

Family	Species	Type of work	Age	Structure	Reference (1)
Loliginidae	<i>Alloteuthis subulata</i>	structure, biomechanics	adult	arms	Bone et al, 1983; Gosline et al, 1983;
	<i>Loligo forbesi</i>	kinematics, physiology	juvenile	tentacles	Thompson & Kier, 1999, 2002, 2001; Kier, 1988;
	<i>Loligo opalescens</i>	ultrastructure, growth	hatch	mantle	Kier & Curtin, 2002; Kier & Smith, 1985;
	<i>Loligo pealeii</i>	swimming, behavior			Preuss et al, 1997; Gilly et al, 1991; Packard, 1969;
	<i>Loligo vulgaris</i>				Mommsen et al, 1981; Johnson et al, 1972;
	<i>Lolliguncula brevis</i>				McGillivray et al, 1999;
	<i>Photoligo sp.</i>				Moltschanewskyj et al 2000;
	<i>Sepioteuthis lessoniana</i>				Moltschanewskyj, 1994; Bartol, 2001a, 2001b;
					Milligan et al, 1997; Finke et al, 1996;
					Otis & Gilly, 1990; Anderson et al, 2000;
					Rogers et al, 1997; Chen et al, 1996.
Ommastrephidae	<i>Illex illecebrosus</i>	biomechanics	adult	arms	Perez et al, 2000;
	<i>Ommastrephes bartramii</i>	ultrastructure	juvenile	tentacles	Mommsen et al, 1981;
	<i>Ommastrephes sp.</i>	growth	hatch	mantle	Shea, 2001, 2004; O'Dor, 1988
	<i>Symplectoteuthis oualanensis</i>				
Gonatidae	<i>Bathyteuthis magister</i>	ultrastructure	adult	mantle	Mommsen et al, 1981
Sepiolidae	<i>Idiosepius pygmaeus</i>	growth	adult	mantle	Pech & Moltschanewskyj et al, 1997
Sepiidae	<i>Sepia elliptica</i>	growth, physiology	adult	mantle	Bone et al, 1983; Bone & Brown, 1994;
	<i>Sepia officinalis</i>	ultrastructure	juvenile	tentacles	Grimaldi et al, 2004a, 2004b;
		swimming behavior	embryo		Martinez & Moltschanewskyj, 1999;
		cell differentiation			Milligan et al, 1997; Moltschanewskyj, 1994;
					Nixon et al, 1998; Rogers et al, 1997
Vampyroteuthidae	<i>Vampyroteuthis infernalis</i>	swimming behavior	adult	arms	Hunt, 1996; Seibel et al, 1999
		biomechanics		fins	
		kinematics		mantle	
Octopodidae	<i>Cirrothauma murrayi</i>	physiology	adult	arms	Graziadei, 1966;
	<i>Eledone cirrhosa</i>	ultrastructure	juvenile	fins	Rogers et al, 1997;
	<i>Opisthoteuthis californiana</i>	swimming behavior	hatch	mantle	Seibel et al, 1999;
	<i>Octopus vulgaris</i>				Villanueva et al, 1996

(1) Partial list of references

CHAPTER II

EMBRYONIC DEVELOPMENT AND ORGANIZATION OF MANTLE, FUNNEL AND FIN MUSCULATURE IN *LOLIGO PEALEI*

Introduction

Although muscle tissue makes up a large percentage of the total body mass of cephalopods (Budelman et al., 1997; Gosline and DeMont, 1985; Hochachka et al., 1978; Martinez and Moltschaniwskyj, 1999) little is known about how muscle tissue develops and organizes during embryogenesis in this group. Previous developmental work in cephalopods has focused on descriptions of comparative morphology and overall formation of body structures and organs (Arnold, 1965; Fields, 1965; Naef, 1928) (Segawa et al., 1988; Shigeno et al., 2001a) rather than on the finer details of muscle development and organization. Moreover, although there have been many recent studies examining the formation and organization of myo-anatomy in invertebrate taxa including molluscs (Wanninger and Haszprunar, 2002a; Wanninger and Haszprunar, 2002b), flatworms (Hooge, 2001; Tyler and Hooge, 2004), phoronids (Santagata, 2001) and gastrotrichs (Hochberg and Litvaitis, 2000), these studies either investigate the formation of specific muscles (such as the larval retractor muscles of gastropods) (Wanninger and Haszprunar, 2002a) or the gross morphology of adults (Hochberg and Litvaitis, 2000; Hooge, 2001; Tyler and Hooge, 2004).

They therefore yield little information relevant to cephalopods, which have species with direct development and lack a larval phase.

The precise three-dimensional arrangement of musculature in the mantle, funnel and fins of cephalopods is the basis for how these structures function during respiration and locomotion (Budelman et al., 1997; Kier, 1988b; Kier, 1992). Although much work has been done on adult and juvenile stages with regard to ultrastructure, morphology and function, (Bone et al., 1981; Martinez and Moltschaniwskyj, 1999; Mommsen et al., 1981; Pecl and Moltschaniwskyj, 1997; Thompson and Kier, 2001b; Thompson and Kier, 2002) (Curtain et al., 2000) there remains a poor understanding of how and when the three-dimensional arrangements of musculature emerge during embryogenesis. Examining these processes is essential for both understanding the functional capabilities of these structures, and for relating the development of form to the development of function. Descriptions (Arnold, 1965; Fields, 1965; Naef, 1928) and observations (Martinez, pers .obs.) of morphological development of the mantle, funnel, and fin structures suggest that muscle development and organization occurs asynchronously in these structures. In order to better understand how and when muscle differentiation and organization occurs, and in particular to determine whether muscle development takes place in the mantle, funnel, and fins at different times, this project investigated the spatial and temporal development and organization of the mantle, funnel, and fin musculature in embryonic *Loligo pealeii*.

Materials and Methods

Experimental Animals

Loligo pealeii egg cases were obtained from the Marine Resource Center, Marine Biological Laboratories, Woods Hole, MA. Egg cases containing embryos at different developmental stages were selected and kept in well-aerated seawater tanks at ambient temperature (13-14°C) at a salinity of 32ppt until needed, approximately 1-2 weeks. Normally developing embryos were selected and staged using Arnold's (Arnold, 1965) staging scheme. Sampling of embryos began with those determined to be at Arnold stage 21 (i.e. embryos with rudimentary mantle and funnel placodes) and continued to stage 30 (hatching) to obtain a developmental series.

Fixation and F-Actin Labeling

While still inside their egg capsules, embryos from Arnold stages 21-30 (Arnold, 1965) were fixed in 4% paraformaldehyde for 1.5 hours (Appendix A). After fixation, embryos were dechorionated and the mantle, funnel, and fins were removed. The individual structures were rinsed with 0.1M Phosphate Buffered Saline (PBS) 3 times for ten minutes each rinse. Specimens were incubated in 0.2% TritonX-100 in PBS (PBT) for 1hour to permeabilize tissue. Specimens were stained with Alexa-488 phalloidin (Molecular Probes, Eugene, OR) in PBT for 1.5-2 hours. Samples were rinsed in 0.1M PBS 3 times for ten minutes each rinse.

4'6-diamidino-2-phenylindole dihydrochloride (DAPI) Labeling

Specimens at stages 24-29 were double labeled with DAPI (Sigma-Aldrich, USA) to view the shape and orientation of muscle cell nuclei. Phalloidin labeled specimens were first rinsed twice with distilled water and then labeled with DAPI for 30 minutes (Appendix A).

Microscopy

For all samples (phalloidin and DAPI), individual structures were mounted on glass slides in Gel Mount antifade mounting medium (Biomedica Corporation, Foster City, CA) and viewed under fluorescence with a Nikon Axiophot compound microscope. Micrographs were captured with a SPOT digital camera mounted on the microscope. Adobe Photoshop v. 7.0 was used to adjust brightness and contrast.

The results of the present study have been divided into three major developmental phases, Phase I/Early (Arnold stages 21-23), Phase II/Middle (Arnold stages 24-26) and Phase III /Late (Arnold stages 27-30), based on the extent of muscle fiber development and distribution prior to hatching.

Results

Gross Morphological Analysis of Muscle Formation

Phase I/Early Phase (Arnold Stages 21-23): Phase I of muscle development and organization begins during organogenesis. The landmark

morphological features of embryos at this phase include the presence of a rudimentary, cap shaped mantle, funnel folds that will begin to fuse and small, paddle shaped fins that have just emerged (Arnold, 1965). A thin epithelial layer (presumptive epidermis) with ciliary tufts surrounds the entire embryo.

Phase I is characterized by the complete lack of elongated muscle fibers in the mantle, funnel and fins. These structures, although present, are not composed of differentiated muscular tissue. Instead, phalloidin staining reveals a reticulated, mesh-like pattern (Fig. II-1a-c). The stained F-actin fibers are configured in a hexagonal array that is characteristic of cortical actin (Bray, 1992) (Fig. II-1a) located underneath the plasma membrane of cells. This reticulation is present in all planes of focus (Fig. II-1a-c) and in all three structures. The prevalence of this reticulated organization suggests that Phase I of muscle development is a period of cell proliferation rather than morphological differentiation of presumptive myoblasts.

Phase II/Middle (Arnold Stages 24-26): At the gross morphological level *L. pealeii* embryos have formed all of their organs and structures and the mantle, funnel and fins are solid structures. The mantle has transitioned from a small cap-shaped structure to a larger, elongated shape as a result of downward growth. The funnel is fully fused into a tube and the fins have grown larger but remain paddle-shaped.

Phase II is a transitional period during which presumptive myoblasts begin to differentiate into myofibers. Prior to Phase II the mantle, funnel and fins

are not composed of differentiated muscle tissue and exist only as scaffolding. During Phase II substantial muscle cell differentiation and organization occur. This differentiation manifests itself as the appearance of distinctive, elongated, muscle fibers. However, emergence of myofibers only occurs in the mantle (Fig. II-1e). At the start of Phase II, the mantle is heavily reticulated and elongated fibers are rare (Fig. II-1d). The funnel (Fig. II-1d) and fins are also heavily reticulated, although they are larger in overall size (Fig. II-1f).

As Phase II progresses there is an increase in both organization and the number of myofibers in the mantle (Fig. II-2a). The muscle fibers that emerge are circularly oriented but not tightly associated with one another, as evidenced by the large spaces between the fibers themselves (Fig. II-1e-g). As the number of muscle fibers increases, less and less of the mantle is reticulated (Fig. II-2a-c). During stage 25, muscle fibers emerge in the funnel (Fig. II-1f). The muscle fibers that first emerge are few but also circularly oriented. The fins remain predominantly reticulated except for one or two short fibers detected in a few specimens (Fig. II-2a).

DAPI stained specimens support the phalloidin evidence that Phase II is transitional. Mantles of embryos at stage 24 (Fig. II-3a) show labeled nuclei in different orientations, illustrating an overall lack of organization in this structure. By the end of Phase II, DAPI stained specimens show nuclei in distinctive circular orientation demonstrating the continued development of circular myofiber organization (Fig. II-3b). Although no cell counts were made, DAPI staining

shows (qualitatively) an increase in both the total number of nuclei and the number of circularly oriented nuclei (Fig. II-3a-b).

The end of Phase II is marked by mantle structures that are composed mainly of differentiated circular muscle fibers. However, the persistence of the reticulated pattern indicates that the mantle is also composed of cells that remain undifferentiated (Fig. II-1f-g; Fig II-2a). The muscle fibers of the funnel also continue to organize and fibers in both circular and longitudinal orientation are distinguishable (Fig. II-2f). However, there is a marked difference in both the extent of development and organization of the myofibers between the funnel and the mantle, but also in the number of fibers present. At the end of Phase II, the funnel is still heavily reticulated. Surprisingly, the funnel retractor muscles are very distinctive and composed largely of elongated myofibers (Fig. II-1f). The fins at the end of Phase II remain essentially undifferentiated, though larger than in Phase I (Fig II-2a).

Phase III/Late Phase (Arnold Stages 27-30): Squid embryos during the late stages of development are growing rapidly, and existing structures are acquiring functional capabilities (i.e. heart beat, mantle contraction) (Arnold, 1965; Fields, 1965; Naef, 1928). Mantle contractions are initially sporadic, but gradually become more frequent and rhythmic (Arnold, 1965; Fields, 1965; Naef, 1928).

Phase III is both a period of growth as well as a period of continued organization and differentiation depending on the structure. Phase III at the

outset appears to be a period of growth and an overall increase in muscle fiber number and density based on the patterns seen in the mantle (Fig. II-4a-b). By the start of Phase III, the mantle is a well formed, muscular structure with muscle fibers in their complex arrangements (Fig. II-2d-f). There is no morphological evidence of remaining undifferentiated myoblasts in the mantle, based on the lack of reticulation (other than in epithelial cells of the skin).

The funnel and the fins change and organize significantly during Phase III. In essence they are overcoming their developmental delay relative to the mantle. The funnel continued to grow throughout Phases I and II and although muscle fibers were evident in the middle of Phase II there was no substantial change in the overall organization and differentiation of the funnel musculature. In Phase III, the funnel finally develops and organizes its muscles into characteristic three-dimensional arrangements; the muscle fibers are in longitudinal, circular and radial orientations (Fig. II-4d). By hatching, the funnel, like the mantle, is a fully formed structure with densely arranged muscles.

The fins (Fig. II-4c), like the funnel, were essentially unchanged during Phases I and II. They got bigger, but without muscle fiber growth or muscle fiber addition. Over the course of four developmental stages (approximately 4-5 days), the fins transition from non-muscular parts to complex three-dimensional muscular structures. By hatching the mantle, funnel, and fins are well developed and organized muscular structures.

Discussion

The present study reveals that muscle development and organization do not occur simultaneously in the mantle, funnel, and fins of *L. pealeii* embryos. Although it was beyond the scope of this study it was noted that the rates of muscle development and organization differ among the three structures studied. This study also suggests that the development of muscle may be dependent in part, not only on the function of a structure but also on when that structure needs to be able to perform that function.

Timing

Most developmental staging tables, including those for cephalopods, assign stages based on easily identifiable morphological features and the timing of their appearance (Arnold, 1965; Fields, 1965; Martinez and Boker, 2003; Naef, 1928; Shardo, 1995; Shigeno et al., 2001a; Shigeno et al., 2001b; Shigeno and Yamamoto, 2002). In cephalopods, morphological landmarks used to determine the extent of embryonic development include the mantle, funnel, and the fins. The mantle is always identifiable first, followed by the funnel and finally the fins, although they are all distinctive structures within a few stages of one another (Arnold, 1965; Fields, 1965; Naef, 1928). Therefore, it is not surprising that the development of the musculature of these structures follows the same sequence. What is surprising is the assumption that these structures are already composed of muscle (Arnold, 1965; Fields, 1965; Naef, 1928) and that the development of their musculature is the same as the emergence of their morphology. Data from

this study show that in *Loligo pealeii*, muscle development and organization do not correspond with the initial morphological emergence of structures. Although the initiation the formation of each structure occurs simultaneously, the rate and timing of muscle development over the course of the embryonic period differ.

When muscle development and organization takes place in these structures seems to correlate not only to their underlying functional roles, but also to when the structures need to be active. At hatching, both respiration and locomotory capabilities must already be in place. The mantle is responsible for both respiration and locomotory functions and must therefore be well developed and functional prior to hatching. The mantle musculature is the first to emerge. Since newly hatched squids must locomote immediately at hatching by jet propulsion the funnel and fins must also be formed and functional, however, because the funnel and fins do not participate in respiration, the development of their musculature need not occur in synchrony with the development of the mantle musculature.

Because the three structures do not all have to be functional during the embryonic period, muscle development and organization can occur at different times and rates. Since the extent of development and function of the mantle is critical, the mantle musculature not only develops first, but also develops over a longer period of time. By comparison the funnel and the fin musculature develop later in embryogenesis and over a shorter period of time. The time it takes for development of the musculature in the mantle is reflected in how well formed the structure is and how well it can function at hatching. At hatching, the mantle is

morphologically and functionally broadly similar to the juvenile and adult structure. The funnel, though functionally not as complex as the mantle, is also well formed and can execute a variety of different movements to steer the animal during jet propulsion. In contrast, although the fins have their three dimensional organization and can function, they are still rudimentary (Boletzky, 1974; Hoar et al., 1994; Okutani, 1987) and poorly developed both morphologically and functionally compared to adult fins. This parallels their functional capabilities and contributions to locomotion in the immediate post-hatching period: The fins at hatching contribute little more than balance during locomotion (Boletzky, 1977; Boletzky, 2003; Vecchione, 1981).

Growth

Another characteristic of gross morphological development is growth of the embryo. Growth in most organisms occurs by hyperplasia (addition of new fibers) or hypertrophy (growth of existing fibers) or a combination of both during different developmental periods (Weatherley et al., 1988) (Goldspink, 1972). In cephalopods, it has been determined that growth occurs by a combination of hyperplasia and hypertrophy at different rates for the duration of the animal's lifespan (Martinez, 2001; Martinez and Moltschaniwskyj, 1999; Pecl and Moltschaniwskyj, 1997). This would suggest that growth during the embryonic period is also a result of hyperplasia and hypertrophy at different rates, however, there is no data to support this in the current literature.

