ULTRASTRUCTURE AND IMMUNOCYTOCHEMISTRY OF THE APODEMES IN THE CHELAE OF THE BLUE CRAB, *CALLINECTES SAPIDUS*

Anne E. Leaser

A Thesis Submitted to the
University of North Carolina Wilmington in Partial Fulfillment
of the Requirements for the Degree of
Master of Science

Department of Biology and Marine Biology
University of North Carolina Wilmington
2010

Approved by

Advisory Committee

Thomas H. Shafer Robert D. Roer

Richard M. Dillaman
Chair

Accepted by

Dean, Graduate School
This thesis has been prepared in a style and format consistent with the journal

The Journal of Structural Biology
# TABLE OF CONTENTS

ABSTRACT ...............................................................................................................................v

ACKNOWLEDGMENTS ........................................................................................................... viii

LIST OF TABLES ................................................................................................................... viii

LIST OF FIGURES ................................................................................................................ ix

CHAPTER 1: ULTRASTRUCTURE AND IMMUNOCYTOCHEMISTRY OF THE APODEMES AND ASSOCIATED TISSUE IN THE CHELAE OF THE BLUE CRAB .......1

1. Introduction .........................................................................................................................1

2. Materials and methods ........................................................................................................ 4

   2.1 Animals and experimental design ................................................................................. 4

   2.2 Light Microscopy ......................................................................................................... 4

   2.3 Immunocytochemistry ................................................................................................. 5

   2.4 Transmission Electron Microscopy .............................................................................. 6

   2.5 Scanning Electron Microscopy .................................................................................... 7

3.0 Results ............................................................................................................................ 8

   3.1 Light Microscopy ......................................................................................................... 8

   3.1.1 Acridine Orange Staining ....................................................................................... 8

   3.1.2 Immunocytochemistry ......................................................................................... 9

   3.2 Transmission Electron Microscopy ............................................................................ 11

   3.3 Scanning Electron Microscopy ................................................................................. 14

   3.4 Ultrastructure of apodeme and associated tissue ..................................................... 15

4. Discussion .......................................................................................................................... 18

   4.1 Cuticle deposition ....................................................................................................... 18

   4.2 Chitin fiber orientation ............................................................................................... 20

   4.3 Cuticle-epithelial boundary ....................................................................................... 21

   4.4 Summary of the results .............................................................................................. 23
CHAPTER 2: EXPRESSION PATTERNS OF THREE PROTEINS CONTAINING THE REBERS-RIDDIFORD CONSENSUS SEQUENCE IN THE BLUE CRAB

1. Introduction

2. Materials and Methods
   2.1 Animals and experimental design
   2.2 Tissue processing
   2.3 Immunocytochemistry

3. Results
   3.1 Negative and positive controls
   3.2 mRNA appearance and antibody staining
   3.3 Antibody binding pattern in the dorsal carapace and branchial chamber
   3.4 Antibody binding pattern in the arthrodial membrane and gills
   3.5 Antibody binding pattern in the apodeme and suture line

4. Discussion
   4.1 Endogenous fluorescence
   4.2 Comparison of antibody results with previous work
   4.3 Finding genes responsible for calcification
   4.4 Summary of the results

LITERATURE CITED
ABSTRACT

In the chelae, the claw, of the blue crab are two invaginations of the cuticle (apodemes) providing attachment sites for muscle. The morphology and composition of intermolt, premolt, postmolt apodemes and associated tissue was revealed using light microscopy, immunocytochemistry, transmission electron microscopy (TEM), and scanning electron microscopy (SEM). Acridine orange (AO) staining of the apodeme differentiated the medial and lateral layers. The fused cuticular regions (median layer) stained red and no stain was observed in the adjacent cuticle (lateral layers). AO staining of the epithelium showed a patchy distribution of red fluorescence indicating enhanced ribosomal activity in portions of the cells during premolt and early postmolt. A β-tubulin antibody and TEM confirmed the presence of bundles of microtubules interspersed with the secretory regions of the cells. TEM also revealed that these microtubules were attached to tonofibrils that anchor the cuticle to the epithelium. The other end of the microtubule bundles forms a terminal z-band with the myofibrils of the muscle. A unidirectional pattern of chitin fibrils along the length of the apodeme was observed with the SEM. The pattern of AO staining as well as other ultrastructural features indicated that the apodemes are a unique cuticle type. Clues as to how muscle maintains function until very late in premolt were also revealed by examination of the cuticle-epithelial boundary throughout the molt cycle.

Three polyclonal antibodies to *C. sapidus* cuticle proteins were used to describe the distribution of the products of the genes CsAMP8.1 and CsCP8.5 as well as the product of the EarlyCP motif at critical points in the molt cycle using their unique amino acid epitopes. They were also used to determine the nature of the apodeme by comparing the localization of each antibody amongst several different regions of the blue crab. Two of the antibodies have been associated with calcified cuticle (CsCP8.5 and the EarlyCP motif) and one (CsAMP8.1)
with uncalcified cuticle (arthrodial membrane). Surprisingly, none of the antibodies stained in the predicted pattern.
ACKNOWLEDGMENTS

I want to take this opportunity to thank and acknowledge all the people who helped me through this Master’s program. Thank you to my chair, Dr. Dillaman, for his unwavering support and believing a girl from Kansas could learn something about blue crabs. Thanks to all my committee members, the blue crab dream team, for their guidance as I developed my thesis. Thanks to Mark Gay and Carolina Priester for teaching and assisting me with my lab work, I could not have done it without you two.

A special thanks to my parents, Ryan, Jodi, and Ben for their love, encouragement, support, and advice throughout the years.

This research was supported by grant #2007-BRG-1251 from the North Carolina Biotechnology Center and grant #IOS-0719123 from the National Science Foundation. Samples were collected at Endurance Seafood in Kill Devil Hills, North Carolina, an amazing facility with terrific people.
### LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Summary of appearance of mRNA and immunocytochemical staining in the dorsal carapace and arthrodial membrane and summary of the immunocytochemical localization pattern for the three antibodies in the gills, branchial chamber, apodeme, and suture line</td>
<td>32</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Transverse section through the thorax of the blue crab showing the location of the apodemes</td>
<td>1</td>
</tr>
<tr>
<td>2. The apodemes from the chela of a blue crab in intermolt with musculature removed</td>
<td>2</td>
</tr>
<tr>
<td>3. Acridine orange staining of the apodeme and associated tissue</td>
<td>9</td>
</tr>
<tr>
<td>4. Localization of β-tubulin in associated tissue throughout the molt cycle</td>
<td>10</td>
</tr>
<tr>
<td>5. Ultrastructure of the apodeme and associated tissue</td>
<td>12</td>
</tr>
<tr>
<td>6. Ultrastructure of the apodeme and associated tissue</td>
<td>13</td>
</tr>
<tr>
<td>7. Chitin fiber orientation in the apodeme</td>
<td>15</td>
</tr>
<tr>
<td>8. Schematic drawing of the apodeme throughout the molt cycle</td>
<td>17</td>
</tr>
<tr>
<td>9. Localization of endogenous fluorescence and non-specific binding of the secondary antibody (negative and positive controls, respectively) in the dorsal carapace, branchial chamber, apodeme, and arthrodial membrane</td>
<td>30</td>
</tr>
<tr>
<td>10. Location of the EarlyCP motif, CsCP8.5, and CsAMP8.1 (three proteins) in the dorsal carapace and branchial chamber using confocal microscopy and a secondary antibody labeled with Alexa 594</td>
<td>34</td>
</tr>
<tr>
<td>11. Location of the three proteins in the arthrodial membrane and the gills</td>
<td>37</td>
</tr>
<tr>
<td>12. Location of the three proteins in the apodeme and suture line</td>
<td>39</td>
</tr>
</tbody>
</table>
CHAPTER 1: ULTRASTRUCTURE AND IMMUNOCYTOCHEMISTRY OF THE APODEMES AND ASSOCIATED TISSUE IN THE CHELAE OF THE BLUE CRAB

1. Introduction

The exoskeleton of the blue crab, Callinectes sapidus, varies tremendously from region to region with respect to the amount of calcification observed. Some regions of the cuticle become completely calcified over the course of the molt cycle, whereas other regions remain uncalcified. Even the sequence of formation differs with each region. For instance, apolysis and deposition of pre-exuvial cuticle layers occurs at D0 in the dorsal carapace while apolysis in the gills doesn’t occur until D2 and deposition of pre-exuvial cuticle layers begins at D3 (Drach and Tchernigovitzeff, 1967; Johnson, 1980; Andrews and Dillaman, 1993; Elliot and Dillaman, 1999). In the crayfish, Procambarus clarkii, apolysis and deposition of pre-exuvial cuticle layers even differs between respiratory and transporting gills (Andrews and Dillaman, 1993).

