INVESTIGATING THE ROLES OF CUTICULAR PROTEINS OF THE BLUE CRAB, *CALLINECTES SAPIDUS* USING RNA INTERFERENCE

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TABLE OF CONTENTS

ABSTRACT iv

ACKNOWLEDGEMENTS vi

LIST OF TABLES vii

LIST OF FIGURES viii

INTRODUCTION 1

METHODS 10

Synthesis of Double Stranded RNAs 10

Organism Collection, dsRNA Injections and Tissue Collection 12

RNA Extractions and Quantitative PCR 14

Tissue Preparation for Microscopy 16

Scanning Electron Microscopy 17

Antibody Preparation 18

Immunohistochemistry 18

Antibody Image Collection 19

RESULTS 19

Mortality Rates 19

mRNA Levels of Target Transcripts 20

Back Scattering Electron Microscopy and EDAX 23

Fluorescence Immunohistochemistry 36

DISCUSSION 46

REFERENCES 57

APPENDIX 61

ABSTRACT

The Atlantic blue crab, *Callinectes sapidus*, is a decapod crustacean that progresses through numerous molt cycles during a life span. A portion of the new exoskeleton is synthesized and deposited during premolt, though calcification of the new cuticle does not begin until after ecdysis. The cuticle of *Callinectes sapidus* is primarily composed of chitin fibers and proteins synthesized by a single layer of hypodermis cells. Of the numerous proteins incorporated into the cuticle, CsEarlyCP1 and the other proteins in the CsEarlyCP family are hypothesized to be regulators or inhibitors of pre-molt calcification based on their expression patterns and homology with proteins in other species. Conversely, other cuticular proteins, most notably CsCP8.5, may act as nucleators of calcification. Since RNA interference (RNAi) can be used as a loss-of-function assay, double-stranded RNAs complementary to *CsEarlyCP1*, to the entire CsEarlyCP family and to *CsCP8.5* were injected into crabs in order to investigate the functional roles of these transcripts. Injection of transcript-targeting dsRNAs led to increased levels of mortality prior to ecdysis compared to the controls. Immunohistochemistry using an antibody specific for the CsEarlyCP family revealed a decrease in the level of CsEarlyCP proteins being incorporated into the dorsal cuticle suggesting that dsRNA injections were silencing the target transcripts. Scanning electron microscopy (SEM) in the back-scattering electron mode coupled with elemental mapping using energy dispersive x-ray analysis suggests that injection of *CsEarlyCP1, EarlyCP Motif* and *CsCP8.5*-targeting dsRNAs resulted in an accumulation of sodium, chloride and low levels of calcium in the pre-exuvial dorsal cuticle. Additionally, immunolocalization of the EarlyCP family in CsCP8.5-targeted samples shows that silencing CsCP8.5 affected the incorporation of the CsEarlyCP proteins into the dorsal cuticle.

This suggests a structural interaction between CsCP8.5 and the CsEarlyCP family and helps to explain why RNAi silencing of both transcripts lead to a similar SEM phenotype.

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LIST OF TABLES

Table Page

1. Primers used in dsRNA synthesis.....11

2. Primers used for qPCR.....15

LIST OF FIGURES

Figure Page

1. Mortality at various times after treatment. For each treatment and control group the percentage of crabs that died in less than 24 hours after injection and first leg sampling (blue bars), within 1 to 4 days after injection (red bars), more than 4 days after injection (green bars), during ecdysis (purple bars), or in postmolt stages (orange bars) is shown..... 22

2. mRNA levels of target transcripts in post-treatment leg samples normalized to the mRNA levels of pre-treatment leg samples measured with qPCR. Blue bars represent the average expression levels of *CsEarlyCP1* in samples from *CsEarlyCP1*-treated, EarlyCP Motif-treated crabs and both control groups. The red bars represent the average expression levels of *CsCP8.5* in samples from *CsCP8.5*-treated and control crabs. Black dots represent the levels of target mRNA for individual crabs.....25

3. SEM of dorsal cuticle from control samples using back scattered electrons (BSE). Most samples from uninjected control crabs (A) and off-target dsRNA injected crabs (B) exhibited a homogeneous BSE signal intensity. However one sample from the uninjected control group (C) and three from the off-target control group (D) exhibited different signal intensities across the cuticle. Arrows indicate the apical edge of the cuticle and arrowheads indicate the basal edge of the cuticle..... 28

4. SEM images of dorsal cuticle from *CsEarlyCP1*-targeted crabs using back-scattered electrons. Four different signal intensity contrast patterns were observed from crabs treated with CsEarlyCP1-targeting dsRNAs. The contrast patterns observed were thin lines of contrast along the apical and basal edges of the cuticle (A), a thin apical line of contrast and thicker basal bands of contrast (B), a thin apical line of contrast and a thick basal band of contrast that in regions extended completely through the interior of the cuticle and fused with the apical line of contrast (C), and punctate crystal-like structures that were present only in the dorsal cuticle of samples (D). One crab did survive to postmolt stages (E) and its cuticle exhibited the expected contrast patterns of calcification previously described for postmolt cuticle. Arrows indicate the apical edge of the cuticle and arrowheads indicate the basal edge of the cuticle.....30

5. SEM images of dorsal cuticle from EarlyCP Motif-targeted crabs using back-scattered electrons. Samples within this treatment group exhibited either thin lines of contrast along the apical and basal edges of the cuticle (A) or homogenous BSE signal intensities lacking contrast (B). Arrows indicate the apical edge of the cuticle and arrowheads indicate the basal edge of the cuticle..... 33

6. SEM images of dorsal cuticle from *CsCP8.5*-targeted crabs using back-scattered electrons. Half of the samples from this sample group exhibited patterns of contrast and three different contrast patterns were observed including: thin apical lines and thick basal bands of contrast (A), thin apical and thin basal lines of contrast (B) and punctate crystal-like structures that were present only in the dorsal cuticle of samples (C). Homogenous BSE signal intensities were observed in the other half of samples collected from this treatment group (D). Arrows indicate the apical edge of the cuticle and arrowheads indicate the basal edge of the cuticle..... 35

7. Analysis of the elemental composition of dorsal cuticle samples from an off-target dsRNA injected control (column A), an uninjected control (column B) and a *CsEarlyCP1*-targeted crab (column C). Samples were imaged with back-scattered electrons (row 1), x-ray mapping of sodium (row 2), x-ray mapping of chloride (row 3) and x-ray mapping of calcium (row 4)..... 38

8. Analysis of the elemental composition of dorsal cuticle samples from *CsCP8.5*-targeted crabs (column A and B) and an EarlyCP Motif-targeted crab (column C). Samples were imaged with back-scattered electrons (row 1), x-ray mapping of sodium (row 2), x-ray mapping of chloride (row 3) and x-ray mapping of calcium (row 4)..... 40

9. Dorsal branchial integument samples immunolabeled with CP8.5 antibody. Branchial cuticle from an uninjected control (A), dorsal cuticle of an uninjected control (B), an off-target dsRNA injected control (C), a CsEarlyCP1-targeted crab (D), an EarlyCP Motif-targeted crab (E) and a CsCP8.5-targeted crab(F). The branchial cuticle is indicated with an asterisk. Arrows indicate the apical edge of the cuticle and arrowheads indicate the basal edge of the cuticle..... 43

10. Dorsal branchial integument samples immunolabeled with EarlyCP Motif antibody. Dorsal cuticle from an uninjected control (A), an off-target dsRNA injected control (B), a CsEarlyCP1-targeted crab that died within 2 days of treatment in ecdysis (C), a CsEarlyCP1-targeted crab that died 5 days after treatment and prior to ecdysis (D), an EarlyCP Motif-targeted crab (E) and a CsCP8.5-targeted crab (F). Arrows indicate the apical edge of the cuticle and arrowheads indicate the basal edge of the cuticle..... 45

INTRODUCTION

The life span of an Atlantic Blue crab, *Callinectes sapidus*, includes numerous molt cycles in which a hard exoskeleton is shed. Because molting occurs at regular intervals, *C. sapidus* serves as a model organism for the study of the processes that control biomineralization and exoskeleton formation. As with many other crustaceans, molting is a critical process for growth. At the beginning of the premolt portion of the cycle, the blue crab separates its hypodermis from its exoskeleton. It then synthesizes two layers of cuticle prior to molting. The old exoskeleton, or exuvia, is shed and the underlying cuticle is expanded through water uptake allowing the crab to increase in size. Shortly after molting, *C. sapidus* absorbs calcium ions from surrounding waters, which are used to mineralize the new exuvia. As with other arthropods, the hard exoskeleton of *C. sapidus* is important for protection from predators, support and locomotion.

Biomineralization is the process through which living organisms form minerals. The phenomenon is extremely widespread and examples of organisms that are capable of mineralization can be found in five of the kingdoms (Lowenstam and Weiner, 1989). Records of biomineralization in evolutionary history date back to examples of vase-shaped tests preserved in rocks from the Grand Canyon dating nearly 700 million years ago and silica biomineralization of scales preserved in 630 million year old rocks from northwest Canada (reviewed in Knoll, 2003). In *C. sapidus*, the organic matrix in which calcification occurs is composed of cross-linked chitin fibers and proteins (Horst, 1990). The cross linking of proteins and chitin is known as sclerotization and occurs in insects and other arthropods. However, in crustaceans hardening of the exoskeleton through sclerotization is followed by the incorporation of inorganic minerals into the organic matrix. In decapod crustaceans, the inorganic salt incorporated into the exoskeleton

is generally calcium carbonate (Lowenstam and Weiner, 1989). A conservative estimate suggests that the calcium carbonate skeleton evolved twenty-eight times within Eucarya (Knoll, 2003).

Addadi and Weiner (1985) observed the importance of aspartic acid-rich proteins acting as nucleators of calcium carbonate in the proper formation of calcite crystals. In mollusk shells, an organic matrix comprised of a scaffold of aspartic rich proteins on β-chitin sheets serves as a surface for calcium carbonate nucleation, calcite crystal formation and growth of the nacreous layer (Addadi and Weiner, 1985; Addadi et al., 2006; Nudelman et al., 2008). Calcite crystals grow vertically until they are limited by β-chitin sheeting and then grow horizontally until further growth is limited by aspartic rich proteins or by contacting crystals from neighboring nucleation sites (Lowenstam and Weiner, 1989; Addadi et al., 2006). The same process of calcite crystal formation has been suggested to occur in the formation of the *C. sapidus* exoskeleton (Roer and Dillaman, 1993).

The intermolt crustacean cuticle is composed of four discrete layers. The outermost layer is the epicuticle, composed of lipoproteins saturated with calcium salts. It is the thinnest of the four cuticle layers. The epicuticle becomes sclerotized, but does not calcify until post-molt stages. The exocuticle lies directly beneath the epicuticle and is comprised of chitin fibers and proteins stacked in layers. The exocuticle is hardened through both sclerotization and calcification. The third layer is the endocuticle, which is the thickest and most heavily calcified layer of the cuticle. Similar to the exocuticle, it is comprised of layers of chitin and proteins; however the endocuticle is never sclerotized but exclusively hardened by calcium salts. The fourth layer of the cuticle is the membranous layer. As the most basal layer of the cuticle, the membranous layer is in contact with the hypodermis. It contains no calcium minerals and is never sclerotized (Roer and Dillaman, 1984). Although not part of the cuticle structure, the

hypodermis is critical in cuticle formation as it is the tissue that secretes the macromolecules that form the organic matrix of the cuticle.

The molt cycle of *C. sapidus* has been studied for nearly a century and a method for describing the molt cycle based on key morphological changes was created in 1939 (Drach,1939). According to this method, the molt cycle is divided into five stages. The first stage in the molt cycle is post-molt (A_1, A_2, B_1, B_2, C_1, C_2, C_3) followed by intermolt (C_4), early premolt (D_o, D_1), late premolt (D_2, D_3, D_4), and ecdysis (E) (Drach and Tchernigovtzeff, 1967). Early premolt (D_0) is characterized by apolysis, or separation of the hypodermis from the cuticle, which is caused by the secretion of molting fluid containing digestive enzymes such as chitinase and chitobiase (Roer and Dillaman, 1984; Priester, 2005). During early premolt stage D_1, a new epicuticle is deposited. During premolt stage D_2, exocuticular deposition takes place. The layers of the cuticle that are synthesized and deposited prior to ecdysis are referred to as pre-exuvial layers. The point in time at which a crab emerges from its exuvia is termed ecdysis (E). During post-molt stages (A-C_3), sclerotization, incorporation of calcium minerals into the pre-exuvial cuticle and endocuticle deposition occurs. The end of post-molt is characterized by termination of calcite crystal formation in the cuticle and membranous layer synthesis and deposition (Roer and Dillaman, 1984). The process of molting must be regulated both temporally and spatially. On the temporal scale, regulation of calcification is important to prevent the hardening of the new cuticle prior to ecdysis; whereas, the spatial scale regulation of calcification is important because mineralization of the entire exoskeleton would inhibit locomotion, nutrient absorption and oxygen transfer. Spatial regions of the cuticle that go through sclerotization but not calcification include cuticle lining the foregut, the joints of appendages, gills and the branchial chamber (Williams et al., 2003).

In order to better understand the processes and mechanisms that control biomineralization and cuticle formation in arthropods, it is necessary to focus on the genes that code for proteins involved in cuticle formation. Numerous crustacean cuticle proteins have been isolated and characterized, as reviewed by Shafer et al. (2006). Twenty-one cuticle proteins have been extracted from the lobster, *Homarus americanus*, and have been found to possess unique motifs found in other arthropods (Anderson, 1998a; Anderson, 1998b; Anderson, 1999; Anderson, 2000). One of these conserved sequences, known as the Rebers-Riddiford (RR) consensus sequence was first extracted and characterized from insect cuticle. The RR consensus amino acid sequence is $Gx_8Gx_6YxAxExGYx_7Px_2P$, where x represents any amino acid (Rebers and Riddiford, 1988). The RR consensus is highly conserved among insects and crustaceans and has been shown to bind chitin *in vitro* (Rebers and Willis, 2001). Since the identification of the RR consensus, three variants of the sequence have been identified in arthropods, RR-1, RR-2, and RR-3 (Andersen, 1998a; Andersen, 1998b; Andersen, 2000). The RR-1 consensus is found in the flexible cuticle of insects and in the non-calcifying arthrodial cuticle of crustaceans (Andersen, 1999). Multiple examples of RR-1 containing crustacean cuticle proteins exist and have been identified in the arthrodial membrane cuticles of *Homarus americanus, Cancer pagurus* and *Marsupeneaus japonicus* (Andersen, 1998b; Andersen, 1999; Watanabe et al., 2000).