Data gathered in this study strongly suggest that the primary mechanism by which loliginid embryos initially accumulate their body mass is a combination of hyperplasia and hypertrophy once the muscle differentiation program has begun. However, initial growth of the embryo appears to be a result of proliferation of undifferentiated myoblasts. Data from this study strongly points to this mechanism during the early phases of structure formation. This is supported by the presence of the reticulated pattern of F-actin seen in Phase I of muscle development. It is further supported because once elongated muscle fibers emerge in the mantle, the reticulated pattern still persists in all three structures and yet, they all continue to increase in size.

Continued growth of the funnel and fins continue even in the absence of differentiated muscle fibers suggests that loliginid embryos initially invest in accumulating size in these structures before differentiating muscle. In contrast, in the mantle both proliferation and differentiation occur simultaneously. The difference may be related to function (though that remains to be investigated). Because the mantle is involved in respiration as well as locomotion, and respiration starts well before hatching, it is critical for the mantle to get larger and differentiate faster than the other structures. Indeed, by Phase III, the musculature of the mantle is well organized into its characteristic arrangement and the only remaining task for the mantle is growth.

Conclusions

In the present study, I document the spatial and temporal development of the musculature of the mantle, funnels and fins in *L. pealeii*. This is the first gross morphological description of muscle development and organization in the pre-hatching phases of squid development. The sequence of muscle development and organization in each of these structures strongly correlates with their ultimate functions, as well as, with when these functions need to occur. The mantle is the first structure in which the musculature develops and organizes, followed by the funnel and then the fins. The rate of muscle development also varies between structures; development is slower in the mantle, but relatively fast in the funnel and the fins. Although muscle develops and organizes asynchronously, by hatching developmental differences in muscular formation among the three structures have been overcome and the mantle, funnel, and fins all have their correct muscular organization and have functional ability. However, during the immediate post-hatching period, their contribution to jet propulsion varies; the mantle and funnel contribute significantly to jet propulsion while the fins contribute little (Boletzky, 1974; Hoar et al., 1994; Okutani, 1987; Vecchione, 1981).

The results of this study do not provide proof that the ultimate functions of the structures examined cause them to develop when they do, indeed this study did not set out to test this: however, my results do suggest that at least to some degree function may influence developmental timing and should be tested further.

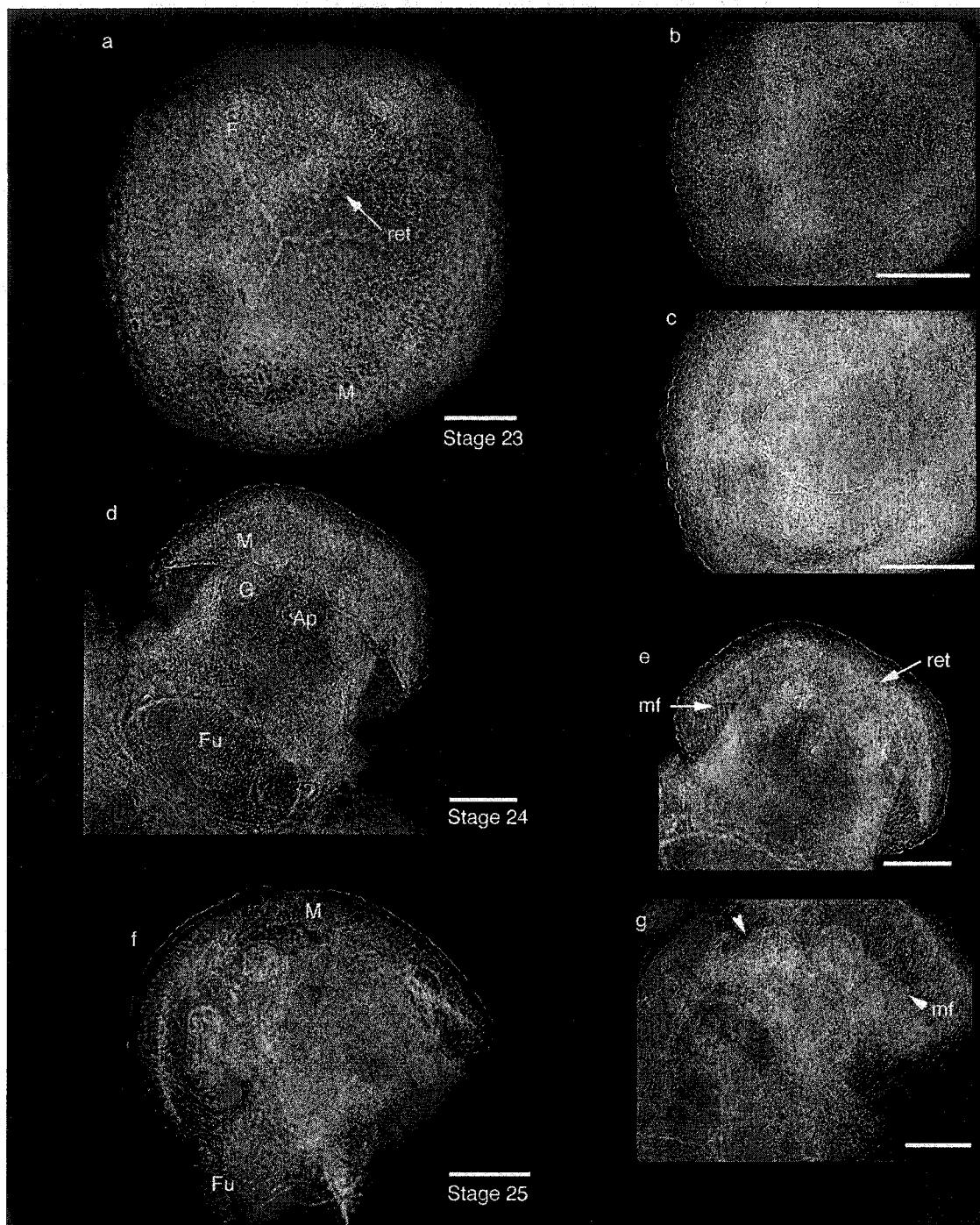


Figure II-1. Emergence of muscle fibers in *L. pealeii* mantle from Arnold Stages 23-25. a-c. Stage 23 mantle with distinct reticulated (ret) pattern of F-actin. d-e. Stage 24 muscle fibers appear in mantle only, reticulated pattern still present, not funnel (Fu) has no muscle fibers. f-g. Stage 25 more muscle fibers present, but reticulation pattern not as prominent (arrowheads), funnel still reticulated. All scale bars 100 μ m.

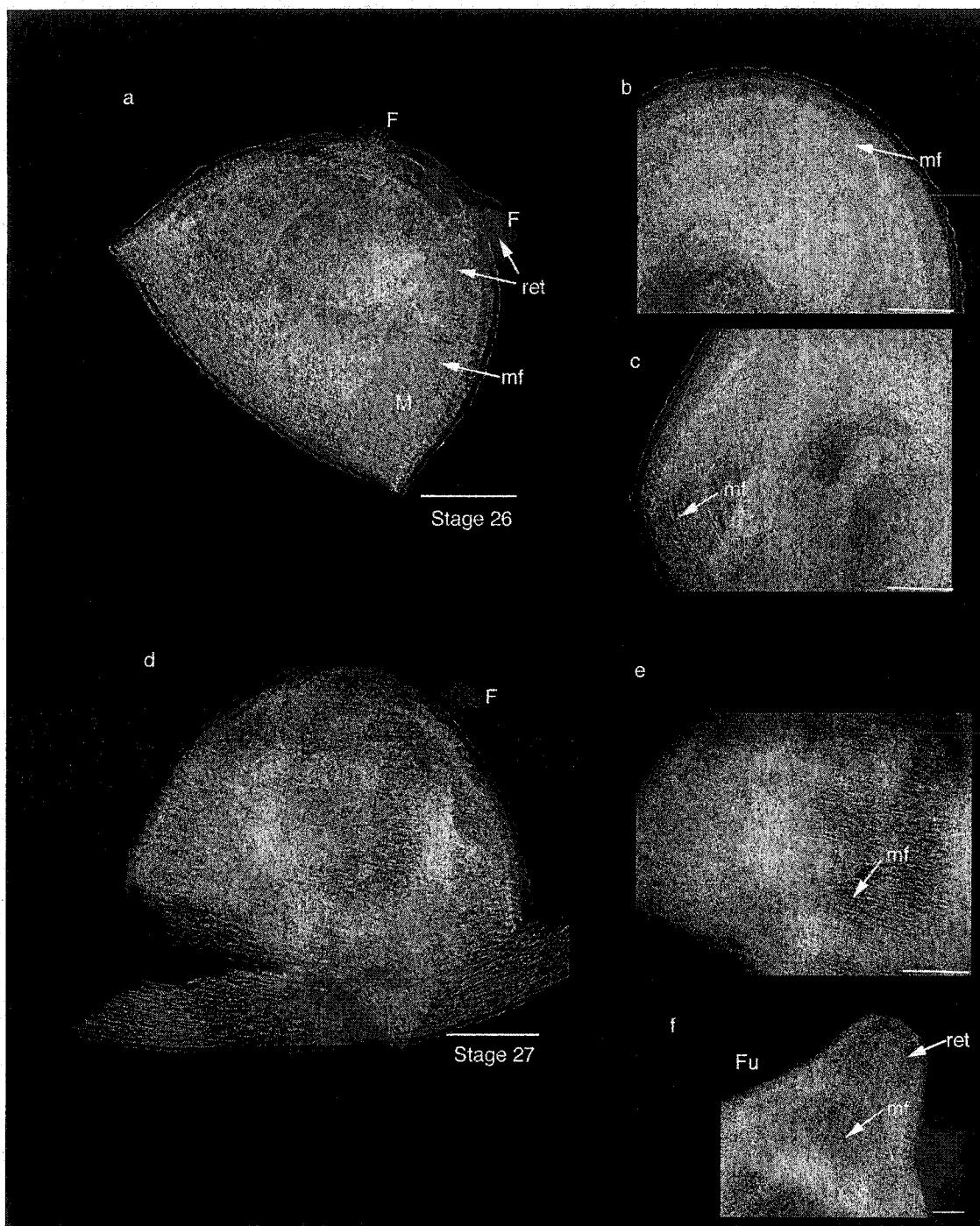


Figure II-2. Further organization and emergence of musculature in *L. pealeii* Arnold stage 26-27. a-c. Continued emergence of elongated muscle fibers in the mantle. Note fins (F) are still undifferentiated but proportionate in size to the rest of the structure. Reticulated pattern still present. d-f. Fins still lack muscle fibers. Muscle fibers mantle have increased in number and density. No reticulation. Funnel shows presence of both muscle fibers and reticulation. Scale bars-100 μ m

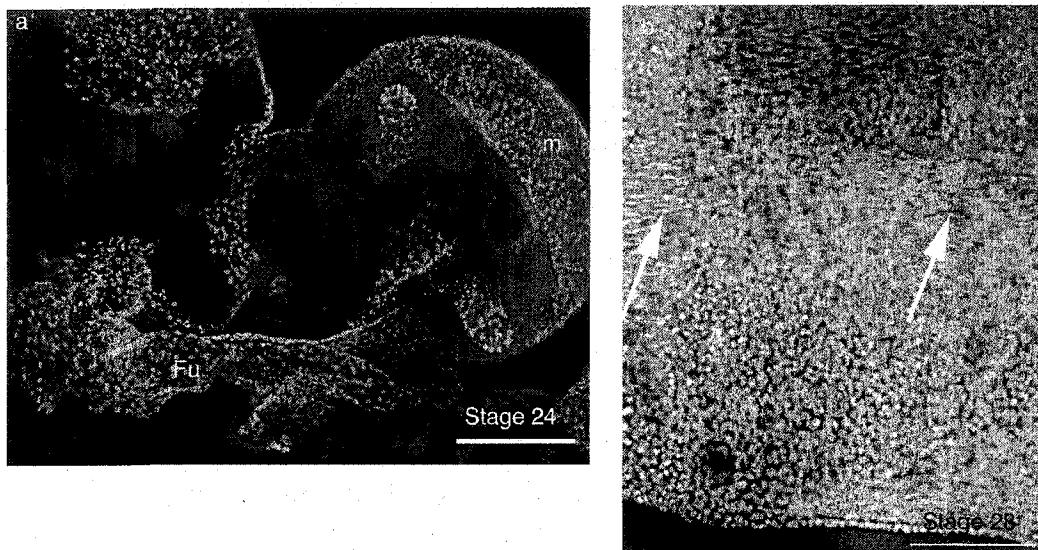


Figure II-3. DAPI stained mantles at Arnold stages 24 and 28. a. Stage 24 mantle is structurally unorganized. DAPI stained nuclei in all orientations. Scale bar is 50 μ m. b. Stage 28 mantle, arrows point to circularly oriented nuclei belonging to circular muscle fibers. Scale bar is 20 μ m.

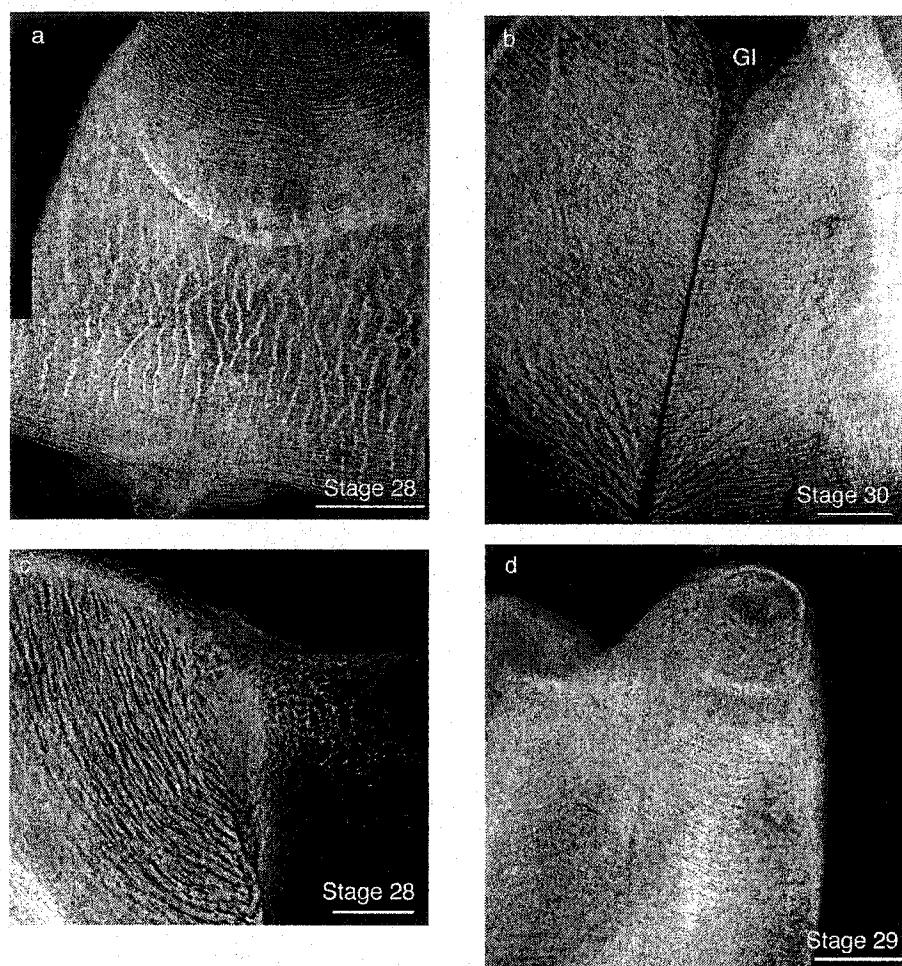


Figure II-4. Final phase of muscle development and organization in mantle, funnel and fins. a-b. Stage 28 and 30 mantles. Note density of circular muscle arrangement and lack of any reticulation. c. Stage 28 fin still has evidence of reticulation as well as muscle fibers in several orientations. d. Stage 29 funnel has muscle fibers in 3 orientations and no reticulation. Scale bars are 100 μ m.

CHAPTER III

ULTRASTRUCTURAL DEVELOPMENT AND ORGANIZATION OF THE MANTLE MUSCULATURE IN *LOLIGO PEALEII*

Introduction

The squid mantle is a highly specialized structure that is used for both respiration and for locomotion. The mantle has a complex and precise arrangement of circular and radial muscles tightly associated with two layers of stiff collagenous tunics and a network of connective tissue fibers within its musculature (Bone et al., 1981; Gosline et al., 1983; Kier, 1988a; Wells, 1988). Additionally, the circular muscle mass is composed of two specialized fiber types with a specific distribution within the mantle. This organization and composition of muscle and connective tissue is the basis for the ability of the mantle to execute both respiration and locomotion (Clarke, 1962; Gosline and Shadwick, 1983a; Gosline and Shadwick, 1983b; Kier, 1988b; MacGillivray et al., 1999; Mommsen et al., 1981; Ward, 1972; Ward and Wainwright, 1972; Wells, 1988).