While much attention has been centered on the structure of the dorsal carapace, there has been little mention of the inner skeletal structure of C. sapidus. Within the first pereiopods of C. sapidus, the chelae, there are structures that have been described as “blade-like tendons” (Cochran, 1935) (Fig. 1). Transmission electron microscopy (TEM) revealed

![Figure 1. Transverse section through the thorax. The musculus abductor dactylopoditis and the musculus adductor dactylopoditis attach the tendons (apodemes) to the dactylopodite. Segments of the first pereiopod from anterior to posterior are: dactylopodite, propodite, carpus (illustration from Cochran, 1935).]
that the “tendon” is formed by the invagination and fusion of two or more segments of the integument (Crawford, 2006; Crawford et al., 2007). This makes them an apodeme, not a tendon; an apodeme being an infolding of the exoskeleton that provides an attachment site for muscles (Oxford, 2000). While a vertebrate tendon attaches muscle to bone (Oxford, 2000) and vertebrates have extracellular tendons composed of collagen, invertebrates have intracellular tendons composed of microtubules that attach muscle to cuticle (Auber, 1963; Lai-Fook, 1967; Reedy and Beall, 1993). There are, in fact, two apodemes located in each chela of *C. sapidus*; a smaller dorsal apodeme that facilitates the opening of the chela and a larger ventral apodeme responsible for closing (Cochran, 1935; Crawford, 2006; Crawford et al., 2007) (Fig. 2). The apodemes do not have the same cuticular structure as the dorsal carapace. The medial layer of the apodeme is formed by the fusion of two or more cuticle layers while the lateral layer is

Figure 2. The apodemes of *C. sapidus* after removal of the muscle using a hot water bath. The dorsal apodeme (D) and ventral apodeme (V) are also shed at ecdysis along with the outer cuticle of the dactylopodite (arrowhead). Insert – The base of the apodeme, where the apodeme is proximal to the dactylopodite, has a complex diamond shape.
similar to the endocuticle and no analog of the exocuticle is present (Crawford, 2006; Crawford et al., 2007). During the molt cycle, *C. sapidus* sheds the two apodemes in the chelae that attach the musculus abductor dactylopoditis and the musculus adductor dactylopoditis to the dactylopodite of the first pereiopod. In order for the crab to free the first pereiopod from the old exoskeleton the muscles may undergo atrophy to reduce the size and allow passage through the basiischial joint located between the carpus and merus of the appendage (Couch, 1837; Couch, 1843; Mykles and Skinner, 1981; Mykles and Skinner, 1990; Mykles, 1992).

Even though the inner skeletal structure of *C. sapidus* has gotten little attention, extensive work has been done on insect apodemes (Reedy and Beall, 1993; Bitsch and Bitsch, 2002). Insect cuticle can usefully be compared to crustacean cuticle due to numerous similarities between them such as the number of cuticle layers, a branching pore canal system, a twisted plywood organization of the chitin-protein fibers, and the highly conserved chitin binding Rebers-Riddiford (RR) consensus sequence (Neville et al., 1969; Neville, 1975; Filshie, 1976; Hadley, 1994; Wynn and Shafer, 2005). Reedy and Beall (1993) examined the myotendon junction in *Drosophila melanogaster* indirect flight muscle and observed that the myofibrils end in a modified terminal z-band, where thick and thin filaments in the sarcomeres are connected to the sarcolemma, forming the shape of a “calligraphic M” that is deeply interdigitated with the tendon cell due to folding of the myotendon junction. Microtubules within the tendon cell attach to the cell membrane near the modified terminal z-band at one end and to the membrane over the tonofibrils at the other end. Tonofibrils are “shafts of dense extracellular material” that attach the tendon cell to the cuticle by anchoring into the cuticle and filling depressions in the tendon cell. Even though the authors do not mention cell boundaries, it was noted that an epithelial cell can form a
cellular tendon in many arthropod muscles (Auber, 1963; Lai-Fook, 1967; Reedy and Beall, 1993).

This investigation was designed to determine the structure of the apodemes in the blue crab as well as the timing of cuticle deposition of the apodemes. In addition, the cuticle-epithelial boundary was examined throughout critical stages in the molt cycle in order to determine how the muscle is attached to the cuticle and how muscle maintains its attachment to the cuticle until very late in premolt.

2. Materials and methods
2.1 Animals and experimental design

Blue crabs were obtained from commercial distributors in Wilmington, NC and Endurance Sea Food, Kill Devil Hills, NC. At Kill Devil Hills the water temperature was 24.8 °C with a salinity of 10 ppt while the salinity of the Intracoastal Waterway in Wilmington was 35 ppt. Crabs were collected and sacrificed at each of the following stages: intermolt (C4), premolt stages D2, D3, D4, and 0, 8, and 24 hours after ecdysis. From each individual crab the apodemes along with their attached musculature were removed from both chelae. Both sexes were used with the majority being females. Carapace width ranged from 11.0 to 16.5 cm. In order to determine the stage of premolt the traditional method of examining the fifth pereiopod, swimming paddle, was used because it is a non-invasive method that permits accuracy along with a cuticle strength/suture failure test (Drach, 1939; Passano, 1960; Johnson, 1980; Mangum, 1985; Freeman et al., 1987; Smith and Chang, 2007).

2.2 Light Microscopy

Apodemes from one chela were removed from a crab at each stage and fixed in 4% paraformaldehyde in 0.2 M cacodylate buffer, pH 7.4. The fixative was changed within 72
hours after collection and again one month after collection. All samples were decalcified in a 10% sodium ethylenediamine tetraacetate (EDTA) in 0.1 M Tris buffer, pH 7.4, for 1 week or until the apodemes were flexible. Once decalcified, the samples were rinsed in 0.1M Tris buffer, pH 7.4, then distilled water (dH2O) and dehydrated through an ascending series of acetone. The samples were then cleared in toluene and embedded in paraffin (Paraplast Plus). Tissue sections 10μm thick were attached to Superfrost Plus Gold slides (Fisher Scientific), deparaffinized, and rehydrated to dH2O (Marlowe and Dillaman, 1995). Acridine orange (AO) staining followed the techniques described by Marlowe and Dillaman (1995). AO slides were examined using an Olympus Fluoview 1000 laser scanning confocal microscope. The slides were excited with the 488 nm laser only, but both the 520 nm and 620 nm emission wavelengths were collected. A stack of approximately 10 optical sections 1μm thick were collected and a composite image was created then saved as a TIFF. Further processing was done using Adobe Photoshop 7.0.

2.3 Immunocytochemistry

Apodemes processed for light microscopy were also used for immunocytochemistry. The E7 monoclonal antibody developed by Michael Klymkowsky was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242. The primary antibody, E7, for β-tubulin was used to identify microtubules along the apodeme. Non-specific binding of the antibody was prevented by a blocking solution consisting of 10% bovine serum albumin in Sörensen’s phosphate buffer saline (PBS), pH 7.4 at room temperature (Presnell and Schreibman, 1997). The sections were then incubated overnight at 4°C with a 1:400 dilution (a final concentration of 112 ng/ml) of the primary antibody in PBS. The primary antibody was visualized using an Alexa Fluor 594 labeled goat anti-mouse
secondary antibody (Invitrogen; Carlsbad, California) diluted to 1:200 (a final concentration of 0.01 μg/ml) in PBS. The blue fluorescent dye 4’,6-diamidino-2-phenylindole (DAPI) (Invitrogen; Carlsbad, California) was used to localize nuclei. The DAPI stock solution of 5 mg/ml was diluted with PBS to give a final concentration of 0.1 μg/ml. Negative controls and positive controls were used to detect any endogenous fluorescence or non-specific binding of the secondary antibody. The negative control was only treated with blocking buffer then rinsed with PBS while the positive control was treated with blocking buffer and secondary antibody then rinsed with PBS. Neither the positive or negative control was treated with the primary antibody. Coverslips were mounted using a 1:9 Tris:glycerol mixture, pH 7.4. The slides were examined with an Olympus Fluoview 1000 laser scanning confocal microscope using 405nm and 543nm excitation wavelengths for the localization of the DAPI and the Alexa Fluor 594 respectively. The 543 nm laser was not used at a laser strength higher than 15% and the 405 laser was not used at a laser strength higher than 1%. The detector sensitivity was not elevated above 600 for either laser. These settings were used for all samples so that valid comparisons could be made amongst the antibodies. Stacks of approximately 10 optical sections 1μm thick were collected and a composite image was created then saved as a TIFF. Further processing was done using Adobe Photoshop 7.0.

2.4 Transmission Electron Microscopy

The apodemes from the remaining chela of the crabs were removed and immediately fixed in a primary fixative solution consisting of 2.5% glutaraldehyde and 1.5% paraformaldehyde in 0.2M cacodylate buffer, pH 7.4. The samples were decalcified in 10% EDTA in 0.1 M Tris buffer, pH 7.4, for 1 week or until the apodemes were flexible. Once decalcified, the tissue was dissected into approximately 2x2 mm pieces before further processing. The samples were rinsed twice with a 0.2M cacodylate buffer solution and were
then post-fixed with 0.5% osmium tetroxide in 0.2M cacodylate buffer, pH 7.4, for 4 hours at room temperature and rinsed with 0.2 M cacodylate buffer, pH 7.4. After the rinses with buffer, the tissue was rinsed twice with distilled water then dehydrated with an ascending series of ethanol (50, 70, 95, 100, 100%). The tissue was infiltrated with Spurr’s epoxy resin (Spurr, 1969) then polymerized for at least 8 hours at 70°C.