In addition to the discovery of RR-1 containing proteins in the non-calcifying cuticles of crustaceans, proteins that are RR-1 like but cannot be classified as RR-1, 2 or 3 due to truncations of the conserved sequence have been identified from the cuticle of the crayfish *Procambarus clarkii*. These novel calcification associated peptides (CAP) from *P. clarkii* are named CAP-1 and CAP-2 and were extracted from the calcifying, post-molt cuticle of the tail-fan blade (Inoue et al., 2001; Inoue et al., 2004). CAP-1 and CAP-2 are thought to be involved

in the nucleation or regulation of $CaCO_3$ in the cuticle after *in vitro* assays were conducted using natural and recombinant proteins (Inoue et al., 2003; Inoue et al., 2004).

More recently, Inoue et al. (2008) identified another novel protein from the *P. clarkii* exoskeleton that possesses the entire RR-1 consensus and is most strongly expressed during the late premolt stage. This protein, named calcification-associated soluble protein (CASP-2), was found to bind to chitin, but inhibited the precipitation of calcium carbonate during *in vitro* assays suggesting a possible role in regulating or inhibiting calcification of the exoskeleton (Inoue et al., 2008). Unlike other RR-1 containing proteins that are found in the non-calcifying arthrodial cuticle of crustaceans, CASP-2 was extracted from cuticle destined to calcify after ecdysis (Inoue et al., 2008).

In *C. sapidus*, the production of an EST database from mRNAs in pre and post-molt crabs has facilitated the identification of crustacean sequence motifs in *C. sapidus* cuticular proteins (Coblentz et al., 2006). To date, thirty-one cuticle protein transcripts have been discovered and characterized through the use of the EST database. Of these thirty-one transcripts, six transcripts from *C. sapidus* contain an RR-1 consensus sequence and are found to be expressed both pre and post-molt in non-calcifying arthrodial membrane (Wynn and Shafer, 2005; Faircloth and Shafer, 2007). When the transcripts were BLASTED and phylogenetic analysis was conducted, all six transcripts were found to be closely related to the RR-1 containing proteins extracted from *H. americanus*, *C. pagurus* and *M. japonicus* (Shafer et al., 2006).

Three of the thirty-one transcripts characterized in *C. sapidus* were found to be locally similar to the calcification-associated proteins found in *P. clarkii* (Wynn and Shafer, 2005; Faircloth and Shafer, 2007). These three proteins (CsCP6.1, CsCP8.2, and CsCP8.5), like the

CAPs contain a truncated RR consensus sequence and therefore cannot be classified as an RR-1, RR-2 or RR-3. These three *C. sapidus* proteins are more similar to CAP-1 than CAP-2 and are hypothesized to be nucleators of $CaCO_3$ because they are synthesized only in hypodermis that produces calcifying cuticle (Wynn and Shafer, 2005; Shafer et al., 2006). However, unlike the CAPs which are expressed only during post-molt, *CsCP8.2* and *CsCP 8.5* are expressed in hypodermis tissue during late premolt stages and only *CsCP6.1* is expressed during post-molt stages.

One transcript in *C. sapidus* that possesses an RR-1 consensus sequence (*CsCP14.1*) has been identified and characterized in calcifying mid-dorsal hypodermis only during premolt stages (Faircloth and Shafer, 2007). It is hypothesized that the six RR-1 proteins found in the arthrodial membrane may be structurally important for the un-calcified matrix and that the one transcript found in mid dorsal hypodermis may prevent calcification of the cuticle prior to ecdysis (Shafer et al., 2006). Recently, six more cuticular protein transcripts from calcifying mid dorsal hypodermis were identified from the EST database that shared high sequence similarity to *CsCP14.1*. Like the *CsCP14.1* transcript, all six transcripts have an RR-1 consensus sequence and are expressed during premolt stages in hypodermis that synthesizes calcifying cuticle. These six new transcripts were named *CsEarlyCP 1* thru *6* (Shafer et al., 2009). The *CsEarlyCP* transcripts and *CsCP14.1* were BLASTED on NCBI to find sequences in other crustaceans with high similarity, one of which was the CASP-2 transcript from *P.clarkii*. Analysis of the sequence alignments between the CsEarlyCP proteins, CsCP 14.1 and the CASP-2 protein revealed a new 14 amino acid motif named, EarlyCP Motif, which was highly conserved among all the transcripts (Shafer et al., 2009). Due to the presence of the motif and high similarity between the *C. sapidus* transcripts and the *P. clarkii* transcript, which was shown to inhibit

calcification *in vitro*, it is hypothesized that all the *CsEarlyCP* transcripts and *CsCP14.1* belong to a single family of transcripts that act as nucleation inhibitors to prevent calcification of the cuticle prior to ecdysis (Shafer et al., 2009). Although these transcripts have been sequenced and their differential expression analyzed, the roles and functions of individual transcripts are still unknown. Functions of a transcript can be hypothesized based on temporal and spatial distribution; however, a loss of function assay could elucidate the role and contribution of individual transcripts to cuticle structure and the process of biomineralization in *C. sapidus.*

Since the discovery of RNA interference (RNAi) by Fire and Mellow in 1998, the mechanism of gene silencing through the use of double stranded RNA has become a widely used tool in a variety of organisms. In their studies with *Caenorhabditis elegans*, Fire et al. (1998) investigated the effects of injecting purified double stranded RNA (dsRNA) directly into the body. The studies conducted by Fire and Mellow demonstrated that the gene of interest (*unc22*) could be silenced, causing a loss of function phenotype, when dsRNA treatments were administered to nematodes through injection, soaking in dsRNA containing solutions, or when fed *Escherichia coli* that expressed the dsRNAs (reviewed in Agrawal et al., 2003). The natural occurrence of RNAi and related processes are hypothesized to be involved in protection of the genome from invasion of mobile genetic elements such as viruses and transposons (Agrawal et al., 2003). Through the exploitation of these endogenous enzymes involved in the process of post-transcriptional gene silencing and translational gene silencing within the cell, studies that aim to suppress the expression of a specific gene target can be conducted. The cellular processes that occur during RNAi begin with dsRNA entering the cell and cleavage of the dsRNA molecule by the Dicer enzyme to produce multiple small interfering RNAs (siRNAs), which are oligonucleotides 21-23 base pairs in length (Agrawal et al., 2003; Yang and Mattes, 2008). The

siRNAs generated by Dicer then bind to a RNA-induced silencing complex (RISC), where it is proposed that a helicase in the complex unwinds the siRNA, activating RISC. The single stranded anti-sense RNA then guides the RISC towards the target mRNA, resulting in endonucleolytic cleavage or suppression of mRNA translation (Agrawal et al., 2003).

While a number of studies utilizing RNAi have been conducted in organisms ranging from protozoans to fish, humans, mice, plants, fungi and numerous insects (Agrawal et al., 2003), very few studies have used RNAi in crustaceans. RNAi in *Drosophila melanogaster* has been conducted in all stages of development, and dsRNA has been administered through injection in adults or larvae. Soaking treatments have been used for studies with cell lines (Agrawal et al., 2003). One study that utilized RNAi in arthropods investigated the role of a chemosensory protein (*csp 5*) in the development of an embryonic integument in the honeybee, *Apis mellifera*. Injection of dsRNA into *A. mellifera* eggs resulted in abnormalities of all body parts where *csp5* was highly expressed, and resulted in embryos that could not hatch or progress to the larval stage due to malformation of the integument (Maleszka et al., 2007). Two separate studies were conducted in the brine shrimp, *Artemia franciscana*,that investigated the effects of RNAi on axis elongation and segmentation by targeting *spalt* and *Hox* mRNA (Copf et al., 2004; Copf et al., 2006). In both studies, injections were administered into the body cavity of *A. franciscana* larvae using a micromanipulator. In the two studies, RNAi treatment resulted in knockdown of the target mRNA and caused phenotypic abnormalities such as missing or malformed appendages, truncated segments, elongated segments and thoracic to genital transformations (Copf et al., 2004; Copf et al., 2006). Another study that used RNAi to study gene silencing in crustaceans investigated farnesoic acid O-methyltransferase (FAMet) in the shrimp, *Litopenaeus vannamei* (Hui et al., 2008). In the study, the authors observed silencing of

FAMet through injection of dsRNA in concentrations of 1μg/10μl into the fifth pereiopod of adult *L. vannamei* in the early stages of the molt cycle. The authors found that individuals injected with dsRNA had suppressed levels of FAMet for at least three days and the shrimp did not progress to the final stage of the molt cycle. The authors also found that mortality among dsRNA injected adults was at 100% (Hui et al., 2008). A few studies using RNAi have been conducted in the pearl oyster, *Pinctada fucata* and in the crayfish *Cherax quadricarinatus* that silenced mRNAs that coded for proteins involved in biomineralization (Shechter et al., 2008; Suzuki et al., 2009). However, to date no literature exists in which RNAi has been used to investigate the roles and functions of cuticle-associated proteins in crustaceans thought to be involved in biomineralization.

In this study, four sets of dsRNA amplicons were synthesized to target *C. sapidus* cuticle mRNAs that were previously sequenced and characterized for their molt cycle expression patterns in order to determine their structural and functional roles in cuticle formation and biomineralization. The first dsRNA amplicon was made complementary to *CsEarlyCP1* (GENEBANK accession number: HM134085), which belongs to a family of transcripts that are hypothesized to act as inhibitors of calcification prior to ecdysis. The dsRNA amplicon was designed complementary to the 3' un-translated region (UTR) of the transcript because it was the region of the transcript that had the lowest sequence similarity to the rest of the *CsEarlyCP* family, and thus allowed for the dsRNA to target only *CsEarlyCP1* (See Appendix). A second dsRNA amplicon was made complementary to the highly conserved 14 amino acid motif, named EarlyCP Motif, that is found in all seven transcripts belonging to the *CsEarlyCP* family (GENEBANK accession numbers: HM134085 thru HM134090) in order to knockout all the transcripts in the group (See Appendix). The third dsRNA amplicon was synthesized

complementary to *CsCP8.5* (GENEBANK accession number: AY752735), which is one of three transcripts hypothesized to act as a nucleator for calcification after ecdysis because of high sequence similarity to the CAP transcripts found in *P. clarkii* (Inuoe et al., 2003; Inuoe et al., 2004). The final dsRNA amplicon set was made complementary to *E.coli* plasmid vectors that were used in the construction of the *C. sapidus* EST library to act as an off-target control so that any structural or mortality effects within treatments could be determined as specific to knockout of target transcripts and not due to side effects caused by injection of dsRNAs. In order to test the hypotheses for each targeted transcript, live premolt crabs were injected with dsRNA treatments and permitted to progress through the molt cycle. Tissue samples were collected at designated times or after death and later analyzed using quantitative PCR (qPCR) to determine target mRNA knockdown followed by scanning electron microscopy (SEM) and immunohistochemistry to analyze the structural and functional effects of the treatments.

MATERIALS AND METHODS

Synthesis of Double Stranded RNAs

In order to synthesize dsRNA, *E.coli* bacterial clones from the hypodermis cDNA library previously constructed (Coblentz et al., 2006) were sampled and grown in Terrific Broth (MO BIO Laboratories Incorporated, Solana Beach, CA, USA) for 1 day followed by plasmid purification with a plasmid mini-prep kit (Promega Corporation, Madison, WI, USA). The sequences of interest in each plasmid were PCR amplified twice using sequence specific primers that allowed the addition of a T7 promoter sequence to one end or the other (Table 1). Primers were designed following the guidelines provided in the RiboMAX T7 Express RNAi system protocol (Promega Corporation, Madison, WI, USA). PCR was run for 35 cycles using annealing

Table 1: Primers used in dsRNA synthesis

Primer Name	Primer Sequence
EarlyCPMotif F	CCCAGCACATCAACATGAAG
EarlyCPMotif R	GATCTGGTCCAGCACGAACT
EarlyCPMotif F w/T7	GGATCCTAATACGACTCACTATAGGCCCAGCACATCAACATGAAG
EarlyCPMotif R w/T7	GGATCCTAATACGACTCACTATAGGGATCTGGTCCAGCACGAACT
EarlyCP1 F	GACAAGACCATGAACAGTGAATGG
EarlyCP1 R	GCAAAACTCTCAAGCAGCAA
EarlyCP1 F w/T7	GGATCCTAATACGACTCACTATAGGGACAAGACCATGAACAGTGAATGG
EarlyCP1 R w/T7	GGATCCTAATACGACTCACTATAGGGCAAAACTCTCAAGCAGCAA
CP8.5 F	TTGCTGACAAGCGTGGATAC
CP8.5 R	TTGGTTTGTGTGAGTGAATAGAGA
CP8.5 F w/T7	GGATCCTAATACGACTCACTATAGGTTGCTGACAAGCGTGGATAC
CP8.5 R w/T7	GGATCCTAATACGACTCACTATAGGTTGGTTTGTGTGAGTGAATAGAGA
off-target F	ACTTTCACCAGCGTTTCTGG
off-target R	CGTTGGAGTCCACGTTCTTT
off-target F w/T7	GGATCCTAATACGACTCACTATAGGACTTTCACCAGCGTTTCTGG
off-target R w/T7	GGATCCTAATACGACTCACTATAGGCGTTGGAGTCCACGTTCTTT

temperatures 5 degrees above the denaturing temperature of the gene-specific parts of the primers. After PCR, gel electrophoresis was run for 45 minutes at 80 volts on a 1.6% agarose gel using 1μl of PCR products and 1 μl of loading dye to ensure amplified products are the correct size. Following gel electrophoresis, PCR products were cleaned and purified using a Wizard SV PCR clean-up kit (Promega Corporation, Madison, WI, USA). The concentration of each sample was measured using 1 μl of cleaned product on a Nanodrop spectrophotometer (Thermo Fisher Scientific Incorporated, Wilmington, DE, USA) set for DNA. A transcription reaction was performed for both the T7-forward and the T7-reverse PCR products. Five micrograms of PCR product was added to a mix containing 50 μl of RiboMAX Express T7 2X buffer (Promega), 10μl of RNA polymerase mix (Promega) and enough nuclease free water for a total volume of 100 μl. The complementary RNAs were annealed following the protocol provided with the RiboMAX T7 Express RNAi system. Purification of dsRNA after transcription reactions also followed the manufacturer protocol. Pellets of purified dsRNA were dissolved in 500μl of nuclease free water and final concentrations were measured on a Nanodrop spectrophotometer. A follow up gel electrophoresis was run after nanodrop measurements to ensure that each sample was a single product.