In the adults, the overall organization of the squid mantle is a dense arrangement of circular muscles divided at regular intervals into blocks by radial muscles (Bone et al., 1981; Gosline et al., 1983; Mommsen et al., 1981). The radial muscles extend from the outer to the inner surface of the mantle. The circular muscle mass is divided into three layers composed of two different

muscle fiber types. The fiber types are metabolically distinct and are distinguishable from one another by the differences in mitochondrial content. There are two layers of oxidative, superficial mitochondrial-rich (SMR) fibers at the inner and outer surfaces of the mantle. The SMR muscles are used during slow jetting, hovering and respiration (Bone et al., 1995a). Between these layers is a central, thicker layer of glycolitic, mitochondria-poor (CMP) fibers (Bone et al., 1981; Hochachka et al., 1975; Mommsen et al., 1981) that are active during escape jetting and rapid locomotion (Bartol, 2001a; Bartol, 2001b) (Bone et al., 1995a).

Although the physiology and ultrastructure of the mantle has been extensively studied in adult squids and to some extent in juveniles (Bone et al., 1995b; Chen et al., 1996; Gilly et al., 1991; Gilly et al., 1996; Gosline and Shadwick, 1983a; Gosline and Shadwick, 1983b; Gosline et al., 1983; MacGillivray et al., 1999; Mommsen et al., 1981; Preuss and Gilly, 2000; Preuss et al., 1997) relevant work on the development and differentiation of muscle during the embryonic period has been largely neglected. At hatching, the mantle morphology and structural organization is similar to the adult form, however, the process by which this is achieved has not been addressed. Similar topics have been examined recently by Grimaldi et al. (2004) on developing tentacles and arms of *Sepia officinalis* using histochemical and molecular techniques, but again little is known with regard to squid mantle formation.

The aim of the present study was to describe the ultrastructural development and differentiation of muscle cells in the mantle of embryonic

L. pealeii. Additionally, I wanted to determine not only where muscle cells first differentiated within the mantle but also when differentiated cells first appeared.

Materials and Methods

The anatomy of the mantle musculature was examined by light and electron microscopy (Appendix B). Prior to fixation, embryos were removed from their egg cases and staged according to Arnold's staging table (Arnold, 1965). Embryos at Arnold stages 22-30 were selected. All embryos were fixed while still inside their chorions in order to prevent excessive tissue damage as a result of handling.

Experimental Animals

Loligo pealeii egg cases were obtained from the Marine Resources center at the Marine Biological Laboratories in Woods Hole, MA. Eggs cases containing embryos at various developmental stages were selected and transferred to well-aerated tanks kept at ambient temperature (15°C) until needed.

Electron and Light Microscopy

Whole embryos were fixed in 2.5% gluteraldehyde for 3 hours and rinsed with 0.2M cacodylate buffer. During the cacodylate buffer rinses, the mantles (and funnels) were removed from the rest of the embryos. Whole embryos and mantles were then post fixed in 4% OsO₄ for two hours at room temperature. Samples were then dehydrated in a graded series of ethanol and infiltrated and

embedded with Embed 812 (EMS) (Appendix C). Samples were sectioned using a MT 6000-XL ultramicrotome for semi-thin ($1\mu\text{m}$) and a RMC-Boekler TM-XL ultramicrotome for ultra-thin ($0.1\mu\text{m}$) sections. Semi-thin sections were mounted on glass slides and stained with Richardson's stain (methylene blue and azure II) (Appendix D). Semi-thin sections were viewed and digitized with an Olympus BX50 compound microscope with an attached Polaroid digital microscope camera and with a Nikon Axiophot compound microscope mounted with a SPOT digital camera. Images were captured using DMC Direct 2.0 and SPOT software for Macintosh, and adjusted for contrast/brightness using Adobe Photoshop v. 6.0 or 7.0.

Ultra-thin sections were mounted on coated grids and stained with uranyl acetate and lead citrate (Appendix D). Sections were viewed and photographed with a JEOL JEM-100S transmission electron microscope. Kodak ESTAR Thick Base TEM film (4489) was developed according to manufacturers instructions. Negatives were scanned at 400 dpi on a HP Scan Jet 6300C with a transparency adapter (ScanJet XPA) and with an Epson Perfection 3200 scanner. Images were stored as .TIF and .JPEG files and adjusted for brightness and contrast with Adobe Photoshop v.6.0 or 7.0.

Definitions and Criteria

The development and differentiation of myoblasts into myocytes and myofibers begins during organogenesis and continues until just prior to hatching at which point development is more a matter of cell growth rather than cell

proliferation. The complex process of muscle cell differentiation in the squid mantle can be divided into three discrete phases consisting of cell proliferation and cell arrangement (Phase I), cell differentiation (Phase II), and cell growth (Phase III). The criteria used to define each phase were based on obvious cytological landmark features including cell and nucleus morphology, as well as presence and absence of mitotic figures, cell division and contractile material. Since this study is entirely morphological and no molecular work was done, the term "differentiation" is defined as the process of a cell acquiring/exhibiting actin and myosin filaments.

Results

Morphological Analysis

Phase I (Stage 21-23, (Arnold, 1965)): Embryos at this stage of development have small, cap-shaped, rudimentary mantles with simple organization and few cell types (Fig. III-1a). The mantle is organized into four easily identifiable layers, a thin epidermal layer, a thin connective tissue layer immediately under the epidermal layer, the mantle tissue layer itself, and a thin epithelial layer beneath the mantle (Fig. III-1a). Except for the thick mantle tissue layer, all others are one cell thick.

During Phase I, the mantle is composed of proliferating, undifferentiated myoblasts (Fig. III-1a,c,e). Cell proliferation is determined by the presence of cells with nuclei containing high content of condensed chromatin as well as cells in different stages of mitosis including cytokinesis. Undifferentiated myoblasts

are identifiable based on the absence of actin and myosin filaments in the cytoplasm and by large ovate, nuclei that are tightly associated with each other (Fig. III-1c) and are pseudostratified. TEM reveals few other organelles within the cell but there is high number of free ribosomes as well as rough endoplasmic reticulum suggesting an increased level of protein synthesis.

Phase II (Stages 24-26, (Arnold, 1965)): The mantles of embryos in Phase II are substantial structures that have been growing in both length and width. Differentiated myoblasts are first detected in stage 24 embryos (Fig. III-1f). Differentiation is determined by the presence of actin and myosin filaments that appear in the apex of some of these cells (Fig. III-1f). Differentiation of myoblasts into myofibers is not uniform within the mantle. Although differentiation is occurring, it is only occurring at the basal laminas of the inner and outer epithelial layer (Fig. III-2b), no evidence of differentiated myoblasts is detectable in the central portion of the mantle layer. Along with the emergence of actin and myosin filaments, mitochondria are also present (Fig. III-1f). Cells that have differentiated have an average of 2 centrally located mitochondria and the nucleus is either displaced from the center or not in the plane of section (Fig. III-2g).

Undifferentiated cells are localized to the central region of the mantle. These cells are morphologically similar to the undifferentiated cells found in Phase I mantles. The cells have large nuclei but are they are oblong shaped rather than rounded (Fig. III-2a, d, g). Cells in the central region have either

dispersed chromatin located at the edge of the nucleus or no chromatin is visible at all. In early Phase II, there are few mitotic figures and cells under going cell division.

Spatially, all cells both differentiated and undifferentiated within the mantle are being organized into rectangular blocks of tissue that reflect the organization of the adult musculature (Fig. III-2c,e). The emergence of "block" organization indicates that the radially oriented muscle fibers are differentiating (Fig. III-2d,g). However, the block organization is not yet well defined, only suggestive of the future organization. In longitudinal sections, the contractile material of the radial muscle fibers is only detected at the ends of the fibers and not in the middle portions of the fibers. Other characteristics of radial fibers such as mitochondrial content are not readily distinguishable (Fig. III-2d).

The muscular organization of circular muscle fibers divided into regular rectangular segments by the radial fibers is readily distinguishable by stage 27 (Fig. III-2e). At this stage differentiated circular muscle fibers are easily identifiable at the basal laminas of the inner and outer tunics. These cells have increased myofibril content and have 2-3 mitochondria in the central portion of the cell. The myofibrils are not organized into recognizable sarcomeric units (Fig. III-2h). Based on this morphology, these circular muscle fibers are of the superficial mitochondria rich fiber (SMR) type. During stage 27, differentiated muscle cells appear in the central zone. These cells are of the central mitochondria poor (CMP) muscle fiber type. The CMP muscle fibers have a lower

myofibril content compared to the SMR fibers and on average have only one mitochondrion in the center of the cell.

Phase III (Stages 27-30, (Arnold, 1965)): The mantles of embryos in Phase III are robust structures with circular and radial muscle fibers arranged in the characteristic adult form. The circular muscle fibers have also differentiated into the two metabolically different fiber types found in the adult, the SMR fibers and the CMP fibers. Phase III is a period of growth and further structural definition. The mantle becomes longer and wider and the muscle fiber arrangement becomes denser. During Phase III growth appears to be by addition of new muscle fibers within in the individual blocks rather than addition of more blocks. The myofibril content on the muscle fibers continues to increase but sarcomeric organization of the myofibrils is rare.

Longitudinal sections show a radical difference in the overall appearance of the mantle structure (Fig. III-3a,b). The inner and outer tunics are well defined and tightly associated with the mantle muscle mass. Whereas in the latter part of Phase II the mantle is clearly organized into muscle blocks, the different zones of circular muscles that are obvious in the adults are not distinguishable. However, this abruptly changes during Phase III with the clear emergence of the three zones of the circular muscle fibers and their respective muscle fiber types (Fig. III-3a-d). The SMR circular fibers are located beneath the inner and outer tunics and the CMP muscle fibers are located in the central region of the mantle.

In addition to the emergence of the different circular muscle zones and overall growth, the vascularization of the mantle also increases. The most noticeable is a large blood vessel that runs through the middle of the mantle muscle mass in the CMP fiber region (Fig. III-3c). The innervation of the mantle musculature is more pronounced as evidenced by the presence of large axonal profiles in association with muscle fiber blocks (Fig. III-3e).

Nervous System: Although examining the development of the nervous system was beyond the scope of this study it must still be briefly addressed. All embryos from stages 24- 30 examined at the TEM level had some degree of neural development within the mantle structure (Fig. III-4). The most conspicuous were the axons from the stellate ganglia, which are ipsilateral structures that are involved in the neural control of the mantle musculature (Young, 1971). Evidence of stellate ganglion axons was seen in stage 24 embryos beneath the skin epithelium (Fig. III-4a). Stage 25 embryos had easily distinguishable stellate ganglia that had more axons within the cluster than in the previous stage (Fig. III-4b). In later stage embryos (stages 27-30), along with the stellate ganglia, other neural structures were evident such as small neuromuscular junctions (Fig. III-4c,d).

Discussion

The mantle musculature in adult loliginid squids is a complex structure. This complexity is the basis for understanding how it can carryout two different

functions, respiration and locomotion (Bone et al., 1995b; Bone et al., 1981; Budelman et al., 1997; Gosline et al., 1983; Kier, 1988b; MacGillivray et al., 1999; Mommsen et al., 1981). There are few cases where one structure is responsible for performing two essentially incompatible functions and the squid mantle has been adapted to do so not only by its intricate arrangement of circular and radial muscle fibers and connective tissue fiber networks, but also by the presence and specific distribution of two metabolically distinct fiber types.

These different fiber types are analogous to the red and white muscle fibers found in vertebrate muscles (Hochachka et al., 1975; Mommsen et al., 1981). In adult squids, muscular activity studies during respiration and locomotion have shown that each function is performed by the activation of one of the two muscle fiber types (Bartol, 2001a; Bartol, 2001b; Gosline and DeMont, 1985; Gosline et al., 1983). The superficial mitochondrial rich fibers (SMR)(=white) are active during aerobic activities such as respiration and hovering. The central mitochondria poor fibers (CMP)(=red) are active during anaerobic activity such as rapid jet propulsion and escape jetting (Bartol, 2001a; Bartol, 2001b; Bone et al., 1995a; Bone et al., 1981; Mommsen et al., 1981). The roles of the SMR and CMP muscles in each of these functions are significant in the adults and their arrangement and distribution reflects this. Examining how the adult ultrastructure in the mantle musculature developed revealed that the structural/mechanical ability for the mantle to function in a dual capacity is established during embryogenesis by the precise sequence of the differentiation process itself.

My results show that differentiation of the mantle muscle fibers and fiber types occurs in a specific sequence and at several levels of organization. Prior to the emergence of differentiated myoblasts the overall organization of the mantle is already present (Fig. III-1a,c,e). There are four distinct layers including the skin, the outer and inner tunics and a thick layer of undifferentiated mantle tissue. Additionally, the presumptive muscle cells begin to align themselves into parallel columns that span the width of the mantle layer from the inner surface to the outer surface. This alignment of the presumptive myoblasts is a precursor to the future organization of the circular and radial muscle fibers into distinct block units. The block organization occurs at the same time that muscle cell differentiation begins although my data suggest that the circular muscles start the differentiation process earlier than the radial fibers. Once muscle differentiation begins (Stage 24), it is not a uniform process within the mantle. Differentiated muscle fibers emerge at the basement membranes of the inner and outer tunics. The central portion of the mantle remains undifferentiated until stage 27. At stage 27 differentiated muscle fibers are detected in the central portion of the mantle. During the later stages of development (28-30) the organization of the mantle begins to take on the appearance of the adult structure with defined segments of circular and radial muscles. Also, the three layers of SMR and CMP fibers are become well defined and by hatching are easily identifiable.

What is interesting about my study is that the patterns that are seen in the adult musculature that directly influence respiratory and locomotory function are laid down in the embryonic period. Not only is the overall organization of the

mantle evident early, but my data show that the zonation of the circular muscles may also be predetermined. The SMR muscle fibers appear first and they appear at both surfaces of the mantle at the same stage. The CMP fibers appear second and significantly later than the SMR fibers. This pattern is significant because it strongly links the emergence of the specific fiber types with specific functions. Therefore, since SMR fibers are responsible for respiratory function and they appear first this would suggest that respiratory ability also emerges first. Similarly, since CMP fibers are active during locomotion, their appearance after SMR fibers indicates that locomotory capabilities emerge after the ability to respire (See Chapter 4).

The emergence of specific fiber types in the mantle at different period in development would suggest that two distinct muscle cell populations exist one giving rise to SMR fibers and the other to CMP fibers. Recent molecular studies looking at muscle differentiation in the arms and tentacles of *Sepia officianalis* have shown the existence of two distinct myoblast populations and that there is also a temporal difference in their differentiation (Grimaldi et al., 2004). Moreover, as was seen in the arms and tentacles of *Sepia*, muscle cell differentiation occurred in specific locations within the structure (Grimaldi et al., 2004). Further exploration into the molecular differentiation of the SMR and CMP fibers is recommended.