Sectioning of tissue was performed with a Reichert-Jung Ultracut E at the desired thickness of 90-100 nm. Sections were post-stained with 2% uranyl acetate in 50% ethanol and Reynolds’ lead citrate (Reynolds, 1963). Afterwards, the tissue was examined using a Philips CM-12 transmission electron microscope (TEM) operated at 80kV and micrographs taken using 3 ¼” X 4” Kodak 4489 electron microscope film. Negatives were digitized with a Microtek ScanMaker 8700 and then processed and labeled using Adobe Photoshop 7.0.

2.5 Scanning Electron Microscopy

Apodemes collected from both chelae were immediately frozen using liquid nitrogen then left intact or fractured either in cross section or longitudinally. Apodemes left intact were placed in the Labconco lyophilizer over night or until samples were dehydrated then kept in a dessicator until further processing. Fractured samples were fixed in 2.5% glutaraldehyde and 1.5% paraformaldehyde in 0.2M cacodylate buffer, pH 7.4, for at least 24 hours. Those samples were rinsed in 0.2M cacodylate buffer then dehydrated through an ascending series of acetone to hexamethyldisilazane (HMDS) and left uncovered in the hood until the HMDS had completely evaporated (Bray et al., 1993). The apodemes left intact had the muscle fibers removed with forceps and the underlying apodeme was subsequently fractured along or across the long axis of the apodeme.

All samples were firmly attached to aluminum stubs with Duco cement (ITW Performance Polymers) so that the cross section of the apodemes was visible. Pieces of air-
dried apodemes from exuvia were also viewed. All samples were sputter-coated with a 6 nm layer of platinum-palladium (80:20) with a Cressington 208 HR Sputter Coater. Samples were examined and photographed with a Philips XL30S FEG scanning electron microscope (SEM) in secondary electron (SE) mode with an accelerating voltage of 5 kV and a spot size of 3. Digital images were processed using Adobe Photoshop 7.0.

3.0 Results
3.1 Light Microscopy
3.1.1 Acridine Orange Staining

With AO staining the cytoplasm of the epithelial/tendon cells along the apodeme fluoresced the same color green as the muscle in intermolt, C₄, with the nuclei staining a combination of yellow and green (Fig. 3a, arrow). The nuclei remained the same color throughout the molt cycle. However, an abrupt change occurred in D₂ when the cytoplasm changed from green to orange/red (Fig. 3b, arrow). The orange/red stain appeared to be concentrated in the apical portion of the cells. The cytoplasm in the apical portion of the cell remained orange/red in D₃ and D₄ (Fig. 3c and d, arrow), but shifted to an intense red beginning at ecdysis (Fig. 3e, arrow) and continued into postmolt (8 and 24 hour) (Fig. 3f and g, arrow). By late postmolt (96 hours), the cytoplasm of the entire cell returned to green (Fig. 3h, arrow).

The chitin fibrils of the lateral layers did not bind the dye, whereas the epicuticles stained green/orange in intermolt (Fig. 3a, arrowhead) and premolt (Fig. 3b, c, and d, arrowhead) and intense green in ecdysis (Fig. 3e, arrowhead). In early postmolt the epicuticle remained green (Fig. 3g, arrowhead). In 96 hours postmolt, a slim black line was observed along both sides of the medial layer where the new lateral layers were forming (Fig. 3h, asterisks).
Figure 3. A-H. Acridine orange staining of the apodeme and associated tissue throughout the molt cycle. Stages (C4, D2, D3, D4, ecdysis, 8, 24, and 96 hours postmolt) are indicated in the upper left corner of the images. Arrow = cytoplasm of epithelium, arrowhead = medial layer of the apodeme, asterisks = lateral layer of the apodeme.

3.1.2 Immunocytochemistry

The negative control (blocking solution only) showed endogenous fluorescence in the medial layer of the apodeme with the 405 nm laser, but not the 543 nm laser (results not shown). The positive control (goat anti-mouse secondary only) had slight background staining, but no specific binding of the secondary antibody in the apodeme (Fig. 4a). The
primary antibody for β-tubulin, a subunit of microtubules, bound continuously along the length of the apodeme throughout the molt cycle from the apical to basal region of the cells (Fig. 4b, c, and d). Breaks in the staining were attributed to DAPI-stained nuclei or components of the cytoplasm that did not bind β-tubulin suggesting microtubules are not the only structure present (Fig. 4b, c, and d). Even so, β-tubulin appeared to be the dominant cell component other than the nucleus.

Figure 4. Antibody staining for β-tubulin (red) and DAPI (blue). Stage in the molt cycle or hours after ecdysis indicated in upper left hand corner. a top. Differential interference contrast (DIC) image of the positive control (goat anti-mouse secondary only). a bottom. Fluorescence image of a top. b. Fluorescence image of intermolt (C4) apodeme with DIC image inserted in top right. c. Fluorescence image of premolt, D2. d. Fluorescence image of 24 hours postmolt.
3.2 Transmission Electron Microscopy

An apodeme that had been fully deposited contained a thin medial layer (ML) that was composed of fused cuticles (ML-a and ML-b) (Fig. 5a). Thick lateral layers (LL-a and LL-b) were observed on either side of the ML (Fig. 5a). A contrast in the intensity of staining was seen between the ML and lateral layers (LL) with the ML staining more intensely. The ML comprised approximately 10% of the total thickness. Tonofibrils ran perpendicular throughout the lateral layers and into the medial layer (Fig. 5a and b, arrowheads). Higher magnification of the cuticle showed the boundary between muscle and apodeme contained microtubules (Mt) along with projections of tonofibrils at the surface of the apodeme (Fig. 5b, Fig. 6b, and d, arrowheads). Interspersed with the microtubules were secretory cell components such as nuclei (N), rough endoplasmic reticulum, and mitochondria (Fig. 5c, d, Fig. 6a and c). Cell boundaries were scarcely ever observed even though multiple nuclei were seen along the length of the apodeme. Throughout the molt cycle a modified terminal z-band was noted where the muscle met the epithelium (Fig. 5c, d, Fig. 6a and c, arrows). In late premolt (D3 and D4) a newly deposited apodeme between the epithelium (E) and old apodeme appeared (Fig. 6a). Higher magnification revealed intact tonofibrils bridging the gap between old and new apodeme (Fig. 6b, arrowheads). The new apodeme being deposited had the same staining intensity observed in the ML. In early (8 hour) postmolt a polymerization zone formed between the epithelium (E) and new apodeme (Fig. 6c and d, asterisks). This zone disappears in intermolt suggesting that deposition of the lateral layers occurs in this space.
Figure 5. Electron micrographs from apodemes during intermolt (b), premolt (a), and postmolt (c and d) (molt cycle stage or hours after ecdysis in upper left corner). ML-a & ML-b = medial layer; LL-a & LL-b = lateral layers, arrowhead = tonofibril; Mt = microtubules; arrow = modified terminal z-band; E = epithelium; N = nucleus; Mu = muscle.
Figure 6. Electron micrographs from apodemes during premolt (a and b) and postmolt (c and d) (molt cycle stage or hours after ecdysis in upper left corner). Arrowhead = tonofibril; Mt = microtubules; arrow = modified terminal z-band; E = epithelium; N = nucleus; Mu = muscle; asterisk = polymerization zone.
3.3 Scanning Electron Microscopy

The only observed difference between fixed apodemes and lyophilized samples was that the fixed apodemes had more interfibrillar material (Fig. 7a, c, and d). When the muscle was removed from the surface of the apodeme with forceps after lyophilisation, SEM of the surface revealed that remnants of muscle fibers were still attached (Fig. 7b, arrow). The muscle cell remnants appeared to be arranged in parallel arrays with the longitudinal axis of the apodeme. Ridges oriented in the same manner as the muscle and chitin fibers were seen on the surface of the apodeme (Fig. 7b, arrowhead). Cross sectional and longitudinal fractures revealed the chitin fibers ran parallel to the long axis in both exuvial apodemes and intermolt apodemes (Fig. 7c and d).

Even though the chitin fibers frayed when broken in the longitudinal fractures, very thin structures could be seen running perpendicular to the axis of the chitin fibers (Fig. 7c, concave arrowhead). Tonofibril width was measured using images from the TEM and compared to the width of the structures seen in SEM. The average width measured in the TEM and SEM was very similar, 63 nm and 56 nm respectively, suggesting the fibrous structures in the SEM were in fact tonofibrils.