Organism Collection, dsRNA Injections and Tissue Collection

Adult *Callinectes sapidus* crabs were collected from Endurance Seafood, a soft shell shedding operation in Kill Devil Hills, NC during May 2009. Molt stages were determined by visually inspecting edges of the swimmeret and by applying pressure to the front suture. Crabs were determined at a late D2 stage if a pink to red line was visible in the gap between the old exuvia and the newly synthesized pre-exuvial cuticle and if the front suture resisted breaking.

When a red line was visible in the swimmeret and the front suture cracked when pressure was applied, the crab was considered stage D3. Crabs were staged as D4 when the front and rear suture lines were naturally broken by the crab and as E when the crab was in the process of molting. All crabs in this study were collected and treated at either late D2 or D3.

Each individual had one of the front walking legs removed through the instigation of limb autotomy and the proximal leg segment was stored in RNAlater (Ambion Incorporated, Austin, TX, USA) for baseline measurements of gene expression. Each crab was then injected with 200 μg dsRNA in a saline injection buffer (0.4M NaCl, 5.4mM KCl, 10 mM CaCl2, 2.6 mM MgCl2, 2 mM NaHCO3, pH 6.8) (Liu et al., 2007) with injection volumes less than 175 μl. Following injection, crabs were placed in numbered cages in shedding tanks. Mortality was monitored, and all crabs surviving 24 hours after treatment were caused to autotomize a second front walking leg. The proximal leg segment was stored in RNAlater for comparison with the first leg removed to quantify the amount of target mRNA change. Crabs that had both front walking legs removed were allowed to progress through the molt and sacrificed at either 10 hours post-molt or were collected after death. At 10 hours post-molt or after death, each crab was dissected and a section of whole dorsal branchial integument located directly above the right side gill chamber was collected, placed in a cryotube, plunged in liquid nitrogen and kept at -70°C until further processing. Crabs that had not progressed through the molt to 10 hours post-molt or died by the third day of sample collection in Kill Devil Hills were placed in numbered plastic containers with holes and placed in large coolers with filtered water from the shedding tanks. The crabs were transported with continuous aeration to the University of North Carolina Wilmington where they were kept in the plastic containers in large holding tanks until reaching D4 when they were

moved to large individual containers to molt. Crabs were again sacrificed at 10 hours post molt or collected after death and dorsobranchial tissue was frozen at -70°C as above.

RNA Extractions and Quantitative PCR

Leg segments from dsRNA-treated and control crabs were homogenized in 900 μl of TRIzol (Invitrogen Corporation, Carlsbad, CA, USA). Samples were then applied to an RNAeasy column (Qiagen Incorporation, Valencia, CA, USA) and manufacturer directions were followed to obtain purified RNA. Total RNA was eluted from the column with 50 μl of nuclease free water. Quantitative and qualitative analysis of purified RNA samples was conducted using an Agilent Bioanalyzer operated with 2100 Expert software and a RNA 6000 Assay Kit (Agilent Technologies, Santa Clara, CA, USA). Following bioanalysis, 0.2μg of total RNA from high quality samples was used for cDNA synthesis. Reverse transcription reactions followed the Superscript II protocol (Invitrogen Corporation, Carlsbad, CA, USA), but included the use of betaine Q-solution (Qiagen Incorporation, Valencia, CA, USA) and slightly elevated reaction temperatures. Reactions were primed with oligo (dT).

Quantitative PCR (qPCR) was then performed using a real-time PCR system, model 7500 (Applied Biosystems Incorporated, Foster City, CA, USA). qPCR primers were designed parts of the transcript sequence outside the dsRNA amplicon regions (Table 2) using Primer Express software version 2.0 (Applied Biosystems Incorporated, Foster City, CA, USA). The following guidelines were used in primer design: qPCR amplicons between 50- 150 bp in length, G/C content of each primer between 30 to 80% , melt temperatures between 58 to 60°C, runs of more than three identical nucleotides avoided, and no more than two G/C bases in the five nucleotides at the 3' end of each primer. qPCR was carried out in 96-well plates with three

Table 2: Primers Used for qPCR

Primer Name	Primer Sequence
EarlyCP1qPCR F	TGCCTAAACACCCTCATTCA
EarlyCP1qPCR R	GCCTCATAGGTGCCATTCAC
CP8.5qPCR F	CCATGAAGTGCACCGCTATTC
CP8.5qPCR R	TCAGAGAAGTCGAAGATGGAGTCA

replicates of each sample. The reaction mixture included 12.5μl of SYBR green master mix (Applied Biosystems Incorporated, Foster City, CA, USA), 1 μl of cDNA (0.5ng), 7.5 ml of water and 4 μl of diluted primers (containing 0.2μM each of the forward and the reverse primer) in each well of the plate. Cycle parameters were 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. A melt temperature dissociation curve was produced at the end of the cycling steps. Each plate contained a set of standards produced from purified *E.coli* vector plasmids containing the complete sequence for *CsCP8.2.* The stock solution of purified vector plasmids was diluted to produce a 10-fold dilution series containing 7 concentrations. All 7 standard concentrations were used on each qPCR plate with primers designed to amplify *CsCP8.2* so that relative expression data collected from samples on different plates would be comparable. qPCR data were analyzed using 7500 System Software (Applied Biosystems Incorporated, Foster City, CA, USA).

Tissue Preparation for Microscopy

Frozen dorsal branchial integument samples were removed from the cryotubes and cut in half. The half of the tissue to be used for scanning electron microscopy was lyophilized for 24 hours until fully dehydrated. The other half was placed into 4% paraformaldehyde fixative in 0.2M sodium cacodylate buffer, pH 7.4 for immunolabeling. The dried samples were embedded in fresh 100% Spurr's epoxy resin (Spurr, 1969) in aluminum dishes. After samples were infiltrated with resin, the aluminum dishes containing tissue were placed in a vacuum oven overnight at 70°C to polymerize. After polymerization, epoxy blocks were cut to remove individual tissue pieces. Each tissue sample was cut in half in order to expose a surface of the tissue. The cut samples were glued on aluminum stubs with the exposed tissue face up and placed into a vacuum desiccator overnight. Once the glue was dried on each stub, the samples

were sanded until a smooth surface was obtained for each sample using increasing grit values of P200, P600, P1200, P1500, and P2000. Sanded sample stubs were sputter-coated with 6 nm of platinum/palladium using a Cressington 200 208HR Sputter coater (Cressington Scientific Instruments Ltd., Watford, England, UK). Mounted samples were stored in a vacuum desiccator until viewed using a Philips XL30S FEG scanning electron microscope (Royal Philips Electronics Incorporated, Amsterdam, Netherlands).

The fixed tissue samples were stored for 2 months with fresh fixative changes occurring once a month. Tissues were then rinsed in distilled water for 30 minutes and dehydrated in an ascending acetone series (50%, 70%, 90%, 100%) with two 30-minute solution changes in each acetone concentration. Tissues were cleared in toluene for two 30-minute changes and then infiltrated with a 1:1 mixture of toluene and paraffin for 1 hour in a vacuum oven at 60°C. This was followed by infiltration of fresh 100% paraffin overnight in the vacuum oven. Tissues were then individually placed into fresh 100% paraffin wax in block molds and allowed to solidify. Paraffin blocks were then sectioned using an American Optical rotary microtome Model 820 (American Optical Manufacturing Company, Southbridge, MA, USA) at a thickness of 10μm. Sections were placed on slides so that each slide contained 4 to 6 sections.

Scanning Electron Microscopy

Back-scattering electron (BSE) images were typically collected at an accelerating voltage of 20Kv with a spot size of 5 and a working distance of 7.5 mm. Energy dispersive x-ray analysis software (EDAX version 3.32) was used to map carbon, oxygen, magnesium, calcium, sodium and chloride found in the cuticle sections. X-ray mapping of these 6 elements was done

at a resolution of 512 X 400 pixels with a dwell time of 100 msec, an amplifier time of 50 μs and a total of 128 frames per sample.

Antibody Preparation

Two sets of antibodies were synthesized by Open Biosystems (Thermo Scientific Open Biosystems, Huntsville, AL, USA) that were specific for CSCP8.5 (DSIFDFSDEDMHL) and a highly conserved 14 amino acid motif present in all members of the CsEarlyCP protein family (FPHPIPQFVLDQIA). Peptides were conjugated with bovine serum albumin (BSA) and injected into rabbits. The immunization schedule included a primary inoculation followed by three booster injections occurring at 14 day intervals. Serum was collected from rabbits on days 56 and 58 after the initial inoculation. Serum was applied to BSA affinity columns and the flow-through was then affinity purified against the antigenic peptide.

Immunohistochemistry

Cuticle sections on slides were washed in toluene twice for 3 minutes followed by 3-minute washes in 100% acetone, 90% acetone, and distilled water and two 2-minute washes in Sorensen's phosphate buffered saline (PBS). Negative control slides for each sample were left in a PBS bath while remaining slides were labeled. Positive control and experimental sections were placed into a hydration chamber and treated with 500 μl goat serum blocking buffer per slide for 1 hour. Blocking buffer was wicked off slides and 500 μl of a 1:100 dilution of primary antibody in a primary dilution buffer (0.25g bovine serum albumin, 25 ml PBS and 0.0125g sodium azide) was applied to all experimental slides for 1 hour. The positive control slides for each treatment were treated with 500 μl of primary dilution buffer without antibody. Slides were rinsed in fresh PBS 3 times for 2 minutes. Positive control and experimental slides

for each sample were transferred to a hydration chamber and treated with 500 μl of a 1:100 dilution goat-anti-rabbit Alexa Fluor 594 (Invitrogen Corporation, Eugene, OR, USA) secondary antibody for 30 minutes. After secondary antibody treatment, the slides received a final rinse in PBS 3 times for 2 minutes. Coverslips were mounted on slides using 1:9 tris glycerol, and the slides were refrigerated until observation.

Antibody Image Collection

Immunolabeled samples were visualized and imaged using an Olympus BX 60 epi-fluorescence microscope with green excitation, achieved by using a DM570 dichroic mirror with a 530-550-nm excitation filter and a 540-nm barrier filter (Olympus America Incorporated, Center Valley, PA, USA). All images were collected at a magnification of 10X for 400 msec using a RT-KE SPOT camera and software version 3.5.6 (Diagnostic Instruments Incorporated, Sterling Heights, MI, USA). Images were edited and prepared for publication using Adobe Photoshop version (7.0); however, no images were digitally altered for contrast or brightness.

RESULTS

Mortality

Some dsRNA injection treatments lead to very rapid mortality compared to the controls (Figure 1). Crabs that were treated with dsRNAs targeting the EarlyCP Motif had 67% mortality during the first 24 hours after treatment. The remaining crabs of this treatment died within one to four days after receiving the injection; none survived to ecdysis. By contrast, the dsRNA that was complementary to *CsEarlyCP1* without targeting the entire EarlyCP family did not cause mortality within the first 24 hours after injection. Instead, this treatment resulted in somewhat delayed mortality, with 50 % of crabs dying between days one and four after injection. Of the

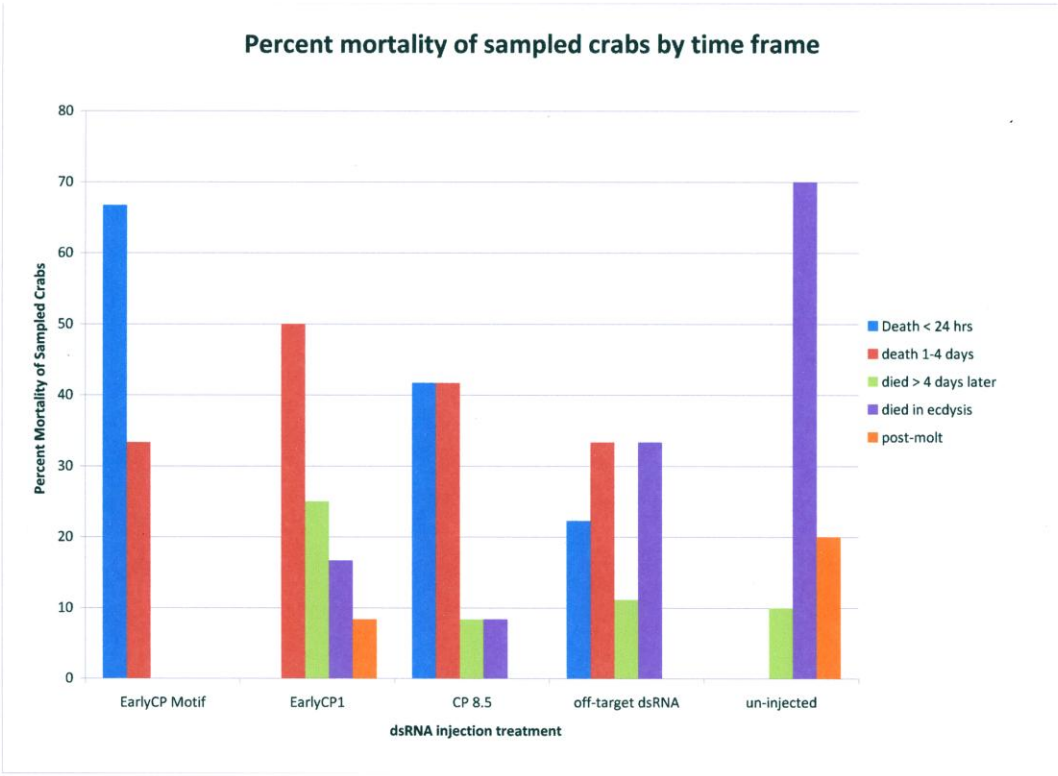
CsEarlyCP1-targeted crabs surviving beyond four days, some died during days four to eight and some died during ecdysis. One crab in this group actually survived ecdysis and was sacrificed 8 to 10 hours post-molt. Injection with dsRNA complementary to the *CsCP8.5* transcript caused a high mortality (42%) during the first 24 hours after treatment. Most of the remaining crabs in this experimental group died during days one to four (42%), though one survived more than four days after injection without beginning to molt and one died in ecdysis.