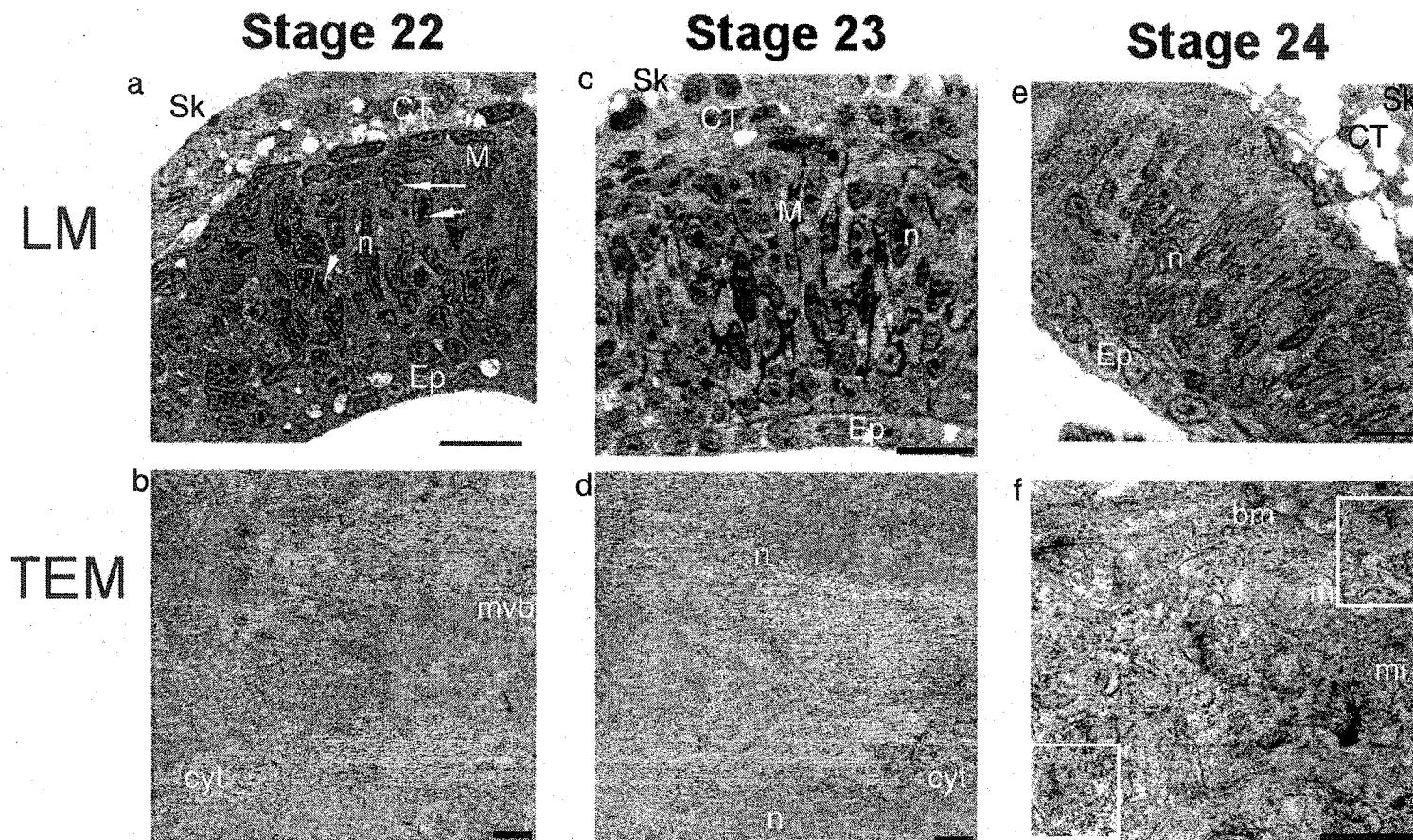


Figure III-1. a,c,e. Early phase of mantle muscle differentiation. All Stages (22-hatch) show general mantle aggregation. Skin (Sk) epithelium, connective tissue layer (Ct), inner surface epithelium (Ep) layers and (M) tissue layer. Mantle has undifferentiated myoblasts in parallel. Nuclei (n) are large, ovate, with condensed chromatin. Stage 24 mantles considerably further organized than Stages 22+23. Scale bars = 10mm. b,d,f. No ultrastructural differentiation of muscle in Stage 22 and 23. Tissue is mostly cytoplasm with many multivesicular bodies. Stage 24 mantle tissue has begun to differentiate. Myofilaments (in boxes) are recognizable beneath the basement membrane (bm) of the connective tissue layer. Scale bars = 1mm. 1mm and 0.5mm respectively.

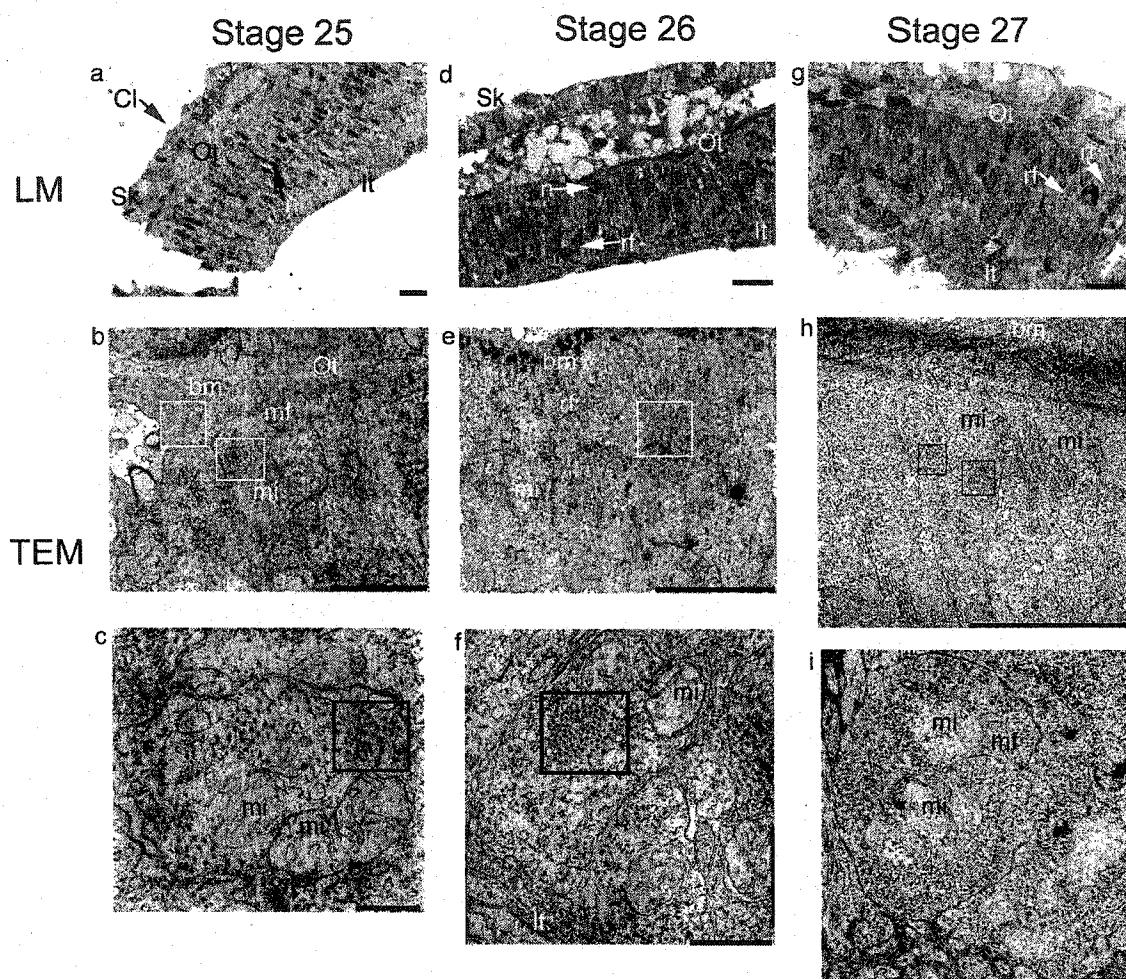


Figure III-2. Further development and organization of mantle muscle ultrastructure. **a,d,g.** Main layers of the mantle more defined. Ciliated tufts (Ci) are present on skin layer. Further alignment of nuclei into parallel arrangement. Outer (Ot) and inner tunics (It) identifiable. Stage 26 mantles have radial fibers (rf) beginning to divide circular muscle mass into blocks. Scale bars 20 μm . **b,e,h.** Ultrastructural differentiation is advanced. Mitochondria (mi) present in differentiated muscle. All muscle fibers located beneath basement membrane of Ot and It. Scale bars 1 μm (b,e) and 5 μm (h). **c,f,i.** High magnification of muscle fiber development. High mitochondrial content. Scale bars 0.5 μm (c) and 1 μm for (f,i).

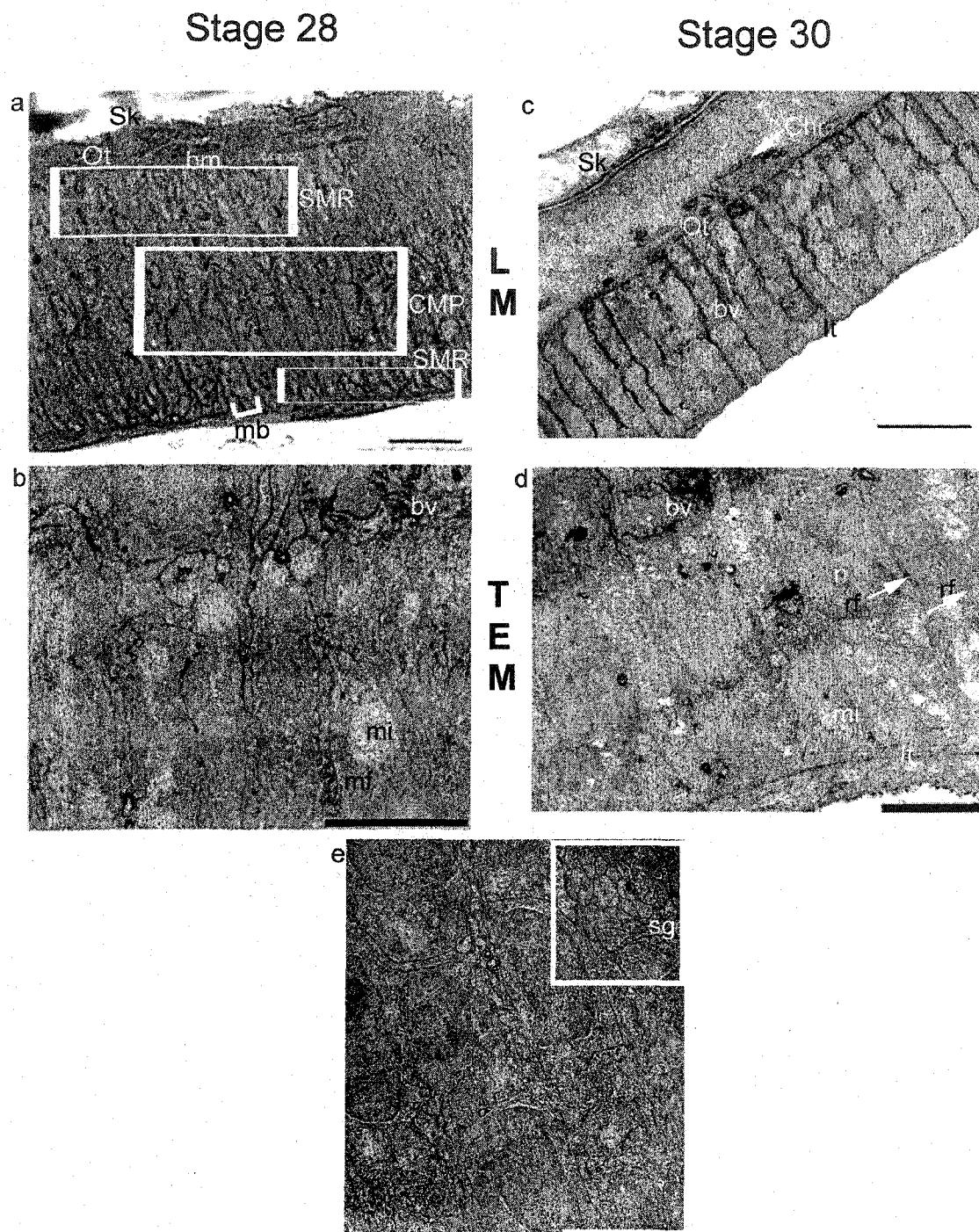


Figure III-3. Late phase differentiation and organization. a. Three layers of different muscle fiber types evident, two SMR layers surrounding a CMP layer. b. CMP fibers characterized by one large mitochondrion surrounded by high number of myofilaments. c. Appearance of blood vessels (bb). d. SMR layer defined by high mitochondrial content. e. Innervation of mantle, evidenced by stellate ganglion (sg). All scale bars 5 μm .

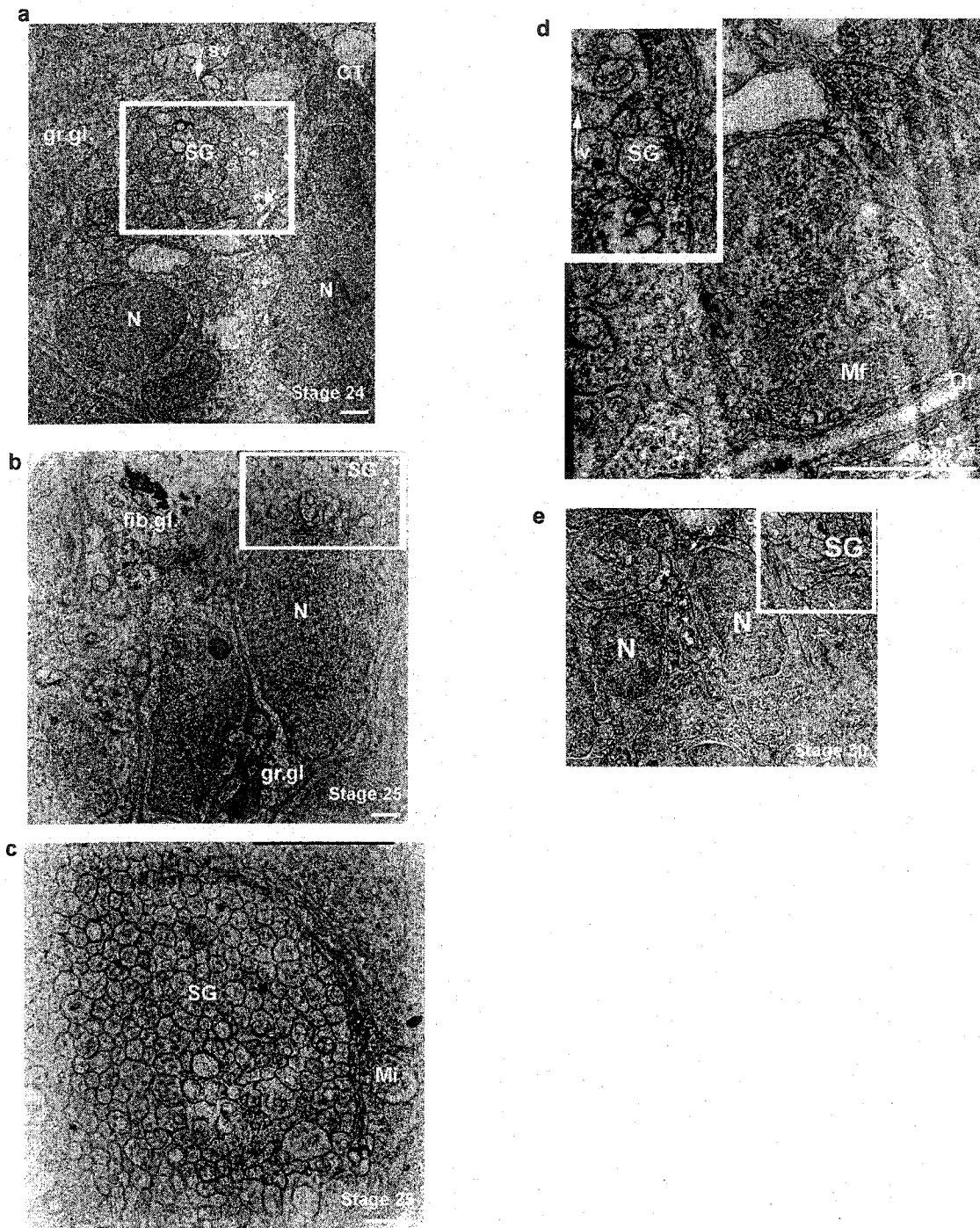


Figure III-4.a-e. Stellate ganglia (Sg) axonal profiles (in boxes) are the most conspicuous neural structures in the developing mantle. a,b. Stages 24 and 25 stellate ganglia surrounded by fibrillar and granular glial cells (fib.gl and gr.gl.). Axons are small in diameter. c. Stage 25, evidence of larger axons (*). d,e. Late stage embryos stellate ganglia tightly associated with differentiated muscle cells. All scale bars are 1 μ m.

CHAPTER IV

EMERGENCE OF RESPIRATORY AND LOCOMOTORY FUNCTIONAL ABILITY IN EMBRYONIC LOLIGINID SQUIDS

Introduction

Squids do not hatch out as miniature replicas of their adult counterparts. They do not experience a radical metamorphosis that involves shedding larval structures and constructing new ones. However, they still have to undergo several life history transitions as they develop from eggs to highly mobile adults. By hatching squids have all of their essential structures and morphologically resemble the adults, (Boletzky, 1974; Naef, 1928; Sweeney et al., 1992) however, newly hatched squids are still undergoing many dramatic changes in function (Thompson and Kier, 2001b; Thompson and Kier, 2002) in order to accommodate rapid growth that maybe related to changes in their immediate Reynolds number environment (Thompson and Kier, 2001b; Thompson and Kier, 2002).

The significant functional changes that occur in the post-hatching period may be correlated with the morphological and physiological changes that take place during the pre-hatching period of development. The acquisition of functional capabilities requires the extensive development of both muscular and

nervous systems (Chen et al., 1996; Gilly et al., 1991; Preuss and Gilly, 2000; Preuss et al., 1997), processes that begin in the early embryonic period and continue through hatching and into the paralarval periods.