At the dactylopodite, the proximal portion of the apodeme has a complex diamond shape (Fig. 2, insert). The chitin fibers in this region appeared to have a complex orientation before becoming parallel to the long axis in the medial and distal portions of the apodeme (not shown). Further research is needed in order to determine the chitin fiber orientation at the proximal portion of the apodeme.
3.4 Ultrastructure of apodeme and associated tissue

The apodeme has two layers: a ML where adjacent cuticles have fused (light grey) and LL on either side composed of chitin fibers that run parallel to the long axis of the apodeme (only one complete lateral layer is depicted) (Fig. 8a, b, and c). The LL extend beyond the ML when both layers are fully deposited (Fig. 8b, insert). Tonofibrils are
anchored deep in the apodeme providing attachment points for microtubules at one end of the epithelium (Fig. 8a, b, and c). The tonofibrils are not depicted as complete throughout the LL into the ML because when sectioned it is extremely difficult to maintain a longitudinal section over that distance. The muscle is molded into finger-like projections forming a modified terminal z-band (dark grey) that provides an attachment point for the microtubules at the other end of the epithelium (Fig. 8a, b, and c). Interspersed within the microtubules are secretory cell components such as nuclei, rough endoplasmic reticulum, and mitochondria (Fig. 3a, b, and c). Cell boundaries were not drawn in the diagram because they remain unclear. In late premolt (D2, D3, and D4), a new ML forms along the lateral edge of the apodeme (light grey) (Fig. 8b and insert). Since the LL of the apodeme extend beyond the length of the ML, the new ML that is deposited along the edge of the LL is longer than the original ML (Fig. 8b, insert). The new and old apodeme are connected to one another by tonofibrils so that the crab can anchor the muscles of the claw during late premolt (Fig. 8b). During ecdysis, the old apodeme releases from the new allowing the old apodeme to be shed in the process. In early postmolt, (~8 hours), the new, longer apodeme has a polymerization zone that lies between the ML and the epithelium and represents the site where the new lateral cuticular layers are being formed (Fig. 8c).
Figure 8. Schematic drawing of the apodeme throughout the molt cycle (modeled after a diagram of the myotendon junction in *Drosophila* from Reedy and Beall, 1993). a = intermolt, b = late premolt, b insert = fluorescence image with antibody that binds the medial layer of the apodeme (red), c = early postmolt.
4. Discussion

4.1 Cuticle deposition

The apodeme has two distinct layers: the ML consists of fused epicuticles and the LL composed mostly of chitin fibrils. During premolt, the ML of the apodeme was deposited along the length of the LL of the old apodeme. The ML of the apodeme appeared to be similar to the epicuticle and exocuticle in the dorsal carapace (the preexuvial layers) because deposition occurs before ecdysis. However, the ML has been shown to have similarities to the epicuticle, not the exocuticle, with AO staining, size comparisons, and lack of lamellae (Crawford, 2006; Crawford et al., 2007). During postmolt the LL of the new apodeme were deposited in a polymerization zone seen between the ML and epithelium. The LL of the apodeme appeared to be similar to the endocuticle and membranous layer because deposition occurs after ecdysis. A previous study has shown the LL are similar to the endocuticle not the membranous layer with AO staining and presence of lamellae (Crawford, 2006; Crawford et al., 2007).

Previous studies have determined AO is a nucleic acid stain that binds DNA and RNA. When bound to DNA, AO emits in the green wavelength, but when bound to RNA a shift occurs in the emission and excitation spectrum causing it to emit in the red wavelength (Armstrong, 1956; von Bertalanffy and Bickis, 1956; Armstrong and Niven, 1957; Marlowe and Dillaman, 1995). Rough endoplasmic reticulum (RER), seen as red staining with AO, was intense in the apical portion of the cells that composed the epithelium in premolt and early post-molt indicating that protein synthesis is elevated during these stages, but declined by approximately 24h postmolt. Lateral layer formation, as seen with TEM, coincides with the decrease in the red AO staining of RER, suggesting that chitin synthesis increases while protein synthesis decreases, but does not cease. The staining pattern of AO indicates the ML and the LL differ in their composition or at least in their total protein/carbohydrate
proportions. Future studies need to be performed in order to determine the actual composition of the two layers.

Other studies have also determined that AO stains muscle cytoplasm, acid mucopolysaccharides, proteoglycans, and glycosaminoglycans (Saunders, 1964; Shepard and Mitchell, 1981; Brandes and Reale, 1990; Marlowe and Dillaman, 1995; Allen et al. 1999; Ohira et al., 1999; Roy et al., 1999). Chitin is a main component in cell walls of fungi and cuticles in invertebrates. It is a polymer formed by covalent β-1,4 linkages of N-acetylglucosamine, a derivative of glucose (Cohen, 2001). Formation of chitin begins intracellularly when chitin synthase units are synthesized then joined into a crystalline fibril. The fibrils are translocated across the plasma membrane in order for crystallization and microfibril formation to occur. The microfibrils then associate with cuticular proteins in arthropods (Cohen, 2001; Raabe et al., 2005a; Raabe et al., 2005b; Raabe et al., 2006). Due to the presence of different glycosaminoglycans in the exoskeleton AO has been used to differentiate cuticular layers (Travis, 1965; Marlowe and Dillaman, 1995). The epicuticle was seen to fluoresce yellow, the exocuticle fluoresced orange/red, and the endocuticle fluoresced green/blue in the carapace of the crayfish, Procambarus clarkii and the blue crab, Callinectes sapidus (Marlowe and Dillaman, 1995). Previously, the LL of the apodeme were found to emit green while the ML emitted red/orange (Crawford, 2006; Crawford et al., 2007). This study supports the staining pattern of the ML, but the LL did not fluoresce. The previous study used Bouin’s fixative, Rossman’s fixative, and Birgus fixative while the current study used paraformaldehyde. This suggests that the methods used in the previous study fixed a molecule within the LL that had an affinity for AO that may not have been fixed in the current study.
4.2 Chitin fiber orientation

Insects such as the cockroach (*Periplaneta americana*), house cricket (*Acheta domesticus*), and locust (*Schistocerca gregaria* and *Locusta migratoria*) were found to have two chitin deposition patterns controlled by a circadian clock: 1. a unidirectional orientation of microfibrils without lamellate layers deposited during the day and 2. helicoidally oriented microfibrils with lamellate layers deposited during the night (Neville and Luke, 1969). Linear arrangement of chitin fibrils has also been found in the cell walls of the spores of the fungi *Trichophyton mentagrophytes* and *Glomus epigaeum* as well as the endocuticle of the horseshoe crab (*Limulus polyphemus*), the endocuticle of sea scorpions (eurypterids), and the chelae of the pedipalps and leg joints of scorpions (*Heterometrus* spp.) (Mutvei, 1977; Pollack et al., 1983; Bonfante-Fasolo and Grippiolo, 1984). However, in all instances where a unidirectional arrangement of chitin was observed, an arcing or helicoidal arrangement was also present in neighboring layers. From the dorsal to ventral edge of the apodeme the chitin fibers ran longitudinally without any rotation from the tip to the middle.

Even though the apodeme is an infolding of the exoskeleton of the chelae, the chitin fiber orientation differs between the two. In the exoskeleton of the dorsal carapace a helicoid or plywood-like structure is formed due to a slight rotation in each layer (lamellae) of the chitin-protein fibers in relation to adjacent layers (Bouligand, 1972; Giraud-Guille, 1984; Neville, 1998; Raabe et al., 2005a; Raabe et al., 2005b; Raabe et al., 2006). However, the orientation of the chitin fibers was more complex at the base of the apodeme due to a complex folding and fusion of cuticular components (Fig. 2 insert). More samples need to be examined before the chitin fiber orientation at the base of the apodeme can be fully elucidated.

A previous study had determined the direction of the muscle fibers and force produced in the chelae of crabs to be along the longitudinal axis of the apodeme (Warner,
Muscle fibers also appeared to be oriented in rows parallel to the long axis of the apodeme with SEM in the current study. The helicoidal chitin arrangement found in the dorsal carapace has been suggested to be mechanically favorable because it provides maximum protection against crushing or piercing (Raabe et al., 2007). In the case of the apodeme, the chitin fibers running parallel with the lines of force produced by the muscle may be the most advantageous arrangement. A helicoidal pattern in the apodeme would cause strain on all layers that were not in parallel with the long axis and that could potentially results in distortion, damage, and separation of those layers.

In addition, pore canals were never observed in the TEM or the SEM. Only chitin fibers with tonofibrils running perpendicularly were noted in the apodeme, which suggests the apodeme may calcify in a different manner than the dorsal carapace (Hequembourg, 2002). The dorsal carapace contains pore canals that extend from the membranous layer to the inner epicuticle. Cytoplasmic extensions of the hypodermal cuticle-secreting cells are located in the pore canals during premolt and postmolt, which permit calcium transport to the distal regions of the exoskeleton for mineralization in early post-molt (Travis, 1963; Compère and Goffinet, 1987; Dillaman et al., 2005). Further research should be performed in order to determine the spatial and temporal pattern of calcification in the apodeme.