The control crabs that received an off-target dsRNA injection complementary to an *E. coli* plasmid had 22% mortality within the first 24 hours, 33.33% mortality during days one to four and 11% mortality more than 4 days after injection. Unlike the targeted treatments, 33.33% of these crabs survived to ecdysis and died while molting. The second control group received no injection, yet was subjected to autotomy of two limbs in the same procedure as the other treatments. In this group there was no mortality within the first 24 hours or during days one to four. Ten percent of the uninjected control crabs died more than 4 days after the first leg was sampled without progressing to ecdysis. The remaining 90% survived to ecdysis, with most dying while molting (70%) but a few successfully emerging to be sampled 8 to 10 hours post-molt.

mRNA Levels of Target Transcripts

For crabs that survived at least one day after initial treatment, total RNA was extracted from a section of one autotomized walking leg immediately before injection and from a section of a second leg 24 hours later. In order to quantify the change in the level of the target transcripts caused by the treatments, the RNA from both leg samples was subjected to reverse

Figure 1: Mortality at various times after treatment. For each treatment and control group the percentage of crabs that died in less than 24 hours after injection and first leg sampling (blue bars), within 1 to 4 days after injection (red bars), more than 4 days after injection (green bars), during ecdysis (purple bars), or in post-molt stages (orange bars) is shown.

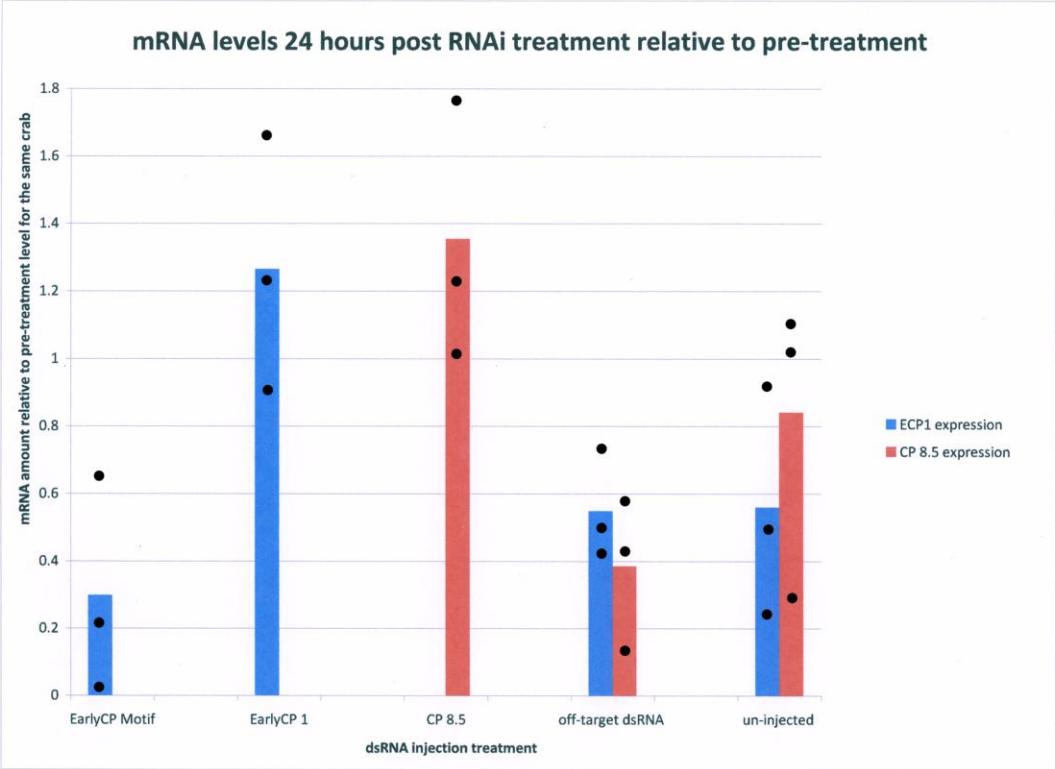

Percent mortality of sampled crabs by time frame
Percent Mortality of Sampled Crabs
80
70
60
50
40
30
20
10
0
EarlyCP Motif
EarlyCP1
CP 8.5
off-target dsRNA
un-injected
dsRNA injection treatment
Death < 24 hrs
death 1-4 days
died > 4 days later
died in ecdysis
post-molt

transcription and qPCR, and the two values were compared. The transcript amount from the pre-treatment sample was set at 1.0 and the post-treatment value from the same crab was normalized to it. Three crabs from each treatment and control group were analyzed in this way. Figure 2 shows the results for individual crabs as well as the treatment averages. Values greater than 1.0 represent an increase in target transcript expression and values less than 1.0 represent a decrease in target transcript expression 24 hours treatment. Crabs treated with dsRNA targeting the motif shared by all transcripts in the *CsEarlyCP* family had a post-treatment to pre-treatment ratio of 0.3, indicating a decrease in *CsEarlyCP1* mRNA. On the other hand, the expression ratios from crabs injected with dsRNA specific for only *CsEarlyCP1* show that this treatment did not decrease the level of *CsEarlyCP1* mRNA. Likewise, crabs treated with dsRNA targeting *CsCP8.5* had an increase of target transcript expression 24 hours after injection. The resulting average expression ratio was 1.35. Thus, in contrast to the EarlyCP Motif-targeting dsRNA, treatments that target only a single transcript did not demonstrate a decrease of mRNA levels in these premolt crabs. For both control groups, the amount of the mRNAs tested was lower for the second sample taken 24 hours after the first. The average ratios for *CsEarlyCP1* were 0.55 and 0.56 for the off-target dsRNA control and uninjected control, respectively. For *CsCP8.5* the ratios were 0.38 and 0.84. This is expected since the levels of both these mRNAs have previously been shown to decrease during late premolt (Faircloth and Shafer, 2007; Shafer et al., 2009).

Back Scattering Electron Microscopy and EDAX

Whole dorsal branchial integument samples were imaged using backscattered electrons (BSE) to determine if the elemental composition of the dorsal cuticle was altered due to dsRNA injections. When the electron beam of the SEM interacts with the specimen, backscattered

Figure 2: mRNA levels of target transcripts in post-treatment leg samples normalized to the mRNA levels of pre-treatment leg samples measured with qPCR. Blue bars represent the average expression levels of *CsEarlyCP1* in samples from *CsEarlyCP1*-treated, EarlyCP Motif-treated crabs and both control groups. The red bars represent the average expression levels of *CsCP8.5* in samples from *CsCP8.5*-treated and control crabs. Black dots represent the levels of target mRNA for individual crabs.


mRNA levels 24 hours post RNAi treatment relative to pre-treatment
mRNA amount relative to pre-treatment level for the same crab
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
EarlyCP Motif
EarlyCP 1
CP 8.5
off-target dsRNA
un-injected
dsRNA injection treatment
ECP1 expression
CP 8.5 expression

electrons are emitted from the specimen in proportion to the atomic number; therefore regions of the specimen with greater signal intensity (brightness) are composed of elements of higher atomic numbers. Twelve samples were imaged for each of the three treatment groups and nine samples were imaged for both the double stranded control group and the uninjected control group.

Most of the tissues samples from the pre-molt RNAi control groups had homogenous backscattered electron signal intensity in all regions of the cuticle (Figure 3A and B), suggesting no accumulation of elements heavier than carbon and oxygen in the pre-exuvial cuticle. However, three of nine samples from the off-target dsRNA control group displayed a contrast pattern consisting of a thin brighter line along the apical edge of the cuticle and a thicker band of contrast extending into the exocuticle from the basal edge (Figure 3C). One sample collected from crabs in the un-injected control group showed thin brighter lines along both the apical and basal edges of the cuticle (Figure 3D). Two of the remaining eight uninjected control samples were dissected from crabs that had molted, so the backscattered electron image exhibited the expected post-molt calcification pattern (Dillaman et al, 2005). No correlation could be found between the unexpected contrast patterns seen in four of 18 pre-molt controls and the stage at injection, the conditions of maintenance, or the stage at which the crabs died.

All twelve samples from crabs targeted specifically for only the *CsEarlyCP1* transcript, exhibited patterns of contrast within the cuticle that suggested the presence of elements of higher atomic mass (Figure 4). Three of these displayed a pattern in which the only the apical and basal edges of the cuticle had a greater signal intensity (Figure 4A). Six other samples had a line of greater contrast along the apical edge of the cuticle and a much thicker band along the basal edge of the cuticle extended into the interior of the exocuticle (Figure 4B). In a few samples the

Figure 3: SEM of dorsal cuticle from control samples using back scattered electrons (BSE). Most samples from uninjected control crabs (A) and off-target dsRNA injected crabs (B) exhibited a homogeneous BSE signal intensity. However one sample from the uninjected control group (C) and three from the off-target control group (D) exhibited different signal intensities across the cuticle. Arrows indicate the apical edge of the cuticle and arrowheads indicate the basal edge of the cuticle.

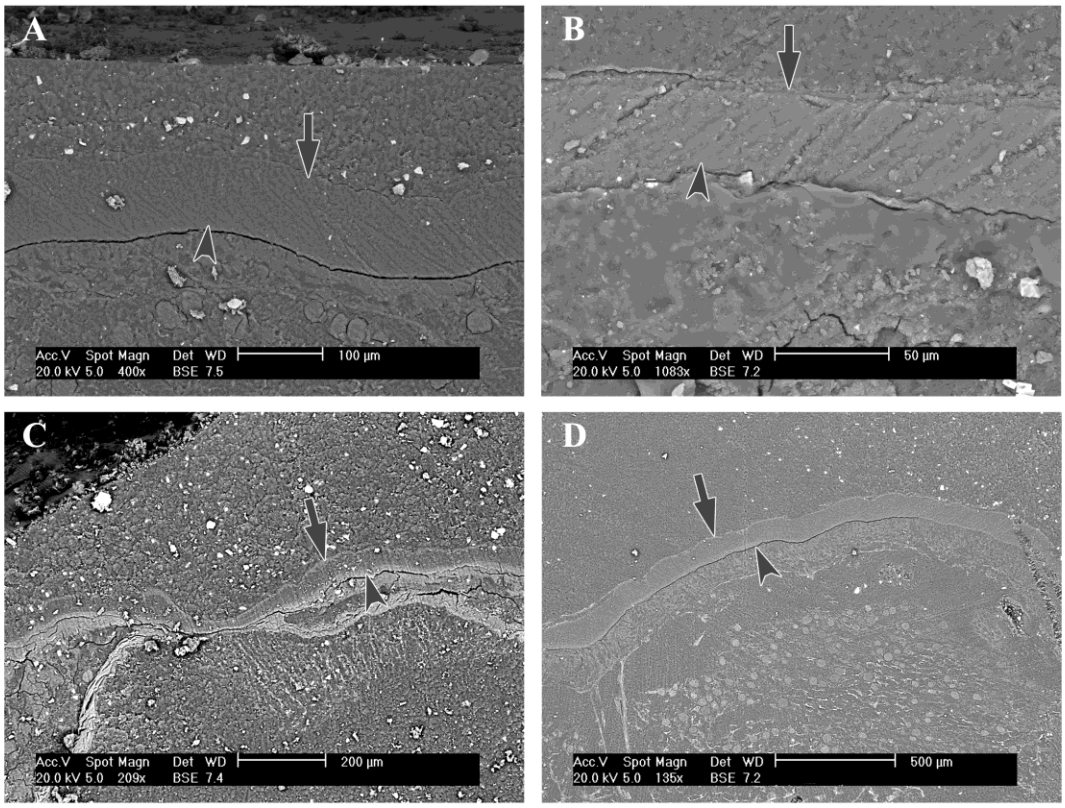
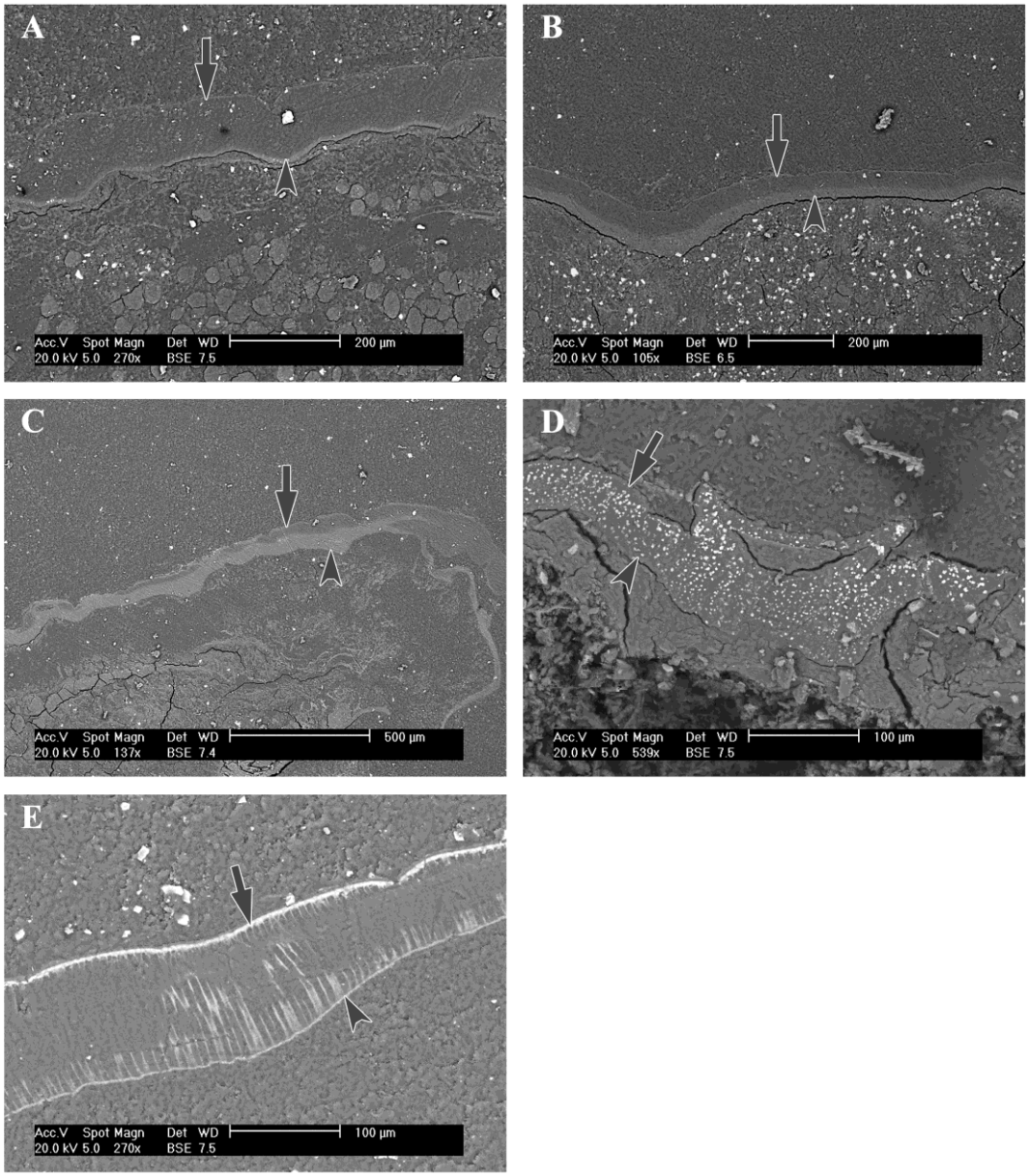