Naef, (Naef, 1928) noted that the mantles of embryonic squids acquire contractile ability early in ontogeny. The mantle initially contracts sporadically and during subsequent stages exhibits coordinated, rhythmic movements suggesting a ventilatory/respiratory function (Naef, 1928), Martinez pers.obs). Additionally, a few stages prior to hatching (Arnold stage 27), embryos can contract the mantle with enough force to create locomotory-like movements both inside the chorion and when manually hatched. Though these movements are readily observable and other cephalopod embryologists have referred to them in passing (Arnold, 1965; Fields, 1965; Naef, 1928), there is no body of work that has attempted to quantify and verify that these early movements are indeed respiration and locomotion and relevant studies only cover the post hatching period and beyond (Bartol, 2001a; Bartol, 2001b; Gilly et al., 1991; Preuss et al., 1997; Thompson and Kier, 2001b; Thompson and Kier, 2002).

The Mantle as a "Muscular-Hydrostat"

Many invertebrates including cephalopods possess hydrostatic skeletons to provide the structural support necessary for locomotion and respiration. Hydrostatic skeletons are generally characterized by having a constant volume

and movements are a result of muscle contractions shuttling the fluid in one dimension and generating a change in one or both of the other two dimensions (Kier, 1988b; Kier, 1992; Wainwright, 1982). Hydrostatic skeletons are commonly composed of muscles arranged in two-dimensional sheets or complex three-dimensional arrays surrounding fluid filled spaces (Kier, 1988b; Kier, 1992; Wainwright, 1982). Structures composed largely of muscle and few, if any, fluid filled cavities such as the arms of cephalopods are called muscular hydrostats because the muscles themselves serve as the incompressible fluid (Kier, 1988b; Kier, 1992; Wainwright, 1982). The squid mantle is an example of a muscular hydrostat. Respiration and locomotory movements are produced by the mantle by the antagonistic action of the radial and circular muscles against the inner and outer collagenous tunics and the intramuscular connective fiber networks (Bone et al., 1981; Gosline and Shadwick, 1983a; Gosline et al., 1983; Kier, 1988b; Ward and Wainwright, 1972; Wells, 1988).

Dual Functions of the Mantle

Respiratory and locomotory movements in adult decapods are easily distinguishable from each other not only qualitatively but also physiologically and mechanically (Bartol, 2001a; Bartol, 2001b; Bone et al., 1981; Gosline et al., 1983; Hochachka et al., 1975; MacGillivray et al., 1999; Mommsen et al., 1981). Respiration and locomotion contractions are similar because they consist of three distinct phases, an inhalant phase, an expulsion phase and a refilling phase (Fig. IV-1). During respiration, the mantle expands and as a result of negative

pressure, water gets sucked into the mantle cavity where it is shuttled back towards the gills. Water is then pushed out through the funnel by the contraction of the mantle (Gosline et al., 1983; Kier, 1988b; Ward and Wainwright, 1972; Wells, 1988; Wells and Wells, 1982). During locomotion, the inhalant and exhalant phases are more pronounced. The expansion of the mantle often is a hyperinflation (a bigger percent change from resting mantle diameter) and the exhalant phase can generate enough force to produce a jet as the water is expelled through the funnel (Gosline et al., 1983; Young, 1938).

Respiration and locomotion movements generated by the mantle may be inherently incompatible with each other (Wells, 1988). The rapid inhalation and expulsion of water into and out of the mantle cavity does not allow enough time for oxygen to be extracted by the gills, particularly during rapid jetting (Wells, 1988). However, the mantle is able to perform rapid jetting and rhythmic respiration because of the arrangement of the circular and radial muscles, different muscle fiber types (SMR or CMP) (Bone et al., 1981; Mommsen et al., 1981; Shadwick, 1994), the presence of connective tissue fiber networks within the musculature and two stiff collagenous inner and outer tunics (Gosline and Shadwick, 1983a)}(Bartol, 2001a; MacGillivray et al., 1999; Ward and Wainwright, 1972).

The mantle can thus function in a dual capacity by using each of these structural components in different combinations. For example, respiratory contractions are produced by the contraction of the circular or radial muscles (Gosline et al., 1983) or collar flaps, however not all of the circular muscles

contribute to the contraction, only the superficial mitochondrial rich muscle fibers may be used (Bartol, 2001a). Moreover, respiratory movements only occur in the anterior portion of the mantle (in the area of the gills) (Packard and Trueman, 1974). In contrast, jetting movements are also generated by the contraction of circular muscles of the central zone (Bartol, 2001a) and the whole mantle is used not just the anterior region (Bone et al., 1981; Mommsen et al., 1981).

In squids, the ability to function must be present at hatching. Therefore, functional capability must be established in the pre-hatching period. Functional capability then, like morphology, must be considered as a process with an ontogeny. The squid mantle must develop the functional capability for locomotion and respiration and the objective of this study was to identify when functional ability began to emerge and to describe its ontogeny into two different functions, respiration and locomotion.

To determine the emergence of mantle functional capability in pre-hatching teuthoid cephalopods I used a combination of modeling and kinematic methods. I examined mantle contraction frequencies, and mantle contraction duration to quantify and distinguish the functional transitions from sporadic movements to coordinated, rhythmic respiratory-like contractions to more powerful locomotory-like contractions.

Material and Methods

Experimental Animals

Egg masses of *Loligo opalescens* were collected by SCUBA in Puget Sound near Old Dash Point, Seattle WA (summers 2002 and 2003) and in Coos Bay, OR (summer 2003). Egg masses were transferred to running seawater tables at the Friday Harbor Laboratories, Friday Harbor, WA and kept at ambient temperatures of approximately 13°C. Embryos in the egg cases collected in Puget Sound in 2002 were at different developmental stages ranging from epiboly (Arnold stage 16) to Arnold Stage 25. Those collected in Puget Sound in 2003 were at Arnold stages 22-25 and those collected from Coos Bay in 2003 were at Arnold stages 27-30. All embryos were kept in the running seawater tables until hatching (1-3 weeks depending on batch) after which they were returned to the ocean.

High Speed Video (HSV)

HSV capture: Individual embryos at Arnold Stages 23-30 were chosen based on the condition of the animal. Only the embryos in the best condition were chosen at which point the chorion was manually removed (artificial hatching). Animals were placed in watch glasses filled with water from the sea tables and observed using a Nikon SMZ1500 dissecting microscope. For studies in 2003 individual animals were placed in chambers made from glass slides and coverslips and sealed with vacuum grease to keep the animal from moving away

from the field of view, and observed under a Nikon SMZ1500 dissecting microscope.

Animals were filmed with either a Red Lake Motion Meter or with a Red Lake Motion Scope high-speed video camera mounted on the dissecting microscopes. All animals were filmed at 500 frames/second with playback at 30 frames/second. Each recording was two seconds (2000 ms) in length. On average 10, two second film clips (of mantle contractions) per animal were recorded.

Digitizing: Contractions were recorded on mini DV cassettes using either a JVC GR-DVL digital camera or a Sony mini DV VCR. Film clips were imported and edited using Apple iMovie software. Once clips were edited they were exported as individual QuickTime movies and then transformed into image stacks of TIFF files. (Every frame of the QuickTime movies is one TIFF file). Only the pertinent TIFF files (frames)-inhalant, exhalant and refilling phases- were saved and analyzed

Measurements From HSV Images: Image files were analyzed using Image J v.3 software (NIH). Measurements of mantle length, mantle width (mid mantle width and mantle aperture) and internal yolk sac width (Fig IV-1) were taken at the three phases of a contraction cycle, inhalation, exhalation, and refilling. Length and frequency of contractions was calculated from the frame counter on the HSV camera.

Model: A mathematical model for mantle function was developed using mantle cavity volume as a proxy for function. Lolinid embryos are small (approximately 1-2mm ML) and measuring volumes of water in the mantle cavity was not practical for the purposes of this study, instead, volumes were calculated using the mathematical model. Values collected from the HSV images were inserted into the model and used to generate volume estimates.

I made the following assumptions to model mantle function at different embryonic stages:

1. Mantle shape remains constant as the animal grows (although morphologically this does change)
2. The mantle is essentially an ellipsoid (actually a prolate spheroid) cut in half with a cylinder in the middle (internal yolk sac).
3. The mantle of embryonic squids functions as a muscular hydrostat as in the adults.

Generalizing the shape of the mantle into two geometric components, a half of an ellipsoid and a cylinder, allowed for the development of a model using the equations to calculate volume for half of an ellipsoid (prolate spheroid) and a cylinder (Fig IV-2).

Mantle cavity volume (M_v) was calculated using the following equations:

$$M_v = V_m - V_y$$

Volume of prolate spheroid (V_m =mantle)= $2/3\pi ab^2$

Volume of cylinder (V_y =internal yolk sac) = $\pi r^2 a$

Where:

a =mantle length (ML)

b =mantle diameter (MD)

r =internal yolk sac diameter (YS)

Therefore:

$$(2/3\pi ab^2 - 2/3\pi a(b-c)^2) - \pi r^2 a$$

$$(Mv = 2/3\pi(ML)(MD/2)^2 - \pi(YS/2)^2(ML))$$

I was looking at changes in mantle cavity volumes (ΔV) between the different phases of a contraction cycle (i.e during a relaxed state vs. a contracted state) therefore changes in mantle cavity volumes (ΔV) were calculated as:

$$\Delta V = [(M_{vr} - M_{yr})] - [(M_{vc} - M_{yc})]$$

Or

$$\Delta V = [(2/3\pi(ML)(MD/2)^2)_R - (\pi(YS/2)^2(ML))_R] - [(2/3\pi(ML)(MD/2)^2)_C - (\pi(YS/2)^2(ML))_C]$$

(Fig IV-2)

The model was used to calculate $\Delta V/\Delta T$ to differentiate between respiratory and locomotory contractions. Measurements from the HSV data were inserted into the mathematical model to generate estimates of mantle cavity volume output during mantle contractions. I predicted two distinct volume outputs one for locomotory-like contractions and one for respiratory-like contractions and these volumes would increase with stage. Because the model was created as a way to quantify differences between observed contractions in embryos, the data were sorted based on the following definitions of what constituted a respiratory-like or locomotory-like contraction. Locomotory-like contractions were defined as any contraction that caused the embryo to cover a distance (i.e. from one side of the watch glass to another). Respiratory-like contractions were defined as any contraction that did not cause the embryo to move from its starting position. The model was run using Microsoft Excel X for Mac.

Mantle Kinematics: To assess mantle function at different developmental stages prior to hatching, I examined the changes in mantle kinematics as the embryos acquired contractile capability. Mantle kinematics were examined during respiratory-like and locomotory-like contractions of pre-hatching *L. opalescens*. Measurements of mantle length (ML), mantle diameter (MD), and mid-mantle diameter (MMD) (Fig IV-1) were made from digitized HSV images using imageJ v.3 (NIH) image analysis software. Mantle length was measured from the junction of the fins to the edge of the mantle. Mid-mantle diameter

(approximately 1/3 of the ML) was measured because observations of "respiring" embryos indicated that this portion of the mantle was also involved in the contraction.

Changes in ML, MD and MMD during respiratory-like or locomotory-like contractions are expressed as the percent change from the corresponding measurements at rest (defined as the period before a contraction begins). On average 10 contractions per squid were analyzed. Mantle contraction durations were determined from the HSV frame counter and mantle contraction frequencies were calculated by counting the number of contractions per second per squid at each of the different stages (Arnold stages 25-30).

Statistics: Mantle kinematics data were analyzed with one-way ANOVA with a post-hoc Tukey's test and linear regression using SYSTAT statistical software.

Results

Part I: Model

The data generated by the model showed that indeed respiration-like and locomotory-like contractions are distinct from each other and this distinction occurs prior to hatching. Respiratory-like movements were discernable at stage 25 but were more frequent by stage 26 while locomotory-like movements consistently emerged by stage 27. Respiratory contraction volumes increased with stage. At stage 26 volume output was 0.2 $\mu\text{l}/\text{second}$ (change in volume/

change in time) and by stage 29/30 the volume output ranged from 1.7 μ l/sec – 2.3 μ l/sec (Fig. IV-3). The volume outputs for locomotory contractions were greater than those for respiratory contractions from 1.5 μ l/sec in stage 27 embryos to 2.1-2.4 μ l/sec in stage 30 embryos.

These data indicate that respiration and locomotion are distinguishable prior to hatching and that volume output also increases with stage. Further, although respiratory volume outputs are lower than locomotory volumes, they increase approximately 2 times more per stage than locomotion outputs. Once an embryo has acquired the ability to locomote (stage 27) the volume output is greater than a respiratory-like contraction.

Part II - Mantle kinematics

Mantle Kinematics During Respiratory-Like Contractions: Published values for changes in mantle length in adult squids during jetting or respiration are minimal (Gosline and Shadwick, 1983a; Gosline and Shadwick, 1983b; Gosline et al., 1983; Ward, 1972); in contrast my results indicate that mantle length is not constant during respiration contractions and does change between stages 26 and 30 (hatching) (Table IV-1). Though ANOVA results indicate that there is no significant percent change in ML length during respiration at any of the stages analyzed, with a P-value of 0.099, it still suggests that there is a noticeable difference in ML changes over the course of development. (When the ANOVA was run only on data from stages 26-29, the P-value = 0.022). More

importantly however, is the range of the percent change over development (Fig IV-4). In the earlier stages, when the average ML at rest was $\geq 1\text{mm}$, the range of percent change in ML was -8%-35% and by hatching the range of percent change is -2%-10% (although there is an outlier point at 20%). Thus, as development progresses the extent of percent change in ML during respiration decreased and approached 0% (no change) by hatching.

Percent changes in mantle diameter during respiration were significantly different ($P=0.017$) from stages 26-30 (Table IV-1). As the embryos developed, the range of mantle diameter change (Fig IV-3) was 3% -15% and by hatching the range of mantle diameter change had increased to 3-51% indicating that mantle width changes are stage dependant. Younger embryos (stages 26-27) can only expand or contract their mantles in a limited manner because they do not have the full complement of differentiated muscles thus younger squids can only experience small mantle width changes. Older embryos (stages 28-30) that possess more muscle fibers that are well differentiated are able to expand and contract the mantle width noticeably more.

Mid-mantle diameter did not have significantly different percent changes at any stage. The range of percent change (10%-35%) (Fig IV-4) remained relatively constant for all stages examined.

Mantle Kinematics During Locomotory-Like Contractions: During locomotory contractions, which can be distinguished by stage 27, there were no statistically significant changes in mantle length ($P=0.117$), mantle diameter

($P=0.757$) or mid-mantle diameter ($P=0.485$) at any stage. However, when the ANOVA was run between stages 27 and 29 the percent change of ML was significant ($P=0.024$) between the two stages, although none of the other dimensions showed significant changes. Regression results do show trends that show that the progression of function acquisition is similar to what occurs during respiration. For example, mantle length percent changes tended to decrease and approach zero with stage (Fig IV-5). Similarly, the range of percent changes of mantle diameter and mid mantle diameter increased with each successive stage. Unlike respiration, mantle kinematic changes during locomotion are not as pronounced. This indicates mantle kinematics become more stable with the onset of locomotion and must become so if the animal is to generate a powerful enough jet to move.

The maximum (hyperinflation) and minimum (contraction) mantle diameters that could be reached by a squid embryo during any type contraction were also measured (Fig IV-6, Fig IV-7). In the younger embryos (Stage 26) the respiratory-like maximum mantle diameter expansion (1.055 mm) was smaller than in the older stage embryos (Stage 30, 2.397 mm). Similar patterns of mantle diameter change were recorded in locomotory-like movements for maximum mantle diameter expansion (Stage 27 = 1.448 mm, Stage 30 = 2.143 mm) and contraction (Stage 27 = 0.947 mm, Stage 30 = 1.246 mm). These results complement those reported by Thompson (2001a, 2001b, 2002) for mantle diameter changes in jetting post-hatching stages of another loliginid squid, *Sepioteuthis lessoniana*. In smaller and younger *S. lessoniana* juveniles,

the mantle diameter changes were higher than in larger older juveniles. Taken together, these data illustrate that the ontogenetic trajectory of mantle kinematics is composed of a gradual increase in mantle diameter changes with a peak attained at hatching and then a decrease during the paralarval period.

The durations of contractions (locomotory-like or respiratory-like) were also calculated (Fig. IV-8). Times were gathered from the start of the inhalant phase of the cycle to the refilling phase. At stage 26, an average respiration contraction took 650 ms to complete. By hatching an average full cycle respiratory contraction took 525 ms to complete. Locomotory-like contraction cycles remain constant, taking on average between 390- 520 ms to complete at any stage beginning at stage 27. A full respiratory-like contraction initially took longer to complete than a locomotory one, though by hatching both contraction types were of equal length.

Average contraction frequencies for both locomotion and respiration increased with stage (Fig. IV-9). The average respiration frequency at stage 26 was 0.6 contractions per second and by hatching an average rate had increased to 1.06 contractions per second. Locomotion contractions were not discernable until stage 27 and the average contraction rate was 0.6 contractions per second and by 1 day post hatching the rate increased to 1.5 locomotory contractions per second.