4.3 Cuticle-epithelial boundary

Immunocytochemical localization of β-tubulin and TEM confirmed the presence of microtubules interspersed with secretory cell components such as nuclei, rough endoplasmic reticulum, and mitochondria along the length of the epithelium forming the apodeme. Previous research of the cuticle-epithelial boundary has either lacked a description of the cells responsible for deposition or a description of cell boundaries (Reedy and Beall, 1993; Bitsch and Bitsch, 2002). Even though there was no mention of cell boundaries, it was noted
that an epithelial cell can form a cellular tendon in many arthropod muscles (Auber, 1963; Lai-Fook, 1967; Reedy and Beall, 1993). In the current study multiple nuclei were located in the epithelium, but cell margins were rarely observed. It remains unclear whether the cells along the apodeme are divided into two types (epithelial cells and tendon cells) or a combination of both. If there are no cell margins, it is possible that the tissue along the apodeme is a type of multinucleated syncytium like skeletal muscle (Ross and Pawlina, 2006).

As described for insects, tonofibrils penetrate deep into the apodeme and are anchoring sites for microtubules at the apical surface of the epithelium while a modified terminal z-band provides an attachment point for the microtubules to the muscle at the proximal surface of the epithelium (Reedy and Beall, 1993). Previous research done on Apterygota and Callinectes sapidus (Caveney, 1969; Modla, 2006) suggested the tonofibrils were formed from secretions by the tendinous epithelial cell while Reedy and Beall (1993) indicated that tonofibrils were formed by a transformation of the microvilli. This study did not find any evidence for the later conclusion. The z-bands in striated muscle are composed of α-actinin (Vigoreaux, 1994; Squire, 1997; Luther et al., 2003). Experiments that caused mutations in actin and α-actinin were found to preferentially affect the link between the muscle and the tendon cell suggesting the finger-like projections are a unique type of z-band (Fyrberg et al., 1990; Sparrow et al., 1991). In late premolt the tonofibrils maintain their continuity, thereby anchoring the new apodeme to the old apodeme resulting in the crab maintaining contractile function of the claw until immediately before ecdysis. Apparently at ecdysis tonofibrils are digested and/or broken. It is critical for the blue crab to maintain function as long as possible and to regain function as soon as possible in order to ensure survival. More research must be completed in order to resolve the exact mechanisms underlying the disconnection of the new apodeme from the old as well as the reacquisition of
motor control while the apodeme continues to be deposited and the muscle mass is reinstated (Couch, 1837; Couch, 1843; Mykles and Skinner, 1981; Mykles and Skinner 1990; Mykles, 1992; West, 1997).

4.4 Summary of the results

In summary, the apodeme appears to be a unique cuticle type in several ways. The unidirectional organization of chitin fibers has not been documented in any other type of cuticle in *C. sapidus*. The temporal deposition pattern is similar to that found in the dorsal carapace, but the spatial deposition may vary due to the differing organization patterns in chitin fibers and a lack of pore canals. Apolysis must also occur in another fashion due to a cuticle-cuticle separation instead of a cuticle-tissue separation. Overall, these unique adaptations of the apodeme make it well designed for its function in the chelae of *C. sapidus*.
CHAPTER 2: EXPRESSION PATTERNS OF THREE PROTEINS CONTAINING THE REBERS-RIDDIFORD CONSENSUS SEQUENCE IN THE BLUE CRAB

1. Introduction

The blue crab, *Callinectes sapidus*, has long been the object of research with regard to its physiology, particularly the events of the molt cycle when the old exoskeleton is shed and a new, larger exoskeleton is formed. The molt cycle has been divided into five stages (A to E) that are defined by the softness or hardness of certain portions of the cuticle in the dorsal carapace (Drach, 1939; Smith and Chang, 2007). In the intermolt stage (C₄) all layers of the dorsal carapace are present (the epicuticle, exocuticle, endocuticle, and membranous layer) (Travis, 1963; Smith and Chang, 2007). Premolt is divided into early and late premolt. In early premolt (D₀ and D₁), the breakdown of the membranous layer occurs in order to allow separation of the cuticle from the hypodermis (apolysis) (Drach, 1939; Jeuniaux, 1959a, 1959b; Green and Neff, 1972; Stevenson, 1972). In late premolt (D₂, D₃, and D₄), the new epicuticle and exocuticle are formed (Drach, 1939; Travis, 1963). Since the matrix of the new epicuticle and exocuticle are synthesized and deposited before ecdysis, they are referred to as pre-exuvial layers (Drach, 1939; Roer and Dillaman, 1984). These pre-exuvial layers do not calcify until after ecdysis, but the epicuticle does tan before ecdysis (Paul and Sharpe, 1916; Krishnan, 1951; Travis, 1963; Travis and Friberg, 1963; Roer and Dillaman, 1984). Ecdysis (stage E) involves the breaking of the ecdysial sutures and the crab shedding its old exoskeleton (Churchill, 1919; Cameron, 1985; Mangum et al., 1985; Mangum, 1992; Neufeld and Cameron, 1994; Compère et al., 1998; Smith and Chang, 2007). The postmolt stages are divided into early and late postmolt. In early postmolt (A₁, A₂, B₁, and B₂) the crab rapidly takes up water in order to expand the enlarged exoskeleton, tans the exocuticle, and begins simultaneous deposition and calcification of the endocuticle (Passano, 1960; Green and Neff, 1972; Vacca and Fingerman, 1975a, 1975b; Mangum et al., 1985). Calcification of the pre-
exuvial cuticle begins in the most distal regions of the cuticle and continues proximally (Travis, 1963; Giraud-Guille, 1984; Compère et al., 1992, 1993; Hequembourg, 2002; Dillaman et al., 2005). In late postmolt (C1, C2, and C3) simultaneous deposition and calcification of the endocuticle is completed, followed by secretion of the uncalcified membranous layer (Travis and Friberg, 1963; Bouligand, 1972; Roer and Dillaman, 1984). The crab enters intermolt (stage C4) when the membranous layer is complete, the cytoplasmic extensions have completely retracted from the calcified cuticle, and net calcium deposition ceases (Travis, 1963; Travis and Friberg, 1963; Hegdahl et al., 1977a, b, and c; Passano, 1960; Roer and Dillaman, 1984; Compère and Goffinet, 1987).

The dorsal carapace, as described above, has been used as a basis for description of various types of cuticle within the crustaceans as well as a point of comparison between arthropod classes such as insecta. However, it is worth noting that cuticle structure and function is not the same throughout the blue crab. For example, the dorsal carapace is used for protection so it is heavily calcified while the gills have a thin uncalcified layer of cuticle because they must form a minimal barrier to gas and ion exchange (Copeland and Fitzjarrell, 1968; Nuefeld et al., 1980; Roer and Dillaman, 1984; Towle, 1984; Burnett and Towle, 1990; Andrews and Dillaman, 1993; Elliott and Dillaman, 1999). Some areas, such as the joints do allow protection, but aren’t calcified in order to permit flexibility (Neville, 1975; Williams et al., 2003). Even the suture line, where the cuticle is preferentially resorbed prior to ecdysis, was found to have a different thickness, external morphology, lectin affinity, and mineral composition from the adjacent calcified carapace (Priester, 2005).

Analysis of extracts of the cuticle in different regions of the blue crab, *C. sapidus*, have shown that those differing regions have differing protein and glycoprotein composition (Vranckx and Durliat, 1980 and 1981; Marlowe et al., 1994; Williams et al., 2003; Wynn and Shafer, 2005; Shafer et al., 2006). Furthermore, many cuticle proteins have also been
characterized by sequence analysis insofar as there are often conserved amino acid sequences upstream and downstream from the chitin-binding Rebers-Riddiford consensus sequence (RR), a highly conserved cuticle protein motif found in insects and crustaceans (Rebers and Riddiford, 1988; Andersen, 1998a and 1998b). Crustaceans have been found to have a variation of the original RR consensus sequence. Conserved residues upstream of the RR sequence (aka RR-1) have been associated with soft (uncalcified) cuticles and conserved residues downstream of the RR sequence (aka RR-2) have been associated with hard (calcified) cuticle (Andersen, 1998b; Andersen, 1999; Endo et al., 2000 and 2004; Watanabe et al., 2000; Inoue et al., 2001, 2003, and 2004; Rebers and Willis, 2001; Togawa et al., 2004).