A
Acc.V Spot Magn Det WD 100 µm
20.0 kV 5.0 400x BSE 7.5
B
Acc.V Spot Magn Det WD 50 µm
20.0 kV 5.0 1083x BSE 7.2
C
Acc.V Spot Magn Det WD 200 µm
20.0 kV 5.0 209x BSE 7.4
D
Acc.V Spot Magn Det WD 500 µm
20.0 kV 5.0 135x BSE 7.2

Figure 4: SEM images of dorsal cuticle from *CsEarlyCP1*-targeted crabs using back-scattered electrons. Four different signal intensity contrast patterns were observed from crabs treated with CsEarlyCP1-targeting dsRNAs. The contrast patterns observed were thin lines of contrast along the apical and basal edges of the cuticle (A), a thin apical line of contrast and thicker basal bands of contrast (B), a thin apical line of contrast and a thick basal band of contrast that in regions extended completely through the interior of the cuticle and fused with the apical line of contrast (C), and punctate crystal-like structures that were present only in the dorsal cuticle of samples (D). One crab did survive to postmolt stages (E) and its cuticle exhibited the expected contrast patterns of calcification previously described for postmolt cuticle. Arrows indicate the apical edge of the cuticle and arrowheads indicate the basal edge of the cuticle.


A
Acc.V Spot Magn Det WD 200 µm
20.0 kV 5.0 270x BSE 7.5
B
Acc.V Spot Magn Det WD 200 µm
20.0 kV 5.0 105x BSE 6.5
C
Acc.V Spot Magn Det WD 500 µm
20.0 kV 5.0 137x BSE 7.4
D
Acc.V Spot Magn Det WD 100 µm
20.0 kV 5.0 539x BSE 7.5
E
Acc.V Spot Magn Det WD 100 µm
20.0 kV 5.0 270x BSE 7.5

lighter basal band extended through the entire cuticle in some regions and fused with the apical line of contrast, creating an appearance of complete fill-in (Figure 4C). Two of the three remaining samples from the *CsEarlyCP1*-targeted treatment displayed a pattern in which regions of the cuticle had punctate bright crystal-like structures (Figure 4D). These crystal-like structures were found only in the dorsal cuticle, eliminating the possibility that they were due to contamination or residual grains of sand paper. The last sample of the *CsEarlyCP1*-targeted group was sampled from a crab that had progressed through ecdysis to post molt the night that it was injected with the dsRNA treatment. Therefore, this sample exhibited the pattern of post-molt calcification described by Dillaman et al. (2005) (Fig 4E).

Four of the twelve samples from the EarlyCP Motif-targeted group had the contrast pattern seen in three samples from the *CsEarlyCP1* treatment group, namely, bright lines that were restricted to the apical and basal edges of the cuticle (Figure 5A). The remaining 8 samples from the treatment group had a homogenous lower signal intensity throughout the cuticle (Figure 5B).

Tissue treated with dsRNA targeting the *CsCP8.5* transcript exhibited the same three contrast patterns that were seen with the samples in the *CsEarlyCP1* treatment group. Of the twelve samples, three displayed the pattern consisting of a bright, thin apical line and a thick basal band that extended up into the interior of the cuticle (Figure 6A). Only one sample in this treatment group had thin bands of contrast along the apical and basal edges of the cuticle (Figure 6B), and 2 samples had the punctate crystal-like structures of higher atomic mass that were restricted to the cuticle (Figure 6C). One sample in particular (Figure 6C) had a high density of the crystal-like structures throughout the cuticle, but they appeared to be most abundant along

Figure 5: SEM images of dorsal cuticle from EarlyCP Motif-targeted crabs using back-scattered electrons. Samples within this treatment group exhibited either thin lines of contrast along the apical and basal edges of the cuticle (A) or homogenous BSE signal intensities lacking contrast (B). Arrows indicate the apical edge of the cuticle and arrowheads indicate the basal edge of the cuticle.

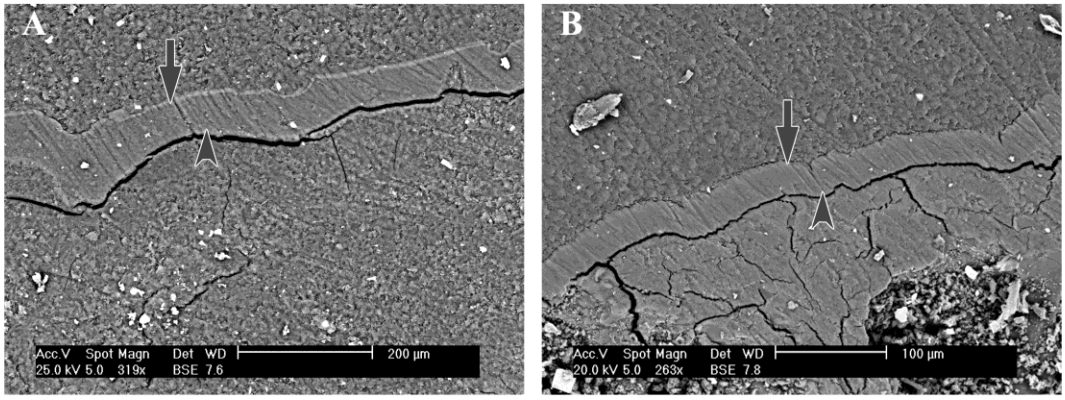
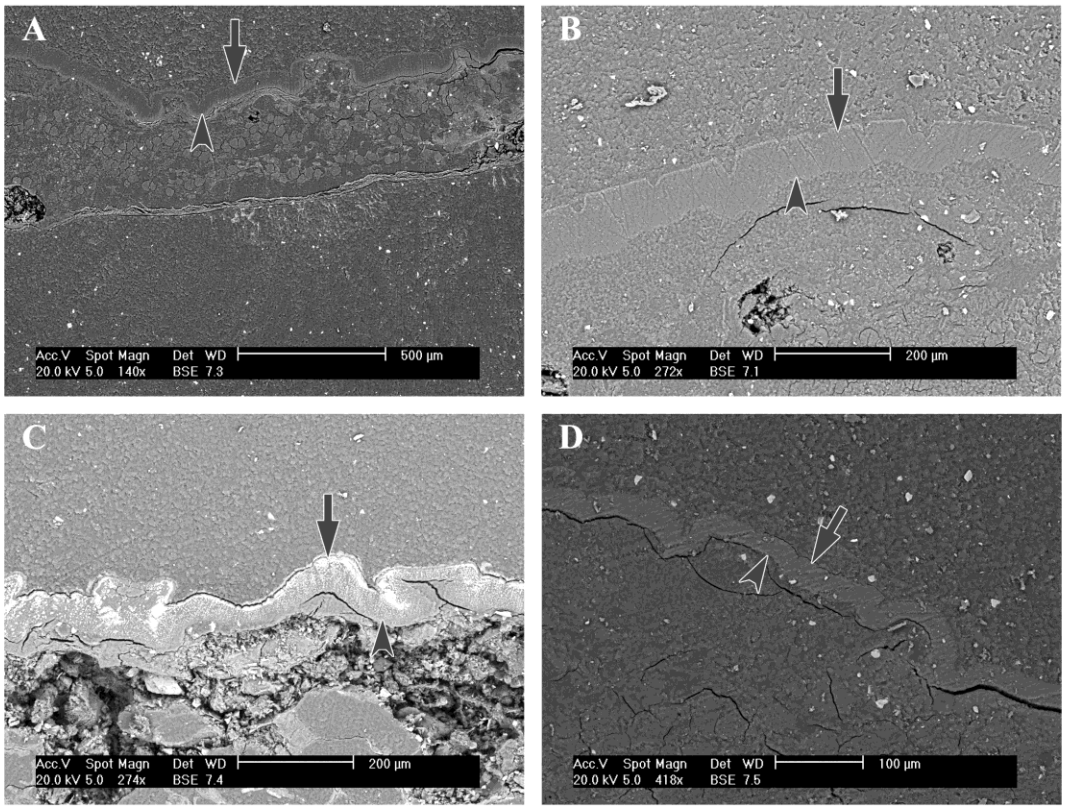

A
Acc.V Spot Magn Det WD 200 µm
25.0 kV 5.0 319x BSE 7.6
B
Acc.V Spot Magn Det WD 100 µm
20.0 kV 5.0 263x BSE 7.8

Figure 6: SEM images of dorsal cuticle from *CsCP8.5*-targeted crabs using back-scattered electrons. Half of the samples from this sample group exhibited patterns of contrast and three different contrast patterns were observed including: thin apical lines and thick basal bands of contrast (A), thin apical and thin basal lines of contrast (B) and punctate crystal-like structures that were present only in the dorsal cuticle of samples (C). Homogenous BSE signal intensities were observed in the other half of samples collected from this treatment group (D). Arrows indicate the apical edge of the cuticle and arrowheads indicate the basal edge of the cuticle.


A
Acc.V Spot Magn Det WD 500 µm
20.0 kV 5.0 140x BSE 7.3
B
Acc.V Spot Magn Det WD 200 µm
20.0 kV 5.0 272x BSE 7.1
C
Acc.V Spot Magn Det WD 200 µm
20.0 kV 5.0 274x BSE 7.4
D
Acc.V Spot Magn Det WD 100 µm
20.0 kV 5.0 418x BSE 7.5

the apical edge of the cuticle. The remaining 6 samples that belonged to the *CsCP8.5* dsRNA treated group exhibited a homogenous composition of the cuticle (Figure 6D).

In order to determine the elemental composition of cuticle samples and identify which higher atomic weight elements were present in samples that had patterns of contrast in the backscattered electron analysis, energy dispersive x-ray analysis (EDAX) was used. Three samples for each treatment group and control groups were analyzed using elemental mapping of carbon, oxygen, chlorine, magnesium, sodium and calcium. In both uninjected controls and off-target dsRNA-injected controls, samples that did not exhibit a contrast pattern had elemental mapping patterns that were also homogenous (Figure 7 A1-A4). However, in the few samples from the control groups that had a contrast pattern, the brighter regions in BSE were composed primarily of sodium and chloride with some calcium present within the pattern (Figure 7 B1-4). Likewise, in samples for the *CsEarlyCP1*, EarlyCP Motif and *CsCP8.5* treatments that possessed BSE contrast patterns had a differential distribution of sodium, chloride and low levels of calcium within the regions of brighter contrast (Figures 7 C1-4 and Figure 8 A1-4, B1-4). In RNAi treatment samples that lacked the contrast patterns, like the controls also lacked a differential distribution of sodium, chloride and calcium (Figure 8 C1-4).

Fluorescence Immunohistochemistry

Following BSE imaging and EDAX, three samples from all treatment and control groups were immunolabeled with antibodies complementary to the EarlyCP Motif and CsCP8.5. Since qPCR was inconsistent within each sample group but mortality data and BSE imaging suggested phenotypic effects due to injection treatments, samples were labeled with antibodies to determine if transcripts were prevented from being translated into proteins, secreted and

Figure 7: Analysis of the elemental composition of dorsal cuticle samples from an off-target dsRNA injected control (column A), an uninjected control (column B) and a *CsEarlyCP1*-targeted crab (column C). Samples were imaged with back-scattered electrons (row 1), x-ray mapping of sodium (row 2), x-ray mapping of chloride (row 3) and x-ray mapping of calcium (row 4).