Discussion

Mantle function in embryonic *L. opalescens* is a gradual process that emerges and differentiates into two distinct types of movement by hatching. The present study shows that mantle function during the embryonic period can be separated into respiration-like and locomotion-like contractions both kinematically and through modeling. Based on these data, I have determined that the onset of respiration by means of mantle contraction begins at stage 26 although at stage 25 mantle contractions are evident, they are infrequent and sporadic and therefore difficult to measure. The onset of locomotion-type contractions of the mantle is stage 27.

Kinematically, the mantle changes with developmental stage in the pre-hatching period of development in four ways, amplitude, frequency, duration of contractions, as well as the percent change of two dimensions, mantle length and mantle diameter. All of these kinematic parameters were different during respiratory-like and locomotory-like contractions though the extent of the difference was variable.

That there were measurable changes in mantle kinematics between respiration and locomotion in pre-hatching squids suggests that mantle function is indeed a differentiation process that begins before hatching (stage 26 for respiration and stage 27 for locomotion) and continues through the post-hatching and juvenile periods of development (Thompson and Kier, 2001a; Thompson and Kier, 2001b; Thompson and Kier, 2002). Moreover, this differentiation of function affects how well pre-hatching embryos use the mantle

as a muscular hydrostatic structure. That the mantle can perform its dual functions prior to hatching does not also mean that it is functioning as an proficient hydrostatic system, instead the ability to function as a muscular hydrostat is also maturing during the pre-hatching stages as indicated by the changes observed in ML and MD during respiration and locomotion.

The ability of the mantle to function as a hydrostat is a property of the mantle that is possible because of the dense, three dimensional arrangement of the circular and radial muscle fibers as well as the close interaction of the muscles with the intramuscular collagenous fiber systems and the two stiff collagenous tunics (Bone et al., 1995b; Bone et al., 1981; Gosline and Shadwick, 1983a; Gosline and Shadwick, 1983b; Gosline et al., 1983; MacGillivray et al., 1999; Ward, 1972; Ward and Wainwright, 1972). My results indicate that prior to hatching this dynamic relationship between muscle fibers, IM systems and the tunics is not present in its entirety. My results complement those of Thompson (Thompson and Kier, 2001a; Thompson and Kier, 2001b; Thompson and Kier, 2002) whose work on the ontogenetic changes of the connective tissue in the mantle of hatchling and juvenile *Sepioteuthis lessoniana*, another member of the loliginid family of squids. Thompson (2001a, 2001b, 2002) have found that at hatching *S.lessoniana* paralarvae do not possess the full complement of IM fibers and that as the paralarvae grow, there is an increase in number of these IM fibers and their arrangement becomes more complex (Thompson and Kier, 2001a; Thompson and Kier, 2001b; Thompson and Kier, 2002). The changes in the IM fiber systems also significantly affect mantle kinematics during jet

propulsion and juvenile squid locomotion begins to reflect that of mature adults indicating a maturing muscular hydrostatic function ((Thompson and Kier, 2001a; Thompson and Kier, 2001b; Thompson and Kier, 2002).

The lack of mature IM systems at hatching, would explain several of my results. First, the percent changes observed in mantle length in both respiratory-like and locomotory-like contractions, but particularly in respiratory-like contractions, decrease with age. The range of ML changes at stage 26 to stage 30 decreases by nearly half such that by hatching ML changes are minimal and approaches zero percent. This is consistent with the numbers found in the literature for adult squids (Table IV-1). Past studies have shown that in order for the mantle to function properly as a muscular hydrostat, mantle length remains more or less constant (-5%-0%) (Gosline and Shadwick, 1983a; Kier, 1988a; Packard and Trueman, 1974; Ward, 1972). A constant mantle length allows for changes in two other dimensions of the mantle, mantle thickness and mantle diameter. If mantle length also changes, the mantle cannot become rigid enough to support a powerful jet through the funnel. My results indicate that this is what may be occurring in pre-hatching squids particularly during respiratory-like contractions. Though my results were not statistically significant for ML changes during locomotory-like contractions, there was a trend that was similar to what was found for the respiratory-like contractions. This in itself is worth noting, because it indicates a maturing dynamic between muscle and connective tissue systems yielding a more mature hydrostat and thus the ability to jet propulse.

With the ability to become stiffer, comes an increase in locomotion-type contractions (starting at stage 27).

Examination of mantle kinematics in loliginid embryos indicated that mantle functional ability is a differentiation process much like morphological differentiation. Furthermore, respiration type and locomotory type movements are not only generated early in development, but are also distinguishable from each other both kinematically and through modeling prior to hatching. Based on kinematic data, pre-hatching squid mantles are not functioning well as muscular hydrostatic systems however, this also changes as the embryos develop and differentiate morphologically.

Table IV-1. Published values of maximum mantle diameter changes compared to values from the current study.

Maximum Mantle Diameter Changes (% Change) In <i>L.opalescens</i>		
Stage	Respiration	Locomotion
26	12.90%	
27	17.20%	32.80%
28	36.73%	
29	18.30%	34.30%
(Hatch) 30	35.00%	36.90%

Maximum Mantle Circumference Changes (%Change) Other Lolinidns			
Stage	<i>L.opalescens</i>	<i>L.vulgaris</i>	<i>S.lessoniana</i>
30(Hatch)	40%-42%*	45%**	45%***
Juvenile			33%***
Adult	30%*	30%**	

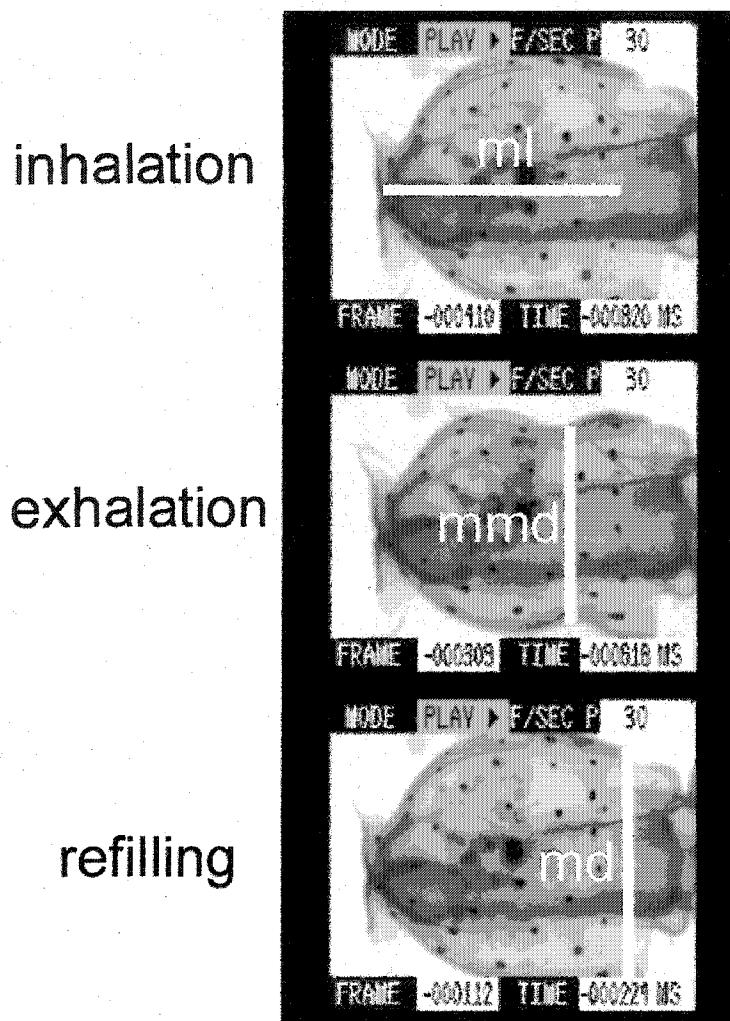


Figure IV-1. Measurements for kinematic and volume results; ml = mantle length, mmd = mid-mantle diameter, md = mantle diameter. Phases of respiratory-like contractions.

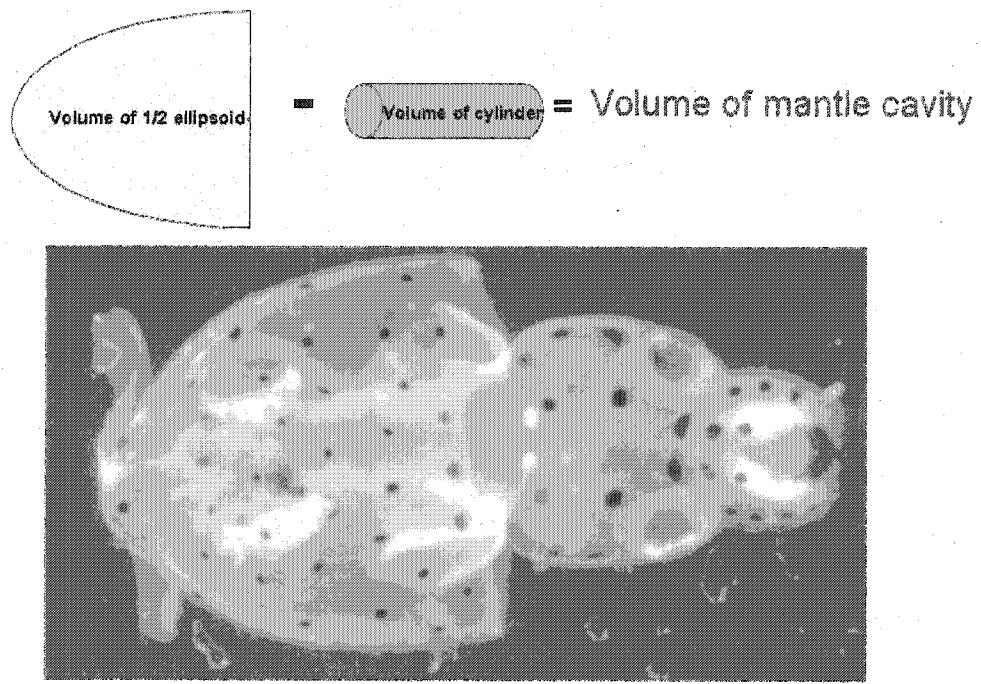


Figure IV-2. Generalization of mantle and internal yolk sac shape for mathematical model.

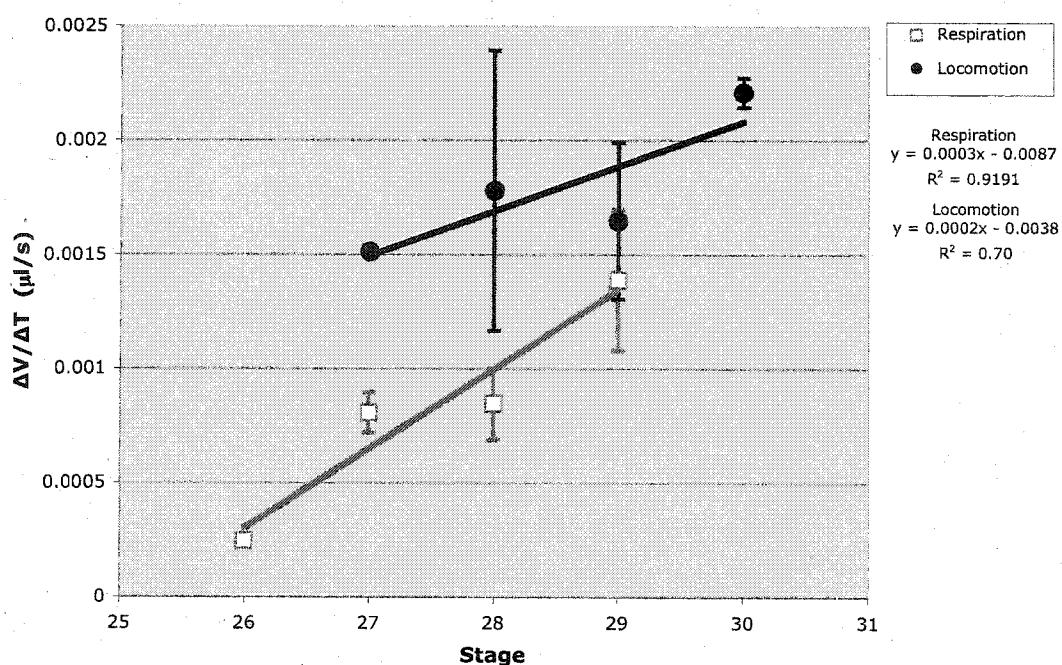


Figure IV-3. Change in volume over change in time per stage.

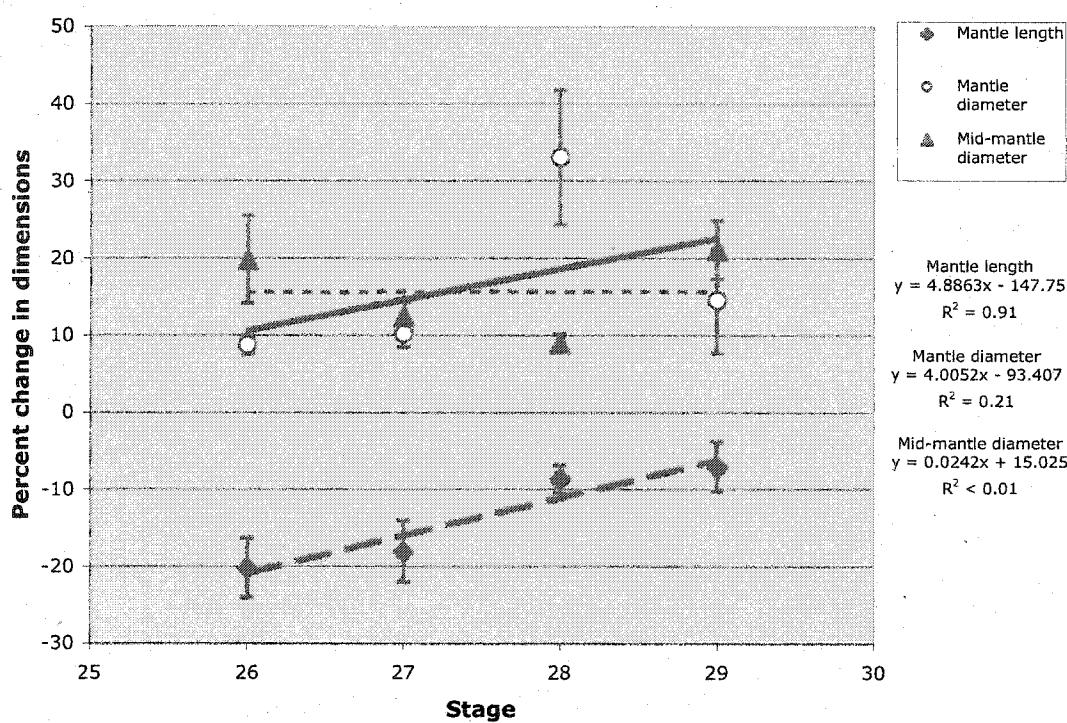


Figure IV-4. Percent change in mantle length (ML), mantle diameter (MD) and mid-mantle diameter (MMD) for respiratory events per stage.

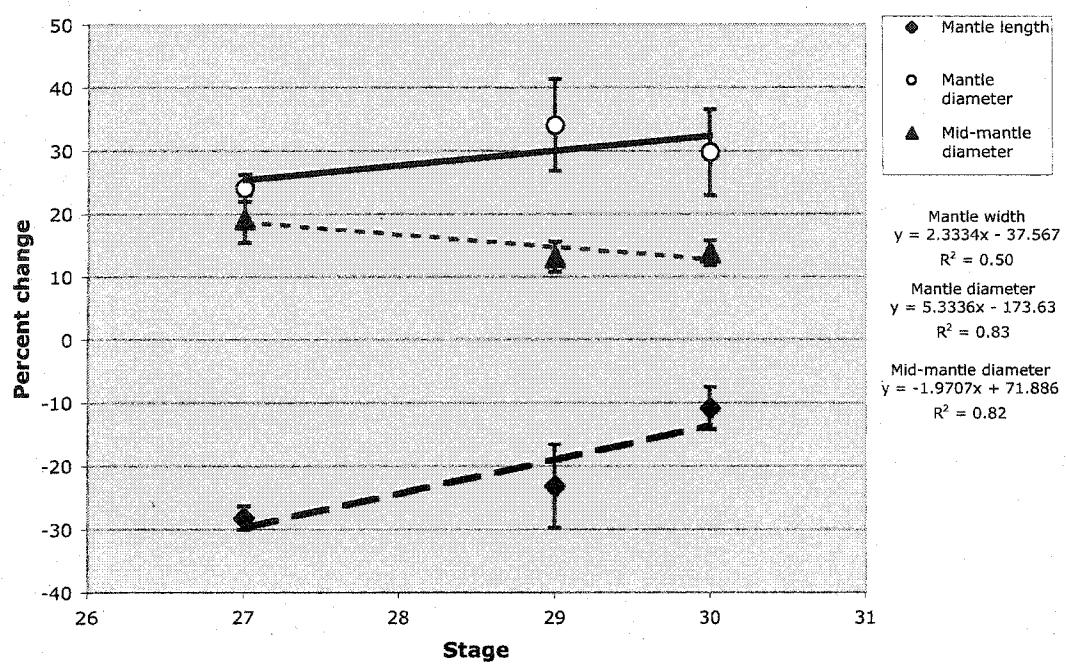


Figure IV-5. Percent change in mantle length (ML), mantle diameter (MD) and mid-mantle diameter (MMD) for locomotory events per stage.