In order to verify the distribution of the gene products of the RR sequence, RR1, and RR2 associated gene sequences, antibodies were created against CsAMP8.1, a cuticle protein expressed in uncalcified cuticle that contains the chitin-binding RR-1 consensus sequence, and against CsCP8.5, a cuticle protein found in calcified cuticle that contains a truncated version of the chitin-binding RR consensus sequence. An antibody was also generated against a conserved region of 14 amino acids that is C-terminal to the chitin-binding RR-1 consensus sequence located within each polypeptide of a complex family of cuticular proteins (the EarlyCP motif). The EarlyCP motif has been found in the blue crab (C. sapidus) as well as the fiddler crab (Uca pugnax), the freshwater crayfish (Procambarus clarkii), and the freshwater amphipod (Gammarus pulex) (Wynn and Shafer, 2005; Shafer et al., 2006; Faircloth and Shafer, 2007; Inoue et al., 2008; Shafer et al., 2009). Through the use of quantitative PCR (qPCR) and Northern blots the spatial and temporal distributions of the messenger RNAs (mRNA) for these particular proteins were determined. CsAMP8.1 was seen to be expressed in the arthrodial membrane hypodermis throughout the molt cycle while CsCP8.5 and the EarlyCP motif are expressed in dorsal carapace hypodermis only in premolt...
(Table 1A) (Wynn and Shafer, 2005; Shafer et al., 2006; Faircloth and Shafer, 2007; Shafer et al., 2009). The current investigation was designed to describe the distribution of the products of the genes CsAMP8.1 and CsCP8.5 as well as the product of the EarlyCP motif at critical points in the molt cycle using antibodies generated against their unique epitopes.

2. Materials and Methods

2.1 Animals and experimental design

Blue crabs were obtained from commercial distributors in Wilmington, NC and Endurance Sea Food, Kill Devil Hills, NC. At Kill Devil Hills the water temperature was 24.8 °C with a salinity of 10 ppt while the salinity of the Intracoastal Waterway in Wilmington was 35 ppt. Crabs were collected and sacrificed at each of the following stages: intermolt (C4), D2, D3, D4, and 0, 8 and 24 hours after ecdysis. From each individual crab six regions were sampled: anterior and posterior gills, dorsal carapace with branchial chamber attached, suture line, arthrodial membrane of the third walking leg, and apodemes with associated musculature. Both sexes were used, but most were females. Carapace widths ranged from 11.0 to 16.5 cm. In order to determine the stage of premolt the traditional non-invasive method of examining the fifth pereiopod, swimming paddle, was used together with a cuticle strength/suture failure test (Drach, 1939; Passano, 1960; Johnson, 1980; Mangum, 1985; Freeman et al., 1987; Smith and Chang, 2007).

2.2 Tissue processing

All tissues removed from the crabs at each stage were fixed in 4% paraformaldehyde in 0.2 M cacodylate buffer (pH 7.4) at room temperature. The fixative was changed after 72 hours and again one month after collection. Dorsal carapace, suture line, the arthrodial membrane of the third walking leg, and apodemes were decalcified in a 10% sodium ethylenediamine tetraacetate (EDTA) in 0.1 M Tris buffer, pH 7.4, for 1 week or until the
samples were flexible. The gills were not decalcified. If decalcified, the samples were rinsed in 0.1M Tris buffer while samples that were not decalcified were placed in 0.2 M cacodylate buffer, both pH 7.4. The samples were then rinsed in distilled water (dH2O) and dehydrated through an ascending series of acetone, cleared in toluene, and embedded in Paraplast Plus (Fisher Scientific). Tissue sections 10μm thick were attached to Superfrost Plus Gold slides (Fisher Scientific), deparaffinized and rehydrated to dH2O (Marlowe and Dillaman, 1995).

2.3 Immunocytochemistry

Primary antibodies generated in rabbits were obtained from Open Biosystems in order to localize the epitopes from CsCP8.5, CsAMP8.1, and the EarlyCP motif (Wynn and Shafer, 2005; Shafer et al., 2009). Open Biosystems used the entire 14 amino acid peptide for the EarlyCP motif and chose the most antigenic peptide for CsAMP8.1 and CsCP8.5. Then peptides were synthesized, conjugated to bovine serum albumin, and injected into rabbits. Blood was removed from the rabbit 56-58 days later, centrifuged, and the serum was purified.

The primary antibodies were visualized using an Alexa Fluor 594 labeled goat anti-rabbit secondary antibody stock obtained from Invitrogen that was diluted 1:100 (a final concentration of 0.02 μg/ml) with PBS. Non-specific binding of the antibodies was prevented by a blocking solution consisting of 2% normal goat serum and triton-x-100 in Sörensen’s phosphate buffer saline (PBS), pH 7.4 (Presnell and Schreibman, 1997). Primary antibody stock was diluted 1:100 (a final concentration of 0.106 μg/ml for CsCP8.5, 0.109 μg/ml for CsAMP8.1, and 0.0294 μg/ml for the EarlyCP motif) with bovine serum albumin and PBS, and sections were exposed to that dilution for 1 hour at room temperature (25°C), pH 7.4. The blue fluorescent dye 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen; Carlsbad, California) was used to localize nuclei. The DAPI stock solution of 5 mg/ml was diluted with PBS to give a final concentration of 0.1 μg/ml. Negative controls and positive
controls were used to detect any endogenous fluorescence as well as non-specific binding of the secondary. The negative control was only treated with blocking buffer then rinsed with PBS. The positive control was treated with blocking buffer and the secondary antibody and then rinsed with PBS. Neither the positive or negative control was treated with the primary antibody. Coverslips were mounted using a 1:9 Tris:glycerol mixture, pH 7.4, to minimize quenching. The slides were examined using an Olympus Fluoview 1000 laser scanning microscope. The 543 nm laser excited the Alexa Fluor 594 and the 405 nm laser excited DAPI. The 543 nm laser was not used at a laser strength higher than 15% and the 405 nm laser was not used at a laser strength higher than 1%. The detector sensitivity was less than 600 for both lasers. These settings were used for all of the samples so that valid comparisons could be made among the various tissues. A stack of approximately 10 optical sections 1μm thick were collected and a composite image was created then saved as a TIFF. Further processing was done using Adobe Photoshop 7.0.

3. Results

3.1 Negative and positive controls

Endogenous fluorescence was observed in the epicuticle and the membranous layer in the dorsal carapace with both the 405 nm and 543 nm laser (Fig. 9a and b, arrows and asterisks). Fluorescence in both was continuous throughout the layers with the epicuticle fluorescing more intensely than the membranous layer (Fig. 9a and b, arrows and asterisks). The medial layer of the apodeme was excited by the 405 nm laser, but not the 543 nm laser (Fig. 9c and d). Endogenous fluorescence was not observed in the arthrodial membrane (Fig. 9e and f). The calcified cuticle surrounding the arthrodial membrane had endogenous fluorescence in the epicuticle (Fig. 9e and f, arrows). Non-specific binding of the secondary antibody did not occur in any region sampled.
Figure 3. Localization of endogenous fluorescence (arrows and asterisks) and non-specific binding of the secondary antibody (negative and positive controls, respectively) in the dorsal carapace, branchial chamber, apodeme, and arthrodial membrane (molt cycle stage or hours after ecdysis in upper left corner). Arrows = epicuticle; asterisks = membranous layer; arrowhead = medial layer of apodeme.
3.2 mRNA appearance and antibody staining

The mRNA levels measured in a previous study and the antibody staining for CsAMP8.1 showed the same pattern for both the dorsal carapace and arthrodial membrane (Table 1A) (Wynn and Shafer, 2005). The mRNA and antibody to CsAMP8.1 appeared throughout the molt cycle in arthrodial membrane and neither were present in the dorsal carapace. The pattern of mRNA levels and antibody staining for the EarlyCP motif in the dorsal carapace was also the same (Table 1A) (Faircloth and Shafer, 2007; Shafer et al. 2009). The mRNA only appeared in the dorsal carapace in premolt while the antibody stained throughout the molt cycle. Even though mRNA was only found in premolt in the dorsal carapace, the antibody localized to the exocuticle, which is a preexuvial layer. The mRNA associated with the exocuticle is made in premolt while deposition is occurring, but mRNA levels drop in postmolt when deposition of the exocuticle has been completed. The antibody staining was always positive because the exocuticle was present in all stages that were investigated. However, some immunocytochemical results varied greatly from the distribution of the observed mRNA levels. The antibody to CsCP8.5 was expected to bind to the dorsal carapace because mRNA was present in premolt and postmolt, but the antibody never bound to any layer in the dorsal carapace throughout the molt cycle (Table 1A) (Wynn and Shafer, 2005). Conversely, antibodies to the EarlyCP motif and CsCP8.5 both bound the arthrodial membrane. They were not expected to bind because mRNA was absent in premolt and postmolt (Table 1A) (Wynn and Shafer, 2005; Faircloth and Shafer, 2007; Shafer et al. 2009).

The EarlyCP motif was the only antibody that bound every region of the crab cuticle examined (Table 1A and B). All three antibodies bound the medial layer of the apodeme and branchial chamber throughout the molt cycle (Table 1B). All antibodies also bound the arthrodial membrane, but the antibody to CsCP8.5 did not bind throughout the molt cycle.
The antibody to CsCP8.5 only bound in postmolt and intermolt (Table 1B). Inconsistent results were observed in the spatial and temporal pattern of the CsAMP8.1 antibody binding in the gills (Table 1B, +/-).