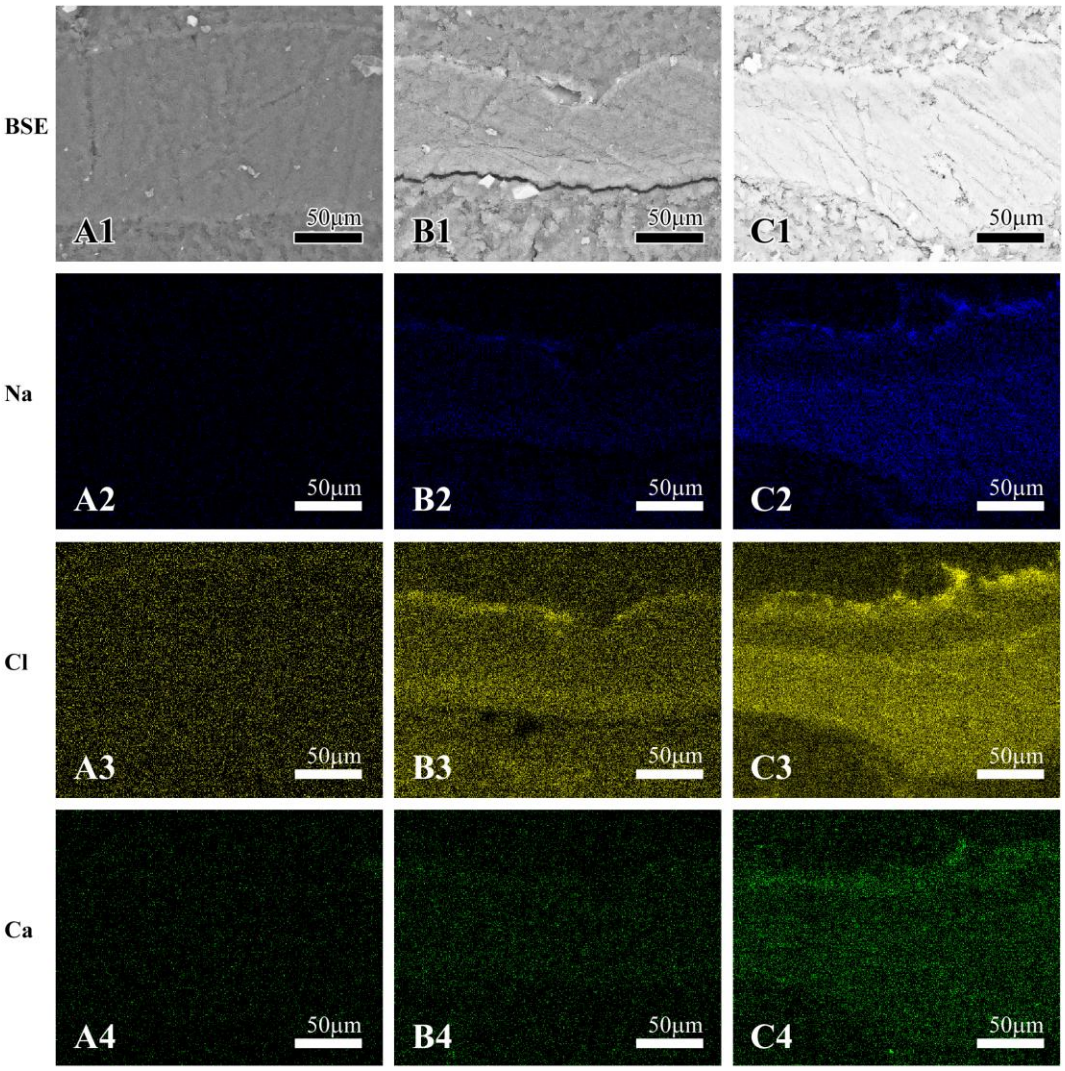
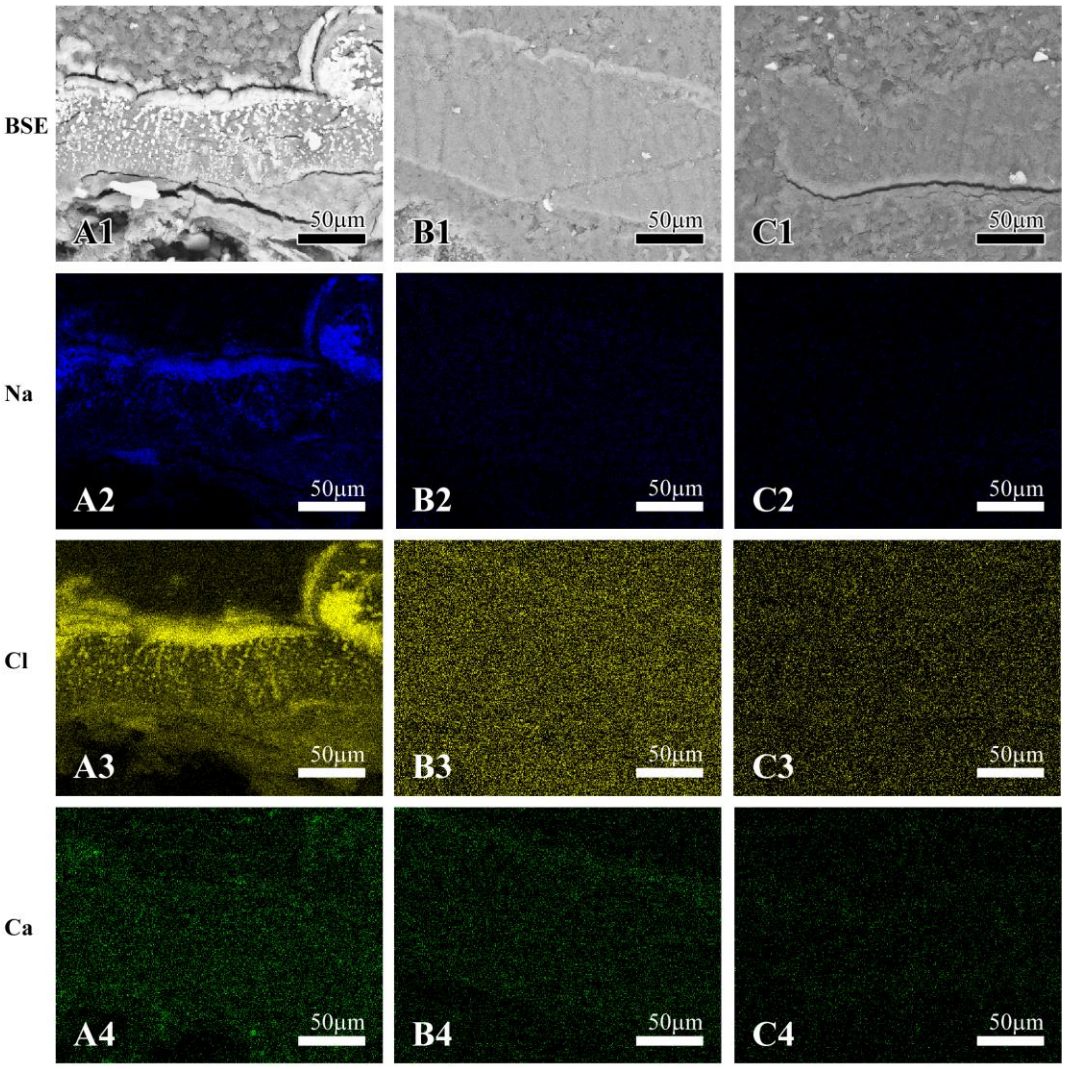

BSE
Na
Cl
Ca
A1
B1
C1
A2
B2
C2
A3
B3
C3
A4
B4
C4
50μm

Figure 8: Analysis of the elemental composition of dorsal cuticle samples from *CsCP8.5*-targeted crabs (column A and B) and an EarlyCP Motif-targeted crab (column C). Samples were imaged with back-scattered electrons (row 1), x-ray mapping of sodium (row 2), x-ray mapping of chloride (row 3) and x-ray mapping of calcium (row 4).


BSE
Na
Cl
Ca
A1
B1
C1
A2
B2
C2
A3
B3
C3
A4
B4
C4
50μm

incorporated into the pre-exuvial cuticle. Samples were labeled with both antibodies regardless of the RNAi treatment to see if injection of dsRNA targeting EarlyCP Motif had an effect on the expression of *CsCP8.5* or vice versa.

For all sample sections from treatment and control groups that were immunolabeled using the CsCP8.5 antibody, fluorescence was observed in cuticle that lined the branchial chamber (Figure 9A). However, little to no fluorescence was observed in dorsal cuticle for all treatment and control samples (Figure 9B-F). Therefore, it could not be determined if the levels of the CSCP8.5 protein in the dorsal cuticle had been reduced due to injections of dsRNA targeting *CsCP8.5*, *CsCPEarly1* or the EarlyCP Motif.

Unlike the CsCP8.5 antibody, the antibody synthesized to bind to all transcripts possessing the EarlyCP Motif labeled both branchial cuticle and dorsal cuticle. Uninjected control samples had a bright fluorescence that was uniform in intensity throughout the entire dorsal cuticle (Figure 10A). Off-target dsRNA-injected samples also had high levels of fluorescence, but fluorescence intensity was sometimes not uniform throughout the dorsal cuticle. In these cases, there was greater fluorescence intensity along the apical edges of the dorsal cuticle and in some regions along the basal edges of the cuticle as well (Figure 10B). Samples from crabs injected with dsRNAs targeting specifically the *CsEarlyCP1* transcript had slightly lower levels of fluorescence relative to controls in samples from crabs that died in the molt (Figure 10C) and much lower levels of fluorescence intensity relative to controls in samples from crabs that survived until a few days after injection treatments but died prior to ecdysis (Figure 10D). The consistent pattern observed in samples from *CsEarlyCP1*-targeted crabs was greater fluorescence intensity along the apical edge of the cuticle and in some regions along the basal edge. The EarlyCP Motif dsRNA injected group exhibited a much greater decrease in

Figure 9: Dorsal branchial integument samples immunolabeled with CP8.5 antibody. Branchial cuticle from an uninjected control (A), dorsal cuticle of an uninjected control (B), an off-target dsRNA injected control (C), a CsEarlyCP1-targeted crab (D), an EarlyCP Motif-targeted crab (E) and a CsCP8.5-targeted crab(F). The branchial cuticle is indicated with an asterisk. Arrows indicate the apical edge of the cuticle and arrowheads indicate the basal edge of the cuticle.