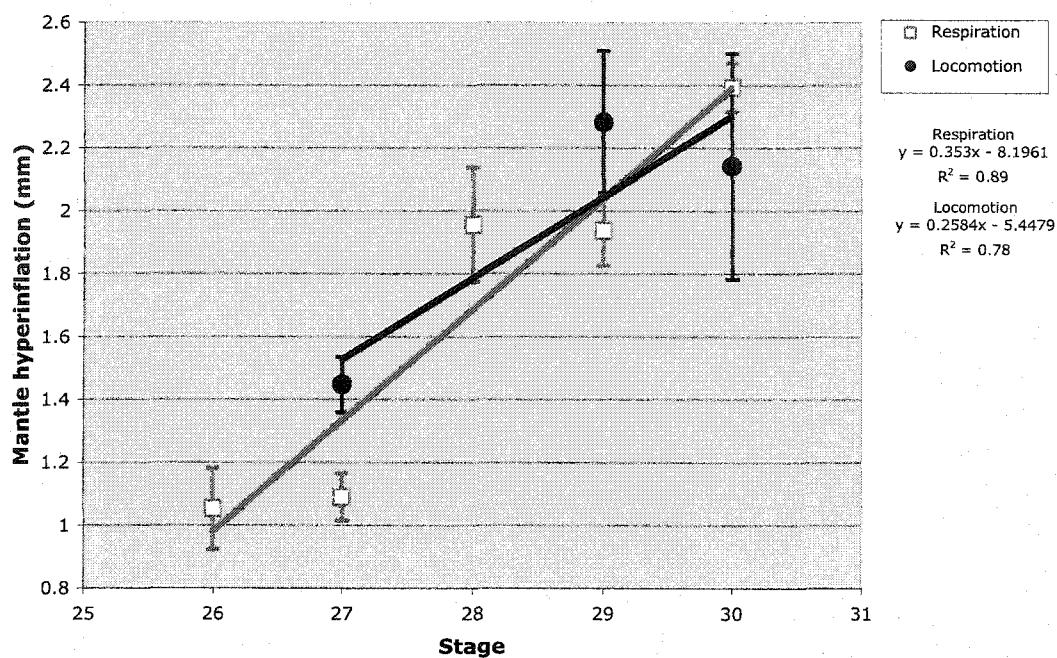


Figure IV-6. Mantle hyperinflation per stage.

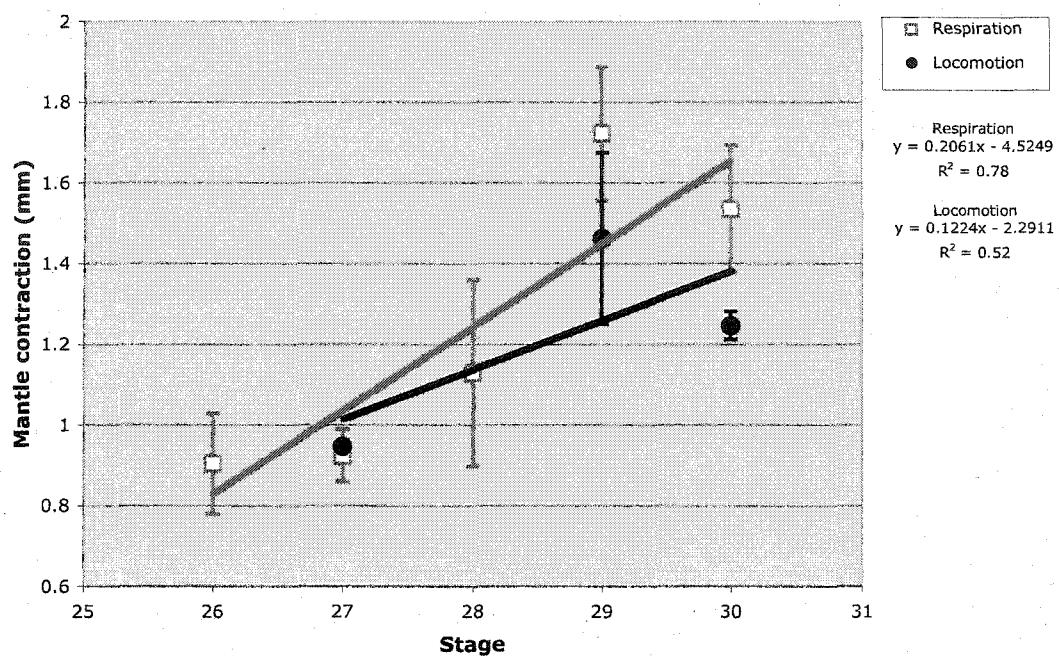


Figure IV-7. Maximum contraction of mantle per stage.

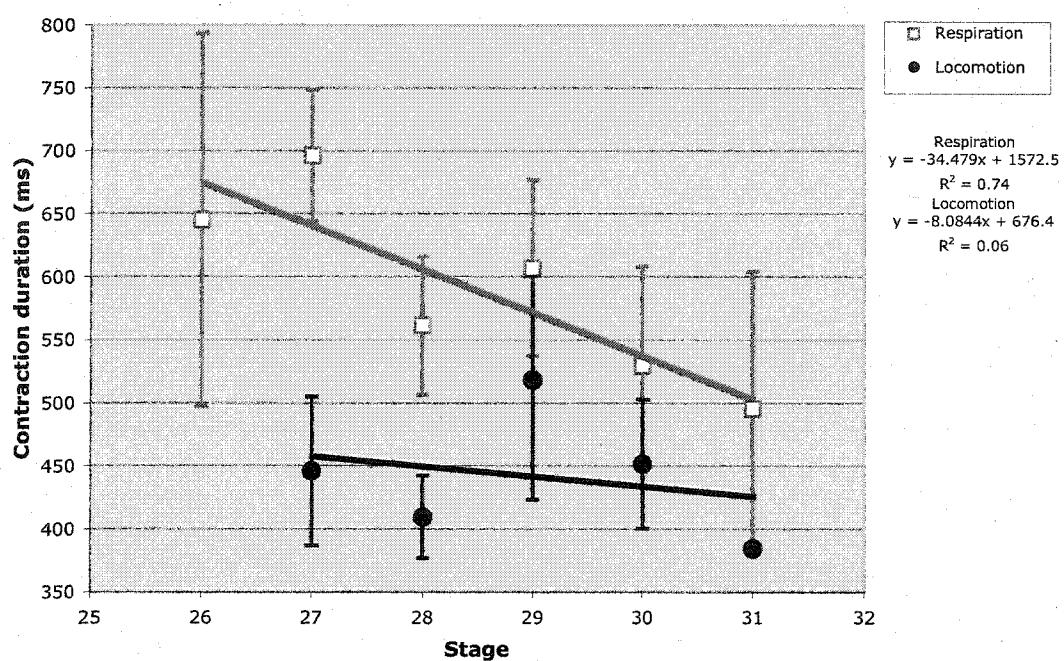


Figure IV-8. Duration of contraction per stage.

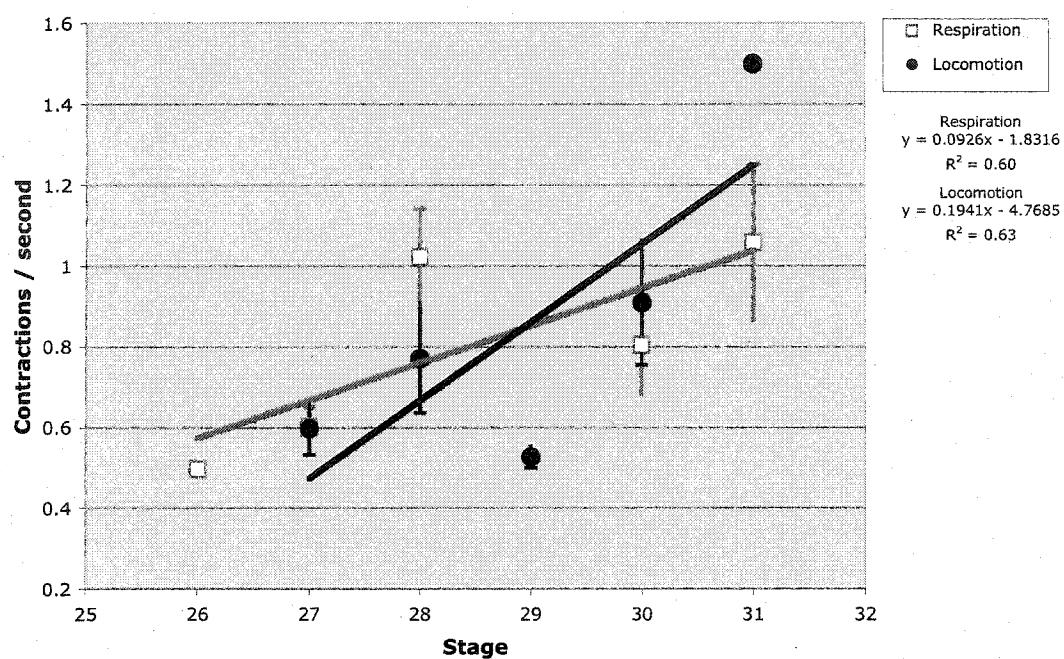


Figure IV-9. Contraction frequency per stage.

CHAPTER V

CONCLUSIONS AND FUTURE WORK

Conclusions

All embryos must go through several functional transitions from seemingly random twitching movements to executing coordinated movements such as respiration and locomotion. These transitions indicate that function, like form, has a definitive ontogeny and should thus be considered as another "structure" that develops and grows over time. As with the ontogeny of form (morphogenesis), the development of function in embryos establishes the foundation for functional ability in juveniles and adults.

Cephalopods do not hatch out as miniature replicas of the adults functionally or morphologically. Although they are more similar to their adult forms than the larvae of indirect developers (e.g. gastropods and bivalves), they still face an important constraint: direct developing embryos must behave as if they were small organisms. Embryos are living entities and must function as such for survival, while at the same time building the structures and accompanying functions that will carry them into adulthood. To do so requires the embryos to operate across a range of environments, each with their own physical and adaptive demands.

Hatching squids encounter a number of life cycle changes as they develop into adults, including growing several orders of magnitude, developing

different modes of both locomotion and prey capture (Thompson and Kier, 2001b; Thompson and Kier, 2002; Vecchione and Shea, 2002) (Chen et al., 1996; Villanueva et al., 1997). Unlike other molluscs with indirect development, squids do not undergo a catastrophic metamorphosis where they shed larval structures and build the adult structures *de novo*. Instead, the structures they use as adults are the same ones that they build during embryogenesis. Thus, these structures need to be operational at hatching and fill the same roles as the adult structures. Because of this, it is difficult to ascertain whether the functional capabilities demonstrated early in the pre-hatching period are of immediate use to the embryo or simply a product of the process of preparing for adulthood. For instance, the mantle of embryonic squids can contract relatively early (stage 25). However, whether these contractions serve a specific purpose for the embryo, or rather, are a result of developing neuromuscular connections required for their eventual post-hatching roles is difficult to determine.

Regardless of the ultimate adaptive purpose of early embryonic movements, functional ability must be established during the pre-hatching stages. The importance of the series of studies described in this dissertation is the detailed examination of the ontogeny both of the structures responsible for locomotion, and of the functional abilities that underlie them. Moreover, doing so in a direct-developing organism means that the embryonic stages examined represent the first steps in the development of the final adult structures and functions, not of a merely larval version. The integrative approach has revealed some interesting developmental correlations, such as the relatively early

emergence of both muscle differentiation and contractile ability in the mantle compared to the fins and funnel.

There are also some surprising disjunctions; for example, structures appear to be present at the gross morphological level well before they show the tissue differentiation (muscle fibers) that is the direct basis of their functional ability. This discrepancy between gross morphology and ultrastructure should serve as reminder that development is much more complex than what can be conveyed in a morphologically-based staging series, no matter how detailed.

Future Directions

The work presented here is an essential descriptive basis for understanding the emergence of functional ability and its structural underpinnings. This study does not, however, directly address function *per se*, nor can the observed correlations test specific hypotheses about the interrelation of emerging form and emerging function. However, several topics requiring further investigation have stemmed from this work. First, evidence from other organisms suggests that morphological development and functional development are interdependent: interfering with either process disrupts the other (Sival, 1993; Sival et al., 1992; Wu et al., 2001). For example, when the early embryonic movements of chicks were disturbed, proper formation and subsequent function of some skeletal structures were significantly affected (Wu et al., 2001). Is the same true in squid embryos? Moreover, is the early ability to contract important for the proper development and differentiation of the mantle? Perhaps these

contractions are of significant use to the embryo itself for other purposes specific to the embryonic period such as aiding in the hatching process or perhaps in forming the neuromuscular connections essential for locomotion. A possible experiment would involve the pharmaceutical or mechanical inhibition of early mantle movements for varying lengths of time in an ontogenetic series of embryos and assessing any morphological and/or functional consequences. The detailed descriptive data provided here for squids are critical to the design and interpretation of such experiments: it is necessary to know the normal course of events in order to measure disruption.

This study did not directly address the issue of growth, which is of central importance to understanding the generation of structures in the embryo, and their transformation to adult morphologies capable of carrying out adult functions. Data from gross morphological and ultrastructural studies presented in chapters 2 and 3 suggest that the mechanism by which these structures are growing is a combination of proliferation of undifferentiated myoblasts and hyperplastic growth (although hypertrophic growth could not be ruled out). Therefore, a more detailed and quantitative ultrastructural examination of the mantle, funnel, and fins is essential to finally resolve this issue. In particular, more comprehensive measurements of the muscle fibers themselves as well as myofibril content must be gathered. Additionally, some of the crucial remaining questions include: Does the mode of growth differ in individual structures at any point during development once the differentiation program has been initiated? (e.g. Does the mantle grow only by hyperplasia while the funnel grows by hypertrophy?) Is muscle growth,

like muscle differentiation, regional: are only certain areas of the mantle, funnel, and fins growing? Finally, if growth in these structures is in fact a result of both hyperplasia and hypertrophy just at different rates what are they? Are the rates comparable in each structure? Understanding patterns and mechanisms of growth in embryos will ultimately allow comparison with the patterns and mechanisms already documented in juveniles (Martinez, 2001; Martinez and Moltschaniwskyj, 1999; Pecl and Moltschaniwskyj, 1997). It will be interesting to see whether such patterns and mechanisms are essentially continuous throughout the life cycle, or whether something qualitatively different is occurring in the earliest stages (in spite of being direct developers, whose ontogeny is a relatively continuous process from embryo to adult).

I focused on the development of musculature in locomotory structures and especially the mantle. An obvious and important complement to this work would be a detailed investigation of the interplay between the developing nervous system, developing connective tissue fiber networks with the developing musculature during the emergence of functional capabilities to determine exactly what roles each play during a contraction cycle. Understanding the relationships between each of these systems would contribute a better understanding of the kinematic changes reported in this study. For example, does contraction frequency increase because of the presence of more muscle or because of more nerves or both? Can the emergence of two distinct contraction types be definitively tied to the emergence of the different muscle fiber types or to the development of one of the connective tissue fiber systems?

This dissertation considered the emergence of functional capability in squid embryos as a developmental process similar to morphological development. By examining the development and differentiation of mantle musculature and its emerging functional abilities, my work provides a basis for understanding how these organisms establish the foundation for specific functions such as respiration and locomotion.

APPENDICES

APPENDIX A

FIXATION AND STAINING PROTOCOLS FOR PHALLOIDIN AND DAPI

A. PREPARATION OF 4% PARAFORMALDEHYDE SOLUTION:

1. 4 grams of paraformaldehyde(powder).
2. 100mls DW.
3. Mix in glass beaker on low heat with stir bar until powder goes into solution (can take up to 8 hours).

B. PREPARATION OF PHALLOIDIN SOLUTIONS:

1. 5x-Stock solution of phosphate buffered saline(pH7.4):
 - a. 40.0 grams NaCl
 - b. 1.0 grams KCl
 - c. 7.2 grams Na₂HPO₄
 - d. 1.2 grams kh₂po₄
 - e. 800 mls distilled deionized water(ddw)
 - f. Dissolve salts in 800 mls ddw.
 - g. Add more ddw until total vol. is 1000 mls.
2. 1x-pbs-working solution (pH7.4): dilute 1part 5xpbs with 4 parts ddw.
3. 0.1m phosphate buffered saline(pbs):
 - a. 10mls 1x pbs
 - b. 90mls ddw
4. 0.2M pbs-triton-x(pbt):
 - a. 20mls triton-x
 - b. 100mls 1x pbs
5. Phalloidin-in dark:
 - a. 10ml phalloidin in microfuge tube.
 - b. Let evaporate for 30 minutes.
 - c. Redissolve in 200ml of pbt.
 - d. Make three tubes of 10ml phalloidin/200ml pbt.