### A.

<table>
<thead>
<tr>
<th>Cuticle Type</th>
<th>Stage</th>
<th>EarlyCP motif*</th>
<th>CsCP8.5†</th>
<th>CsAMP8.1†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dorsal Carapace</td>
<td>Intermolt</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>Premolt</td>
<td>+ (ex)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Postmolt</td>
<td>- (ex)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Arthrodial Membrane</td>
<td>Intermolt</td>
<td>N/A</td>
<td>+</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>Premolt</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Postmolt</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

### B.

<table>
<thead>
<tr>
<th>Cuticle Type</th>
<th>Stage</th>
<th>EarlyCP motif</th>
<th>CsCP8.5</th>
<th>CsAMP8.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Branchial Chamber</td>
<td>Intermolt</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Premolt</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Postmolt</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Gills</td>
<td>Intermolt</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Premolt</td>
<td>+</td>
<td>-</td>
<td>+/-</td>
</tr>
<tr>
<td></td>
<td>Postmolt</td>
<td>+</td>
<td>-</td>
<td>+/-</td>
</tr>
<tr>
<td>Apodeme</td>
<td>Intermolt</td>
<td>+ (ml)</td>
<td>+ (ml)</td>
<td>+ (ml)</td>
</tr>
<tr>
<td></td>
<td>Premolt</td>
<td>+ (ml)</td>
<td>+ (ml)</td>
<td>+ (ml)</td>
</tr>
<tr>
<td></td>
<td>Postmolt</td>
<td>+ (ml)</td>
<td>+ (ml)</td>
<td>+ (ml)</td>
</tr>
<tr>
<td>Suture Line</td>
<td>Intermolt</td>
<td>+ (ex)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Premolt</td>
<td>+ (ex)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Postmolt</td>
<td>+ (ex)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 1A. Summary of appearance of mRNA, molecular biology (MB), when available and immunocytochemistry (IMC) in the dorsal carapace and arthrodial membrane (Wynn and Shafer, 2005†; Shafer et al. 2009*). N/A = data not available. B. Summary of the immunocytochemistry localization pattern for the three antibodies in additional crab parts. +/- = inconsistent results, ex = exocuticle, and ml = medial layer.

3.3 Antibody binding pattern in the dorsal carapace and branchial chamber

The antibody to the EarlyCP motif was limited to the exocuticle (Fig. 10a asterisks) throughout the molt cycle while the antibody to CsCP8.5 unexpectedly did not appear in any
layer of the dorsal carapace (Fig. 10c) despite the fact that mRNA was present in premolt and postmolt (Table 1A). The antibody to CsAMP8.1 did not bind to the dorsal carapace (Fig. 10e) and this was expected because CsAMP8.1 mRNA was not present in the dorsal carapace in either premolt or postmolt (Table 1A). Binding of the antibody to the EarlyCP motif was continuous throughout the exocuticle. This was also expected because the EarlyCP motif mRNA was found in the dorsal carapace in premolt (Table 1A). Just like the controls, endogenous fluorescence was observed in the epicuticle (Fig. 10a, c, and e). Unexpectedly, all three antibodies bound the entire branchial chamber cuticle throughout the molt cycle (Fig 10b, d, and f, arrows). Antibodies to CsCP8.5 and the EarlyCP motif were not predicted to have an affinity for uncalcified cuticle because mRNA was not present for either gene in arthrodial membrane in premolt or postmolt (Table 1A).
Figure 4. Location of the EarlyCP motif, CsCP8.5, and CsAMP8.1 in the dorsal carapace (asterisks) and branchial chamber (arrows) using confocal microscopy and a secondary antibody labeled with Alexa 594 (molt cycle stage or hours after ecdysis in upper left corner).
3.4 Antibody binding pattern in the arthrodial membrane and gills

Hematoxylin and eosin staining was performed in order to verify that the sections of walking leg contained calcified cuticle and arthrodial membrane (results not shown) (Groat, 1949; Presnell and Schriebman, 1997; Williams et al., 2003). Identification was also determined based on thickening of the cuticle found on either side of the arthrodial membrane (Williams et al., 2003, Faircloth and Shafer, 2007). Antibodies to the EarlyCP motif and CsAMP8.1 bound the arthrodial membrane of the walking leg throughout the molt cycle while the antibody to CsCP8.5 only bound the arthrodial membrane in intermolt and postmolt (Fig. 11c, top and bottom). The antibody to CsAMP8.1 was expected to bind to arthrodial membrane because mRNA was present in premolt and postmolt (Table 1A). However, the antibodies to the EarlyCP motif and CsCP8.5 were not expected to bind to arthrodial membrane because mRNA was absent in both premolt and postmolt (Table 1A). While a previous study found preexuvial and postexuvial layers of the arthrodial membrane could be differentiated with periodic-acid Schiff’s reaction (PAS) staining (Williams et al., 2003), no differentiation within the cuticle was observed any of these antibodies.

The blue crab has phyllobranchiate gills containing pairs of lamellae, flattened sacs, with a central stem that is hourglass shaped in cross section (Johnson, 1980; Taylor and Taylor, 1992). The efferent and afferent channels are located at each end of the hourglass (Johnson, 1980; Taylor and Taylor, 1992). When cut tangentially multiple lamellae can be seen as finger-like projections branching from off the central stem (Johnson, 1980). The lamellae contain an epicuticle, exocuticle, endocuticle, and membranous layer (Johnson, 1980). A very thin layer of endogenous fluorescence was observed in the epicuticle on the tip of the finger-like projections (results not shown). The antibody to the EarlyCP motif bound to the efferent/afferent vessel and lamellae lining throughout the molt cycle in both anterior and posterior gills (Fig. 11b, asterisks and arrows). The antibody to CsCP8.5 did not
bind the gills and the antibody to CsAMP8.1 showed inconsistent results with the
efferent/afferent vessel lining due to odd patterns in fluorescence at varying stages (Fig. 11f,
top and bottom). None of the antibodies differentiated between anterior and posterior gills.
Furthermore, both proximal and distal portions of the gill were also examined, but no
difference in antibody localization was observed.
Figure 5. Location of the EarlyCP motif, CsCP8.5, and CsAMP8.1 in the arthrodial membrane of the walking leg and the gills (molt cycle stage or hours after ecdysis in upper left corner).  

- a. Above the arrowhead is arthrodial membrane and below is calcified cuticle.
- b. Asterisks designate efferent/afferent vessel lining while arrows designate lamellae lining.
- c top. Above the arrowhead is calcified cuticle and below is arthrodial membrane.
- c bottom. Left of the arrowhead is arthrodial membrane and to the right is calcified cuticle.
- d. Efferent/afferent vessel lining with lamellae branching off.
- e. above the arrowhead is calcified cuticle and below is arthrodial membrane.
- f top and bottom. Efferent/afferent vessel lining with lamellae.
- f bottom. Efferent/afferent vessel lining.
3.5 Antibody binding pattern in the apodeme and suture line

The apodeme was found to consist of a medial layer composed of fused cuticles surrounded on either side by lateral layers. All three antibodies bound to the medial layer of the apodeme (Fig. 12a, c, and e). In Fig. 12a and 12c the antibodies to the EarlyCP motif and CsCP8.5 were observed in the medial layer (arrowhead) as well as both sides of the apodeme where the new medial layer was forming (arrows). The antibody to CsAMP8.1 also bound the sides of the apodeme in the same premolt stages as the antibodies to the EarlyCP motif and CsCP8.5 (results not shown). Cross sections of the medial layer revealed three rays near the dactylopodite where the apodeme fused cuticular components to form a diamond shape (Fig. 12c, arrowheads) and was seen to have various smaller rays throughout (Fig. 12e, oval). The smaller dorsal apodeme and larger ventral apodeme as well as the proximal and distal portions of each were examined, but the antibody staining did not reveal any differences in composition.

The antibody to the EarlyCP motif bound the exocuticle of the dorsal carapace as well as the region of the suture line throughout the molt cycle (Fig. 12b, middle asterisk). The antibodies to CsCP8.5 and CsAMP8.1 did not bind any layers of the dorsal carapace including the suture line (Fig. 12d and f). Even though the suture line has been shown to have differing properties from the surrounding cuticle (Priester, 2005) none of these antibodies differentiated the suture line from the adjacent dorsal carapace.
Figure 6. Location of the EarlyCP motif, CsCP8.5, and CsAMP8.1 in the apodeme (arrowheads and arrows) and suture line (asterisks) (molt cycle stage or hours after ecdysis in upper left corner). The oval indicates rays of the medial layer.
4. Discussion

4.1 Endogenous fluorescence

In order to understand the pattern of staining of fluorescently labeled antibodies, it is necessary to first determine the endogenous fluorescence in the tissue. Furthermore, it is important to know that endogenous fluorescence is often influenced by the fixative. Without negative controls, endogenous fluorescence can be mistaken for localization of the antibody (a false positive). The epicuticle of the dorsal carapace and gills fluoresced at the same wavelength as the secondary antibody. Due to a broad range of endogenous fluorescence seen in the epicuticle, methods that do not utilize fluorescence such as colloidal gold or 3,3'-diaminobenzidine (DAB) labeling should be used in order to determine whether or not the three antibodies had an affinity to the epicuticle. Positive controls are also necessary in order to identify if there is any non-specific binding of the secondary antibody. Fortunately, the blocking buffer used with all of the primary antibodies prevented all non-specific binding of the secondary antibody. Likewise, the use of paraformaldehyde as the fixative rather than glutaraldehyde prevented general autofluorescence of the tissues (Baschong et al., 2001; Chandler and Roberson, 2009).