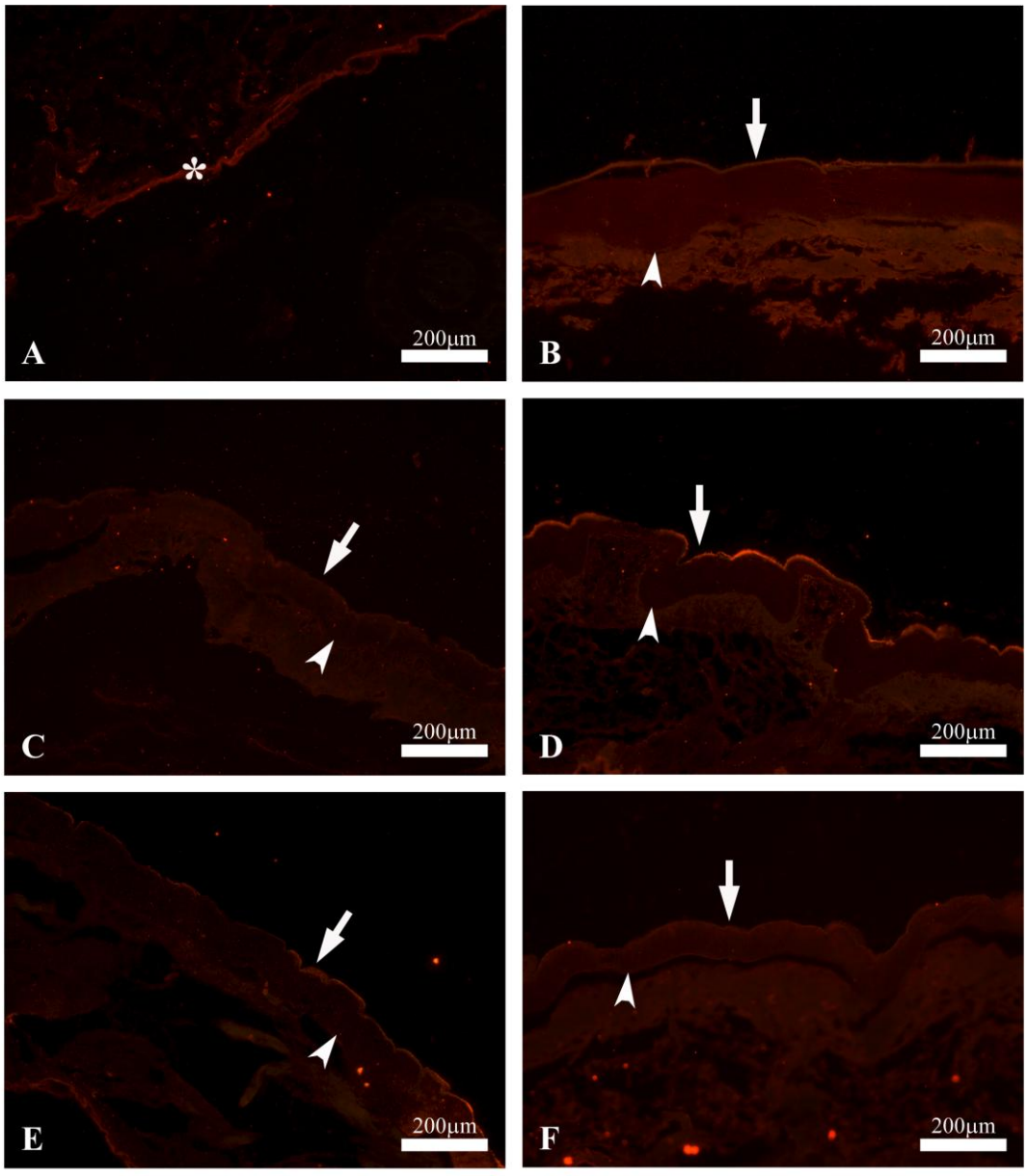
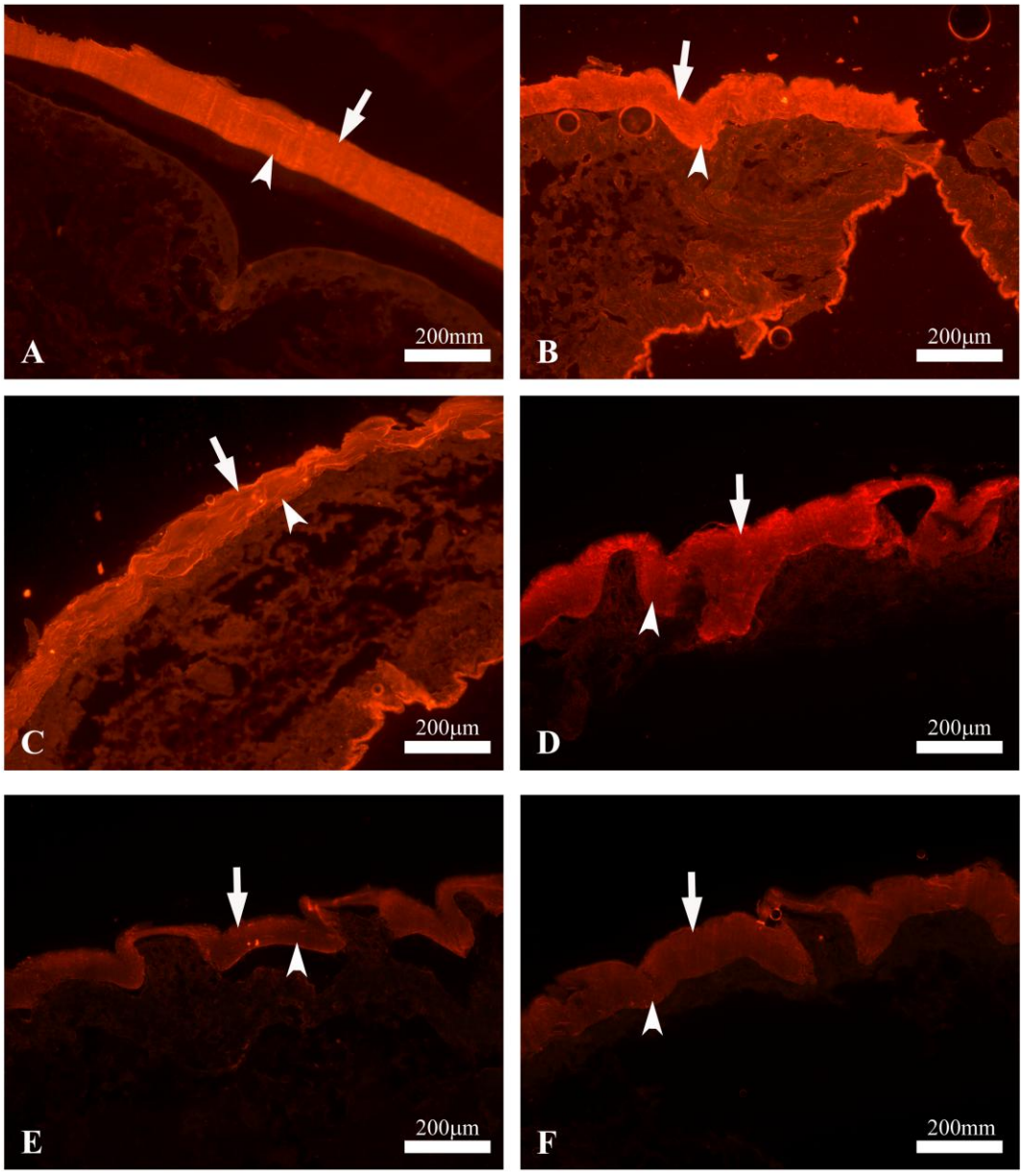

A
200μm
B
200μm
C
200μm
D
200μm
E
200μm
F
200μm

Figure 10: Dorsal branchial integument samples immunolabeled with EarlyCP Motif antibody. Dorsal cuticle from an uninjected control (A), an off-target dsRNA injected control (B), a CsEarlyCP1-targeted crab that died within 2 days of treatment in ecdysis (C), a CsEarlyCP1-targeted crab that died 5 days after treatment and prior to ecdysis (D), an EarlyCP Motif-targeted crab (E) and a CsCP8.5-targeted crab (F). Arrows indicate the apical edge of the cuticle and arrowheads indicate the basal edge of the cuticle.


A
200mm
B
200μm
C
200μm
D
200μm
E
200μm
F
200mm

fluorescence intensity relative to the controls (Figure 10E) than did the *EarlyCP1*-targeted group. Crabs in the *EarlyCP1* treatment group received dsRNA that targeted only one of member of the *CsEarlyCP* family, but the EarlyCP Motif antibody is able to bind to the other members of the *CsEarlyCP* family. However, in the EarlyCP Motif treatment, the dsRNA was complementary to the conserved motif, and thus targeted the whole family of transcripts, resulting in a greater decrease in fluorescence intensity. Figure 10E shows a sample that was treated with dsRNAs targeting the EarlyCP Motif and labeled with the EarlyCP Motif antibody. As can be seen in the image, the fluorescence intensity of samples within the EarlyCP Motif group is much lower than that of controls and fluorescence is primarily restricted to the apical and basal edges of the cuticle with minimal fluorescence in the interior regions of the cuticle.

Samples that were treated with dsRNAs targeting CsCP8.5 and labeled with EarlyCP Motif antibodies also displayed a great decrease in fluorescence relative to the control samples (Figure 10F). The *CsCP8.5*-targeted samples had about an equal decrease in fluorescence relative to controls as the samples that were treated with dsRNAs targeting the entire EarlyCP Motif family, suggesting an interaction between these cuticular proteins. The most intense levels of fluorescence that were seen in *CsCP8.5* dsRNA treated samples were restricted to the apical edge of the cuticle, while much lower levels of fluorescence were observed at the basal edges and interior regions of the cuticle (Figure 10F).

DISCUSSION

This study investigated the effects of injecting dsRNAs that were synthesized to target transcripts coding for cuticular proteins on the pre-exuvial cuticle of blue crabs. One of the dsRNA treatments targeted the *CsEarlyCP1* transcript while a second dsRNA treatment targeted the EarlyCP Motif, which is a 14 amino acid sequence found in all the transcripts that are

members of the *CsEarlyCP* family, including *CsEarlyCP1*. The seven transcripts of the *CsEarlyCP* family are transcribed in hypodermis that produces calcifying cuticle during premolt and expression of the transcripts peaks during the early D3 stage (Shafer et al., 2009). However, this family of transcripts is unique because while it is found in calcifying cuticle, these proteins posses a RR-1 consensus, which is primarily found in the flexible, non-calcifying cuticle of insects and crustaceans. To date, the only other protein that has been found in calcifying cuticle and possesses a RR-1 consensus is CASP-2, which was extracted from the crayfish, *P. clarkii.* Characterization and *in vitro* assays of CASP-2 have lead to the hypothesis that CASP-2 may regulate or inhibit calcification of the exoskeleton (Inoue et al., 2008). Although *in vitro* assays have not been conducted for any of the *CsEarlyCP* transcripts, the time of expression, possession of a RR-1 consensus and high sequence similarities to CASP-2 including the 14 amino acid residues constituting the EarlyCP Motif, have lead to the hypothesis that these transcripts may act as inhibitors of pre-mature calcification in the blue crab cuticle. A cuticular protein model previously proposed by Coblentz et al. (1998) suggested that in the blue crab cuticle some proteins bound to the chitin fibers of the organic matrix may serve as sites for nucleation of calcium carbonate during post-ecdysial stages. This model also proposes that prior to ecdysis, a shielding protein is present that interacts with the nucleating proteins and blocks the nucleation sites on the nucleating protein from binding calcium carbonate and therefore acts as an inhibitor of pre-mature calcification. During ecdysis, some alteration of the shielding protein occurs that allows sites on the nucleating protein to be exposed and subsequently permits calcification of the cuticle (Coblentz et al., 1998). Following this model, it is hypothesized that the seven transcripts of the *CsEarlyCP* family are shielding proteins that interact with nucleating proteins in the cuticle to prevent pre-mature calcification.

In this study, it was found that crabs treated with EarlyCP Motif-targeting dsRNA experienced high mortality during the first 24 hours after treatment and that the surviving 33% died within four days post-treatment. No crabs within this treatment group survived to ecdysis or postmolt stages. However, when crabs were treated with *EarlyCP1*-targeting dsRNA, which targeted only a single transcript instead of all seven *CsEarlyCP* transcripts, no mortality occurred during the first 24 hours, but 50% of the crabs died during days one to four, 25 % died more than four days after treatment and a few survived to ecdysis and post-molt stages. This is in contrast to the control treatments, where most crabs survived until ecdysis when death was presumed to be due to handling and leg autotomy rather than RNAi. The early mortality that was seen in EarlyCP Motif treated crabs but not *CsEarlyCP1* treated crabs suggests that targeting and suppressing expression of the entire family of EarlyCP Motif proteins is lethal to crabs and that this family of proteins is critical during the molt cycle. However, when only one transcript of the family is targeted and suppressed mortality did not occur as suddenly, but the treatment still lead to death within a few days. Since 75% of the crabs in the *CsEarlyCP1* treatment died prior to reaching ecdysis, the dsRNA injections were still negatively affecting crabs suggesting that suppressing even one transcript may be enough to disrupt proper cuticle formation.

qPCR was conducted in order to measure the levels of target transcript change in post-treatment samples relative to the pre-treatment levels of target transcript from the same crab to determine if injection of dsRNAs resulted in mRNA knockdown. Leg samples from three of the crabs in the EarlyCP Motif- targeting treatment that survived beyond the first 24 hours were used for qPCR analysis since they were the few individuals that survived long enough to get a second leg sample. In these few surviving individuals the level of *CsEarlyCP1* mRNA in post-treatment legs had greatly decreased compared to the pre-treatment levels. Previous studies investigating

the expression patterns of the transcripts in the *CsEarlyCP* family have demonstrated that transcript levels begin to naturally decrease during the D3 stage (Faircloth and Shafer, 2007; Shafer et al., 2009), and this decrease was observed in control samples that were un-injected and injected with off-target dsRNA. However, the decrease in the *CsEarlyCP1* transcript that was observed in the EarlyCP Motif-targeted samples was much greater than the decrease observed in all the control samples. The decrease of *CsEarlyCP1* mRNA observed in surviving crab samples and the very early mortality of crabs within this treatment suggests that the dsRNA injections were very effective in silencing the family of *CsEarlyCP* transcripts, which ultimately lead to the death of all crabs within four days. qPCR data collected from the leg samples of crabs in the *CsEarlyCP1*-targeted group demonstrated an increase of target transcript levels in post-treatment legs relative to the mRNA levels in pre-treatment legs. However, since 50% of the crabs in this treatment died within one to four days of treatment, the dsRNA injections were still affecting individuals within this treatment group, despite the increase in target transcript levels after treatment. Two gene silencing pathways have been proposed for RNAi. In one, a complementary RNA strand binds to the target mRNA and results in cleavage of the transcript. However in the second proposed pathway, when dsRNAs are introduced, the complementary strand binds to the target mRNA and blocks its translation without cleaving it (Hannon, 2002; Agrawal et al., 2003). Therefore, it is hypothesized that crabs treated with CsEarlyCP1-targeted dsRNAs experienced an increase in target transcript after treatment because instead of being cleaved, the transcript was blocked from being translated and that having the dsRNA anti-sense strand bound to the target mRNA also prevented the transcript from being naturally degraded by the cell, thus leading to an accumulation of the transcript within 24 hours of treatment.

Since qPCR data indicated a decrease of target transcript in EarlyCP Motif-targeted crabs but an increase in *CsEarlyCP1*-targeted crabs, immunohistochemistry was conducted to determine if the levels of CsEarlyCP proteins incorporated into the cuticle were being affected by dsRNA injections. Antibodies were synthesized complementary to the EarlyCP Motif so that the localization and presence of all the CsEarlyCP proteins in the cuticle could be investigated. Sections of whole dorsal branchial integument from off-target and uninjected control crabs that were immuolabeled with EarlyCP Motif antibody exhibited a bright fluorescence that was uniform in intensity throughout the entire dorsal cuticle (Figure 10A). However, samples treated with EarlyCP-Motif-targeting dsRNA exhibited a great decrease in fluorescence intensity (Figure 10) relative to the controls. The bright band of fluorescence at the apical edge of the cuticle suggests that prior to dsRNA injection the CsEarlyCP proteins were being translated and incorporated into the pre-exuvial cuticle. However, where the fluorescence decreases in the interior regions and at the basal edge of the cuticle indicates where the proteins were inhibited from being translated and incorporated. The drastic decrease in fluorescence for EarlyCP Motif–targeted samples supports the qPCR data, which indicated a decrease in the target transcript. Whereas the qPCR primer set detected only *CsEarlyCP1*, the antibody staining suggesting that protein expression of the entire family of *CsEarlyCP* genes was affected. Samples from crabs treated with *CsEarlyCP1*-targeting dsRNAs that were immunolabeled with the EarlyCP Motif antibody exhibited only a slight decrease in fluorescence in crabs that died shortly after treatment and a slightly greater decrease in crabs that survived longer after treatment relative to the fluorescence of control samples. These data support the hypothesis that despite an increase in target transcript levels indicated by qPCR, the amount of CsEarlyCP1 protein being incorporated into the pre-exuvial cuticle decreased relative to the controls and that translation was being

suppressed. The decrease observed in *CsEarlyCP1*-targeted samples was not as great as the decrease observed in EarlyCP Motif-targeted samples, presumably due to the fact that the EarlyCP1 dsRNA treatment targeted only the *CsEarlyCP1* transcript but the EarlyCP Motif antibody was synthesized complementary to all the proteins in the family. Seven members of the *CsEarlyCP* family have been sequenced so far, but it is possible that as many as six to seven other transcripts exist that could be detected by the EarlyCP1 Motif (unpublished data).