C. PREPARATION OF DAPI (DAPI IS SOLUBLE IN DW):

1. Stock solution of dapi (store in dark):
 - a. 1mg dapi/1ml dw.
 - b. Dilute 100x for working solution.
 - c. 10ml/90mls dw.
2. Staining protocol-phalloidin:
 - a. Fix tissue in 4%paraformaldehyde 2 hrs.
 - b. Wash 3 times with 0.1M pbs-20 minutes each rinse.
 - c. Permeabilize in pbt- 1hr.

3. Stain with phalloidin in dark-1hr.
4. Wash 3 times with 0.1mpbs-15 minutes each rinse.
 - a. Mount tissue on glass slides with gelmount;coverslip.
 - b. Slides will not fade if kept in dark and in freezer for 2 weeks.
5. Staining protocol-dapi:
 - a. After staining with phalloidin rinse tissue 2times with dw.
 - b. Add dapi-20minutes-1 hr. depending on tissue size.
 - c. Mount tissue as above.

APPENDIX B

FIXATION PROTOCOLS FOR THICK (1.0 μM) AND THIN (0.1 μM) SECTIONS

A. PREPARATION OF A 3% GLUTERALDEHYDE (GTA) SOLUTION:

1. Stock solution of GTA is 25% in glass vials.
2. 1.5 mls of the 25% stock solution, bring to 6.1 mls with seawater (SW).
This yields a 6% GTA solution.
3. Mix 1:1 with 0.2M cacodylate buffer, pH7.2
4. Add 2-3 drops of 30% H_2O_2 per ml of concentrated GTA used.

B. BUFFER SOLUTION:

1. Add 4.28 grams of sodium cacodylate to 100ml DW or 2.14 grams cacodylate/50 ml of water.
2. Above yields a 0.2M buffer solution; it will be diluted before use to 0.1M.
3. pH with concentrated HCL to pH7.2.

C. TISSUE PREPARATION:

1. Pieces of tissue to be fixed are placed in a puddle of fixative on a wax sheet. Cut tissue into small pieces –approximately 1-2mm diameter.
2. Place pieces of tissue into glass vials with 3% GTA and fix for 3 hours at room temp.

D. RINSE:

1. Rinse 3 times every fifteen minutes with cacodylate buffer solution.
2. Dilute stock buffer by half before use so that rinse buffer will be 0.1M concentration.

E. POSTFIXATION:

1. Osmium tetroxide solution:
 - a. Take stock solution of 4% OsO_4 and dilute one to one with DW to make a 2% solution.
 - b. Mix 2% OsO_4 one to one with 0.2M sodium cacodylate buffer (pH 7.2) to get 1% OsO_4 solution.
 - c. After final rinse of fixative, replace rinse buffer with approximately 2mls of 1% OsO_4 and fix at room temperature for 2 hrs.
2. Rinse as described above for 1 hour as 4 fifteen minute rinses.

F. DEHYDRATION

1. Alcohol-ethanol only (ETOH).
 - a. 50% ETOH-10 minutes.
 - b. 70% ETOH-10 minutes.
 - c. 90% ETOH-10 minutes.
 - d. 100% Acetone- one change, 10 minutes.
2. Acetone transition solvent.
 - a. 100% acetone replaces the 100% Acetone from above-10 minutes.
 - b. A second change of 100% acetone as above.

APPENDIX C

EMBED-812 EMBEDDING PROTOCOL FOR THICK AND THIN SECTIONS

A. RESIN RECIPE (FROM EMS):

(If mixing a small quantity, mix everything together including accelerator and keep frozen maximum of two weeks.)

1. Mix A: 5mls Embed-812 and 8mls DDSA
2. Mix B: 8mls Embed-812 and 7mls NMA

Pour both A and B into a glass jar and mix vigorously. Make sure you get all mixture from vials because about a ml will be left behind.

3. Add 0.5mls of DMP-30(accelerant) to mixture-mixture will darken to an almost orange color.
4. Mix vigorously.
5. Place jar in vacuum for about 30 minute.
6. Suck mixture into a 30 ml syringe. Try to limit bubbling.

B. INITIAL INFILTRATION:

1. Make a 1:1 dilution of resin with 100% acetone. Stir solution until it is clear (yellow but clear).
2. Replace the 100% acetone with resin/acetone mix and place vial with caps on a rotator overnight.
3. After overnight rotation, remove cap vial and let acetone evaporate 8 hrs.
4. Remove specimens from vials onto paper towels on aluminum foil.
5. New resin put into gelatin capsules with syringe.
6. Place specimens into capsules with wood spoons.
7. Fill capsules to almost full.
8. Place under vacuum for 2 hrs.

C. POLYMERIZATION:

1. New resin; use syringe and place one drop of resin at the end of each plastic mold trough.
2. Transfer tissue, one piece per trough with wood spoon. When each trough has one piece of tissue in it, use syringe to fill up troughs to the brim with resin.
3. Place molds in 60° oven 12-18 hrss. Once hardened blocks can be easily removed.
4. Mark blocks to ID them with paper labels.

APPENDIX D

STAINING PROTOCOLS FOR THICK (1.0 μM) AND THIN (0.1 μM) SECTIONS

THICK SECTION STAINING:

A. STOCK SOLUTIONS (OR CAN USE EMS PREMADE STAIN)

1. 1% Azure II
5 g Azure II in 500 ml DI water
2. % Methylene Blue in 1% sodium borate
Mix 5 g sodium borate in 500 ml DI.
Add 5 g of Methylene Blue.
 - a. Store stock solution separately in glass flasks.
 - b. Make working solution:
Mix stains in 1:1 proportion in small beaker.
Store in 10 ml syringe with 0.45um filter on tip.

B. STAINING SECTIONS

1. Use working solution (1:1 methylene blue: azure II).
2. Drop stain onto dry slide - enough to cover all sections.
3. Waft through the Bunsen burner flame (roughly 5x).
 - a. Will see some smoke & the edges will turn a yellow-green.
 - b. Don't let the stain dry on the sections.
4. Pour off excess stain down the drain.
5. Plunge cooling slide into beaker of DI water & swoosh to get rid of stain.
 - a. May want to run under DI at sink.
 - b. Start stream of water on edge and let it wash over sections.

C. DRY SLIDE

1. Waft stained & rinsed slide under Bunsen burner flame until dry.

THIN SECTION STAINING

A. SOLUTIONS

1. Boiled millipore-filtered distilled water:
 - a. Fill 2 L flask to 1.5 L with DI water or Ultrapure water with 0.2 μm filter.
 - b. Boil for 30 min on ringstand or hotplate-gets rid of CO_2 .
 - c. Cool.
 - d. Filter through 0.2 μm Nalgene filter.

2. Uranyl Acetate- 5%
 - a. 0.5g UA in 10 ml DW:
 1. Want a saturated solution.
 2. Stir for 15 minutes to get UA crystals into solution.
 3. Filter 3x.
 - a. 1x: our pour from beaker - try not to get crystals onto filter.
 1. Decant into second beaker.
 2. Rinse excess crystals off filter by filtering 25mls DW.
 3. Discard into waste rinse UA container.
 4. Get rid of excess crystals in original beaker - dissolve in DI.
 5. Use enough DI to just dissolve crystals.
 6. Discard into waste UA container.
 - b. 2x: pour from beaker, decant into second beaker
 1. rinse filter if necessary
 - c. 3x: pour from beaker, decant into cleaned ground glass container
 3. Lead citrate:
 - a. 25 ml DW.
 - b. 0.125 g lead citrate.
 - c. 1 pellet NaOH.
 1. Cover with parafilm and stir gently.
 2. Want everything to dissolve but NO AIR IN SOLUTION.
 3. Filter 2x as above (or run through (2) 0.2 μ m syring tip filters).
 4. Store in tall flask on the door of the 'fridge.
 4. NaOH stock solution (0.2 M NaOH):
 - a. 8 g of NaOH pellets in 100 ml DW.
 - b. Stir.
 - c. Store in refrigerator.
 5. NaOH working solution (0.02 M NaOH):
 - a. 10 ml stock NaOH + 90 ml DW.
 - b. Stored in squirt bottle in refrigerator.

B. STAINING

1. STAIN WITH URANYL ACETATE:
 - a. Place square of parafilm on countertop.
 - b. Put 5 drops of UA on parafilm & cover w/petri dish to minimize CO₂ exposure.
 - c. Float 1 grid on each drop of UA - tissue side down.
 1. Keep track of which grid is which!
 - d. Cover with petri dish.
 - e. Cover with box (UA is light sensitive).
 - f. Heat area with lamp (may help improve stain penetration).

- g. Stain for 20-30 min.(depends on tissue).
 - h. Rinse tissue 3x with DW.
 1. Dry with Whatman filter paper.
2. STAIN WITH LEAD CITRATE:
 - a. Put 5-6 pellets on a new square of parafilm & cover with petri dish.
 - b. Put one large "puddle" of lead citrate next to the pellets.
 - c. Submerge grids in lead citrate - put them on the parafilm w/sections facing up.
 - d. Stain for 5-10 min.
 - e. Rinse grids with DW.
 1. Dry with Whatman paper.
 - f. Return to grid holder.

APPENDIX E

DEVELOPING TEM NEGATIVES (FROM BRYN MAWR COLLEGE-GARDINER LAB)

A. PREPARE STOCK DEVELOPING SOLUTIONS:

1. D19-(good for several months):
 - a. Use hot tap H₂O - 48°C.
 - b. Put 3800ml hot H₂O in 4L beaker w/stirrer (kept under sink).
 - c. Put stirrer on - stir until get "tornado".
 - d. Slowly empty bag into beaker.
 - e. Transfer to jug & wash beaker immediately.
 - f. Cool to room temperature - OK to have flocculant material on surface.
 - g. Keep stock (& working) solutions in incubator at 20 °C.
2. Rapid Fix (=Hypo)-(good for several months):
Follow circled recipe on instruction package - makes 3.8L.
3. Prepare HypoClear stock solution-(good for several months):
 - a. Put 3800ml tap H₂O in 4L beaker w/stirrer (kept under sink).
 - b. Put stirrer on - fast enough to get "tornado".
 - c. Slowly empty bag into beaker.
 - d. Transfer to jug & wash beaker immediately.
 - e. Let cool to room temperature.

B. PREPARE TUBS WITH WORKING SOLUTIONS:

Arrange tubs so they don't drain over the edge - push back 5cm from edge.

Tub 1: 1300mL stock D19 + 2600mL tap H₂O.

Must be at 19-20degC

Developer is bad when turns amber-brown.

Roughly 100 negatives or 2 weeks.

Tub 2 - Rinse water (room temp).

Tub 3 - Rapid Fix: full strength (room temp).

Use Hypo Check to test for "goodness".

Couple of drops into Rapid Fix.

Clear - OK, Cloudy / Precipitate - bad.

Tub 4 - 800mL stock HypoClear + 3200mL H₂O (room temp).

Tub 5- Rinse water (room temp).

FROM HERE ON, WORK WITH SAFE LIGHT ON

C. DEVELOP FILM

1. Put negatives into holders every other slot

2. Developing procedure:

Tub 1 D19	4min	a) tap holder against edge to remove air bubbles
Tub 2 rinse	1min	
Tub 3 Rapid Fix	4 min	
Tub 2 rinse	1 min	b) agitate up & down constantly throughout procedure
Tub 4 HypoClear	2 min	
Tub 5 rinse	5 min	

3. When all film has been processed:

- a. Rinse each holder with DI water hose.
- b. Empty 1 rinse tub.
- c. Put 2 capfuls of photoflo.
- d. Let fill with water.
- e. Dip all negatives 2-3x in photoflo.
- f. Let dry completely, in holders on top of water chiller.
- g. Put in glycine envelopes for long-term storage.

APPENDIX F

LIST OF *L.PEALEII* SPECIMENS USED FOR LIGHT (LM) AND TRANSMISSION ELECTRON MICROSCOPY(TEM)

STAGE	BLOCK#	THICKNESS	ANALYSIS	STAGE	BLOCK#	THICKNESS	ANALYSIS
22	3	0.1μm	TEM	25	11	1μm/0.1μm	LM/TEM
22	4	1μm	LM	25	6a	1μm	LM
22	6	1μm/0.1μm	LM/TEM	25	12	0.1μm	TEM
22	7	1μm	LM	26	1	1μm/0.1μm	LM/TEM
23	8	1μm	LM	26	2	1μm	LM
23	9	1μm	LM	26	3	1μm/0.1μm	LM/TEM
23	10	1μm	LM	26	5	1μm/0.1μm	LM/TEM
23	11	1μm	LM	27	3	1μm	LM
23	12	1μm	LM	27	6	1μm/0.1μm	LM/TEM
23	9a	1μm	LM	27	8	1μm	LM
23	11a	1μm	LM	27	10	1μm/0.1μm	LM/TEM
23	10a	1μm	LM	27	11	1μm/0.1μm	LM/TEM
24	1	1μm	LM	27	12	1μm/0.1μm	LM/TEM
24	2	1μm	LM	27	8a	1μm	LM
24	3	1μm	LM	28	1	1μm/0.1μm	LM/TEM
24	4	1μm	LM	28	7	1μm	LM
24	5	1μm/0.1μm	LM/TEM	28	12	1μm/0.1μm	LM/TEM
24	6	1μm/0.1μm	LM/TEM	29	9	1μm	LM
24	6a	1μm	LM	29	10	1μm	LM
24	7	1μm	LM	29	9a	1μm	LM
25	2	1μm	LM	30	15	1μm/0.1μm	LM/TEM
25	3	1μm/0.1μm	LM/TEM	30	16	1μm	LM
25	6	1μm	LM	30+	3A	1μm	LM
25	7	1μm	LM	30+	3C	1μm	LM

APPENDIX G

NUMBER AND STAGES OF *L.OPALESCENS* EMBRYOS MEASURED FOR KINEMATIC AND MODEL DATA

SQ#STG	Contractions		DATE	SQ#STG	Contractions		
	meas.	Locomotion			meas.	Locomotion	
2 25	4	0	0	7.16.03	2 28	26	0 26
3 26	5	0	0		5 28	20	0 20
5 26	5	0	5		8 28	14	8 6
1 26	2	0	2		10 28	12	2 10
3 26	6	0	6		7 28	11	5 6
4 26	2	0	2		8 28	18	1 17
3 27	15	1	14		10 28	13	0 13
7 27	11	4	7		5 28	19	5 14
8 27	5	0	5	7.26.02	3 28	6	2 4
4 27	6	0	6		5 28	8	0 8
6 27	5	0	5		9 28	6	0 6
7 27	10	2	8	7.23.02	10 28	10	0 10
9 27	18	0	18		11 28	4	0 4
6 27	4	0	4		13 28	7	0 7
7 27	8	0	8		16 28	4	1 3
9 27	9	0	9	6.28.03	2 29	8	6 2
3 27	5	0	5		4 29	17	6 11
8 27	4	0	4	6.29.03	1 29	12	5 7
6 27	5	0	5		2 29	13	1 12
8 27	4	0	4		3 29	14	2 12
10 27	3	0	3		6 29	8	3 5
1 27	2	0	2		2 29	4	2 2
2 27	5	0	5		7 29	2	0 2
1 27	12	0	12		7.2 29	6	6 0
5 28	16	0	16		7 29	4	2 2
3 28	19	18	1	7.22.03	1 30	14	8 6
5 28	14	6	8		2 30	14	9 5
				7.19.03	2 31	9	2 7
					5 31	9	3 6
				7.25.02	1 31	9	4 5
					6 31	7	4 3
					4 31	15	14 1
					11 31	24	24 0
					6 35	10	4 6
				7.17.03	1 35	21	8 13
					4 35	15	5 10

APPENDIX H

LIST OF ABBREVIATIONS

Ap	anal papilla
bm	basement membrane
bv	blood vessel
CMP	central mitochondria poor
Ci	cilia
CT	connective tissue
cyt	cytoplasm
Ep	epithelium
fib.gl.	fibrous glial cell
F	fin
Fu	funnel
G	gills
Gl	gladius
gr.gl.	granular glial cell
It	inner tunic
iys	internal yolk sac
ml	mantle length
M	mantle
md	mantle diameter
mmd	mid-mantle diameter
mi	mitochondria
mvb	multi-vesiculate bodies
mf	muscle fibers
n	nucleus
Ot	outer tunic
ret	reticulation
rf	radial fiber
Sk	skin
sg	stelate ganglion
SMR	superficial mitochondria rich

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