4.1.1 Comparison of the dorsal carapace and apodeme

Even though the apodeme is an infolding of the calcified cuticle of the chela there are inherent differences between the apodeme and the calcified cuticle. Due to size similarities and lack of lamellae the medial layer of the apodeme has been compared to the epicuticle (Crawford, 2006; Crawford et al., 2007). However, endogenous fluorescence varied between the epicuticle and medial layer of the apodeme. The epicuticle was excited by both the 405 nm and 543 nm lasers while the medial layer of the apodeme was only excited by the 405 nm laser. Several accounts have indicated that the epicuticle in the dorsal carapace is not a homogenous layer (Travis, 1963; Hegdahl et al., 1977c; Roer and Dillaman, 1984; Compère,
Instead, the epicuticle has been described as being bilaminar with a thin outer epicuticle and a thicker inner epicuticle. Vertical canals along with calcium crystals have been observed in the inner epicuticle, but not the outer epicuticle (Compère, 1995; Hegdahl et al., 1977c; Roer and Dillaman, 1984). Future studies are needed to determine whether or not the medial layer of the apodeme is a homogeneous or heterogeneous layer.

Another comparison was made between the lateral layers and the endocuticle due to similarities in AO staining and a presence of lamellae (Crawford, 2006; Crawford et al., 2007). This comparison was further supported by localization of the antibody to the EarlyCP motif in the exocuticle, but not to the lateral layers of the apodeme or the endocuticle. Antibody staining with the EarlyCP motif and AO staining also indicated that the apodeme lacked a layer equivalent to the exocuticle of the dorsal carapace.

4.2 Comparison of antibody results with previous work

Previous work using qPCR and Northern blots determined the distribution of CsAMP8.1 mRNA to be in the arthrodial membrane hypodermis throughout the molt cycle while CsCP8.5 and the EarlyCP motif mRNA’s in the dorsal carapace hypodermis in premolt. CsAMP8.1 mRNA was not detected in the dorsal hypodermis while CsCP8.5 and the EarlyCP motif mRNA’s were not found in the arthrodial membrane at any point in the molt cycle (Wynn and Shafer, 2005; Shafer et al., 2009). Consequently, antibodies created against CsCP8.5 and the EarlyCP motif were expected to bind the dorsal carapace and possibly the apodeme and suture line because they are all calcified cuticle. Likewise, the antibody created against CsAMP8.1 was expected to bind arthrodial membrane and possibly the gills and branchial chamber because they are all uncalcified cuticle.

However, none of the antibodies stained in such an expanded manner so as to make them useful as a predictor of calcified and uncalcified cuticle. The antibody to the EarlyCP
motif bound every region of the blue crab tested indicating it is a very ubiquitous epitope, yet the antibody differentiated between layers in the dorsal carapace. All three polyclonal antibodies (CsCAMP8.1, CsCP8.5, and the EarlyCP motif) bound to the medial layer of the apodeme, the branchial chamber, and the arthrodial membrane. There are several possible explanations for the differences observed between the molecular biology and immunocytochemistry.

First, the EarlyCP motif is downstream of the RR-1 that has been associated with uncalcified cuticle. Due to the RR-1 sequence, the EarlyCP motif should be found in uncalcified cuticle such as the arthrodial membrane. However, the expression pattern of each protein within the family may differ. The qPCR levels of four of the proteins within the family (CsCP14.1, EarlyCP1, EarlyCP2, and EarlyCP6) have been investigated throughout the molt cycle, but not all thirteen of the members (EarlyCP3, EarlyCP4, EarlyCP6, and 6 others). The mRNA levels and protein levels for all the proteins need to be examined throughout the molt cycle in order to determine the temporal and spatial distribution of all the members of this complex family.

Secondly, the previous study (Shafer et al. 2009) that found the EarlyCP motif mRNA only to be in the dorsal carapace did consistently find extremely low levels of the EarlyCP motif mRNA in the arthrodial membrane, but considered it negligible when compared to levels of the EarlyCP motif mRNA levels found in the dorsal carapace. Even though the mRNA levels were extremely low, this may explain why the antibody to the EarlyCP motif was observed in arthrodial membrane and other uncalcified cuticle. Studies have shown that there is no predictable relationship between mRNA and protein abundance (Gygi et al., 1999; Griffen et al., 2002; Washburn et al., 2003). Several ideas have been proposed in an attempt to explain this lack of relationship such as posttranscriptional regulatory mechanisms that regulate gene expression specifically or globally (i.e. mRNA decay rate and modification of
initiation factors) as well as stimuli that perturb protein expression (Day and Tuite, 1998; Gygi et al., 1999; Ideker et al., 2001; Griffin et al., 2002). In other words, while levels of mRNA may seem negligible, if the mRNA decay rate is slow enough this may allow the mRNA time to make a lot of protein.

Thirdly, all three antibodies bound to the apodeme, branchial chamber, and arthrodial membrane suggesting that these antibodies are not as specific to cuticle types as originally proposed. In other words, the antibodies to CsCP8.5 and the EarlyCP motif may not necessarily be associated with calcified cuticle (e.g. dorsal carapace), nor the antibody to CsAMP8.1 with uncalcified cuticle (e.g. arthrodial membrane).

Even though little work has been done on the apodeme in the blue crab, the medial layer has been shown to be similar to the epicuticle in the dorsal carapace. The epicuticle is known to be hardened by phenolic tanning (Travis, 1965; Roer and Dillaman, 1984). Despite the fact that the epicuticle does not contain chitin or lamellar organization, calcium crystals have been observed indicating the occurrence of post-ecdysial calcification (Hegdahl et al., 1977c; Roer and Dillaman, 1984). Nonetheless, future work is needed to determine if the medial layer of the apodeme is calcified or uncalcified cuticle. If the medial layer of the apodeme was determined to be uncalcified then CsAMP8.1 would be the only antibody that truly differentiated between cuticle types in a predictable manner. The previous notion that sequences upstream of RR are associated with uncalcified cuticle and sequences downstream of RR are associated with calcified cuticle may not be as simple as it first appeared (Andersen, 1998b; Andersen, 1999; Endo et al., 2000 and 2004; Watanabe et al., 2000; Inoue et al., 2001, 2003, and 2004; Rebers and Willis, 2001; Togawa et al., 2004).

Lastly, the antibodies used in this study were polyclonal antibodies indicating it is likely that there was more than one antibody present. This may account for inconsistencies seen between the molecular biology and immunocytochemistry data. However, all three
antibodies were made in a rabbit and then the serum was removed and run through an affinity column in order to purify them. An antibody with only part of the pre-chosen epitope may have bound the antigen in the affinity column, but it seems unlikely that the antibody with only part of the pre-chosen epitope would have a high enough affinity to bind a very different cuticle epitope. Consequently, any epitope bound would be very similar so one might expect that the cuticle protein would also be very similar.

4.3 Finding genes responsible for calcification

Antibodies against CsCP8.5 and the EarlyCP motif were created in an attempt to determine which genes are responsible for calcification in the blue crab. A technique called RNA interference, RNAi, could be used to eliminate expression of the gene or in other words cause “knockdown” of a gene. Researchers have successfully caused knockdown in the common fruit fly (Drosophila melanogaster), roundworm (Caenorhabditis elegans), plants, and more recently, mammalian cell cultures and mice (Stoutjesdijk et al., 2002; Kamath and Ahringer, 2003; Patkaniowska and Tuschl, 2003; Boutros, 2004; Palliser et al., 2006). Even though the chitin-binding Rebers-Riddiford consensus sequence (RR) gives clues as to which sequences may be found in calcified or uncalcified cuticle, the process of finding an amino acid sequence that is specific to one cuticle type has proven challenging.

4.4 Summary of the results

This study investigated binding of three antibodies (CsCP8.5, the EarlyCP motif, and CsAMP8.1) to a variety of cuticular structures. Although it was hoped that these antibodies would allow one to differentiate calcified and uncalcified cuticle, the actual staining pattern did not validate that prediction. Several factors are discussed that may be responsible for the differences seen in the molecular biology and immunocytochemistry data. In summary, it
appears that epitopes localized by these antibodies are shared by calcified and uncalcified cuticle.
LITERATURE CITED


46


Fyrberg, E., Mahaffey, J., Bond, B., Davidson, N., 1982. Transcripts of the six Drosophila actin genes accumulate in a stage and tissue specific manner. Cell 33, 115-123.


Patkaniowska, A. Tuschl, T. 2003. Gene silencing by synthetic siRNA duplexes in


Watanabe, T., Persson, P., Endo, H., Kono, M., 2000. Molecular analysis of two genes,