In order to visualize the physical effects of silencing the *CsEarlyCP* transcripts on the pre-exuvial cuticle, samples were imaged using back-scattered electrons. Whole dorsal branchial integument samples that were obtained from crabs treated with EarlyCP Motif-targeting dsRNAs were mostly similar to the controls, which exhibited a homogenous low backscattered electron signal intensity in all regions of the cuticle. However, four of twelve samples possessed regions of contrast that were restricted to the apical and basal edges of the cuticle, which indicated the accumulation of elements heavier than carbon and oxygen in the pre-exuvial cuticle. All twelve samples from the *CsEarlyCP1*-targeted treatment group exhibited regions of contrast within the pre-exuvial cuticle indicating that all twelve samples accumulated elements of higher atomic numbers. Since the samples obtained from crabs surviving for longer periods of time after treatment injections of *CsEarlyCP1* or EarlyCP Motif had more exaggerated patterns of contrast within the cuticle, it is hypothesized that the samples that lacked contrast patterns in the EarlyCP Motif group died too quickly after treatment for the accumulation of heavier elements to occur. Targeting only a single transcript, as was done in the *CsEarlyCP1*-targeted group, allowed the phenotypic effects to be displayed prior to death of the crab, which occurred within a few days. In order to identify the ions that were being deposited into the pre-exuvial cuticle following targeted dsRNA injections, EDAX was conducted on three samples from each treatment. It was

supposed that the patterns were caused by calcium deposition since the *CsEarlyCP* transcripts targeted by RNAi were believed to be inhibitors of calcification, and thus silencing them would cause premature calcification. However, the EDAX analysis revealed that the regions of bright contrast were primarily comprised of sodium and chloride ions, with a small amount of calcium present as well. This was true for all samples that exhibited contrast in the *CsEarlyCP1*-targeted and EarlyCP Motif-targeted treatment groups and the few samples from control groups that exhibited regions of contrast. However, when the pattern of contrast was not present in BSE images, there was no differential composition of sodium, chloride or calcium seen in the elemental mapping. It is not clear why sodium and chloride are deposited in the cuticle in a differential pattern prior to molting, even in the samples that received off-target dsRNA injections. However, since no previous research has been conducted on what happens in the cuticle when proteins are excluded, it is possible that altering the protein composition of the cuticle may affect the ion affinity of the remaining proteins and thus result in sodium and chloride binding being favored over calcium. It is clear though that further research needs to be conducted in order to understand what mechanisms allowed and controlled the accumulation of sodium and chloride in the cuticle in a distinct pattern when crabs were treated with dsRNAs.

The third dsRNA treatment used in this study targeted the *CsCP8.5* transcript. This mRNA has been shown to be expressed only during premolt stages and only in hypodermis that produces calcifying cuticle (Wynn and Shafer, 2005). The *CsCP8.5* sequence shows significant similarity to the CAP-1and CAP-2 proteins extracted from *P. clarkii* (Inoue et al., 2001; Inoue et al., 2003). CAP-1 and CAP-2 are thought to be involved in the nucleation or regulation of $CaCO_3$ in the cuticle based on *in vitro* assays using natural and recombinant proteins (Inoue et al., 2001; Inoue et al., 2003; Inoue et al., 2004). Like the CAP proteins, *CsCP8.5* has a truncated

RR consensus and therefore cannot be classified as a RR-1, RR-2 or RR-3. However, based on its sequence similarities to the CAP proteins and the fact that this transcript is expressed only in hypodermis producing calcifying cuticle, it is hypothesized that CsCP8.5 may be a nucleator of calcification after ecdysis. It could be one of the nucleating proteins that is deposited into the cuticle during premolt and is prevented from binding calcium ions by a shielding protein until after ecdysis as proposed by Coblentz et al., (1998).

The highest mortality for the crabs treated with *CsCP8.5*-targeting dsRNAs occurred within the first 24 hours post-treatment and between days one and four post-treatment. The high mortality seen after injection suggests that *CsCP8.5* may be critical in cuticle formation and thus survival of the premolt crab. qPCR data from *CsCP8.5*-targeted crabs surviving the first 24 hours indicated an increase in *CsCP8.5* mRNA levels in post-treatment legs relative to the mRNA levels in pre-treatment legs. However, samples from off-target and uninjected control crabs that were analyzed for *CsCP8.5* expression on average exhibited a decrease in target transcript levels as was expected since *CsCP8.5* mRNA levels naturally begin to decrease during the D3 premolt stage. Therefore, as suggested above for crabs treated with *CsEarlyCP1*-targeting dsRNAs, it may be that crabs targeted for only this single transcript have the blocked from being translated but not cleaved. Possibly, the complementary strand bound to the target mRNA prevents the transcript from being naturally degraded in the tissue, thus leading to an accumulation within 24 hours of treatment.

In order to determine if injection of *CsCP8.5*-targeting dsRNAs caused a decrease in translation and protein incorporation into the pre-exuvial cuticle, dorsal branchial integument samples from *CsCP8.5*-targeted crabs and samples from both control groups were immunolabeled with an antibody that was synthesized complementary to a region of CsCP8.5.

However, the CsCP8.5 antibody did not label any region of the dorsal cuticle in either control or treatment samples and no conclusions could be made as to whether *CsCP8.5* was silenced and prevented from being incorporated into the cuticle.

Despite a lack of evidence showing knockdown in crabs treated with dsRNAs targeting *CsCP8.5*, the high mortality observed within the first 24 hours and between days one and four post-treatment suggest that *CsCP8.5*-targeting dsRNAs were affecting the crabs. Therefore, whole dorsal branchial integument samples were also analyzed using back-scattered electrons to visualize any physical effects of injections on the pre-exuvial cuticle. BSE imaging of samples from *CsCP8.5*-targeted crabs revealed that six out of twelve samples exhibited the same patterns of contrast observed in samples from crabs treated with *CsEarlyCP1*-targeting dsRNAs. The remaining six samples from the *CsCP8.5* treatment group exhibited no patterns of contrast within the cuticle and were mostly sampled from crabs that died within the first 24 hours after injection. It is hypothesized that, like EarlyCP Motif-targeted samples that did not exhibit a pattern, *CsCP8.5*-targeted samples that did not exhibit regions of contrast died too quickly after treatment for the accumulation of higher atomic elements to occur. EDAX analysis conducted on samples from *CsCP8.5*-targeted crabs, again showed that when the contrast patterns are present, they are composed primarily of sodium and chloride, with small amounts of calcium present and that when the contrast patterns were very light or absent, a homogenous distribution of ions throughout the sample was observed.

Since the patterns of contrast observed in the *CsCP8.5*-targeted samples were highly similar to those observed in *CsEarlyCP1* and EarlyCP Motif-targeted samples, it was hypothesized that silencing *CsCP8.5* may have affected the ability of *CsEarlyCP* transcripts to be incorporated and bound to the cuticle. Such a possibility is consistent with the model of

Coblentz et al. (1998). In order to test this hypothesis, whole dorsal branchial samples from crabs that were treated with *CsCP8.5*-targeting dsRNAs were immunolabeled with the EarlyCP Motif antibody and compared to control samples. It was observed that the level of fluorescence was greatly decreased in samples from crabs treated with *CsCP8.5*-targeting dsRNAs relative to the level of fluorescence observed in control samples (Figure 10F). The decrease of fluorescence in *CsCP8.5*-targeted samples was as great as the decrease observed in samples from crabs injected with EarlyCP Motif-targeting dsRNA. These results support the hypothesis, that targeting and suppressing *CsCP8.5* prevented CsEarlyCP proteins from being incorporated into the pre-exuvial cuticle after *CsCP8.5*-targeting injections were administered. Therefore the similar pattern of contrast observed in BSE images for *CsEarlyCP1*, CsEarlyCP Motif and *CsCP8.5*-targeted treatments coupled with the immunofluorescence data that demonstrates a decrease in CsEarlyCP proteins in the cuticle when *CsCP8.5* is suppressed suggests that these proteins have a structural interaction in the cuticle. Based on the results collected in this study, I propose that CsCP8.5 is the nucleating protein and that the CsEarlyCP proteins are the shielding proteins in the model described by Coblentz et al. (1998) for the blue crab cuticle. In addition, I hypothesize that CsEarlyCP proteins are dependent upon the presence of CsCP8.5 in the cuticle in order to be incorporated into the cuticle.

Previous studies have been conducted that utilize RNAi to investigate the functional roles of proteins hypothesized to be involved in the process of biomineralization. The protein GAP65 from the gastrolith of the crayfish *Cherax quadricarinatus* was silenced through targeted dsRNA injections and resulted in structural and morphological deformities of the chitinous-amorphous calcium carbonate (ACC) extracellular matrix (Shechter et al., 2008). The structure of ACC spherules in the gastrolith was altered and their packing was less dense. Another study

investigated Pif, a hypothesized biomineralization protein in the pearl oyster, *Pinctada fucata*. The results strongly suggested that Pif is involved in regulating calcification and nacre formation because RNAi silencing of the protein caused altered calcium carbonate crystal structure *in vivo* and lead to deformities within the nacreous layers (Suzuki et al., 2009). However, while other studies have looked at biomineralization *in vivo* using RNAi, this was the first study conducted using RNAi that investigated multiple cuticular proteins hypothesized to be involved in the inhibition and nucleation of calcification of the crustacean cuticle.

The results from this study suggest that CsCP8.5 and the family of CsEarlyCP proteins may have a structural interaction similar to the model proposed by Coblentz et al (1998) where CsCP8.5 may potentially be a nucleating protein and CsEarlyCP proteins may act as shielding proteins to prevent cuticle calcification during premolt stages. The use of dsRNA in this study has demonstrated that difficulties may exist when attempting to silence a single protein in a structure comprised of an array of proteins potentially involved in interactions with one another. Further research on the interactions between CP8.5 and the CsEarlyCP family is required to understand the structural and functional roles of each protein in the cuticle. This study also demonstrated that when crabs were injected with dsRNAs targeting cuticular protein transcripts, sodium and chloride ions were deposited into the pre-exuvial cuticle in a differential pattern that could not be explained. This deposition of sodium and chloride in the pre-exuvial cuticle warrants further investigation to determine if the deposition of these ions is a direct or an indirect effect of a decrease of CP8.5 and CsEarlyCP proteins in the cuticle.

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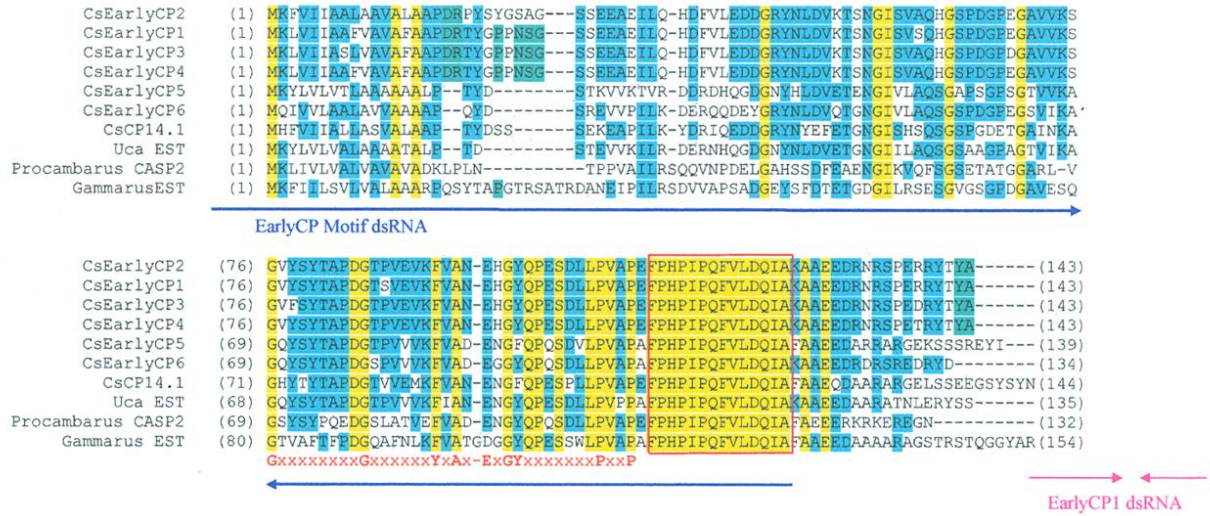
APPENDIX

Appendix. Amino acid sequence alignment of the CsEarlyCP family from *Callinectes sapidus* with similar cuticular protein sequences from other crustaceans. Amino acids highlighted in yellow are shared by all transcripts in the alignment. Amino acids highlighted in blue are shared by most of the transcripts and amino acids highlighted in green are shared by half of the transcripts. The RR consensus is in red text below the alignment and the EarlyCP Motif is indicated by the red box. The blue arrow below the sequence alignment indicates the region of the transcript that the EarlyCP Motif dsRNA amplicon was synthesized complementary to. The magenta arrows indicate the region of *CsEarlyCP1* that was used as a template in the synthesis of the *CsEarlyCP1*-targeting dsRNA amplicon.


CsEarlyCP2 (1) MKFVIIAALAAVALAAPDRPYSYGSAG---SSEEAEILQ-HDFVLEDDGRYNLDVKTSNGISVAQHGSPDGPEGAVVKS
CsEarlyCP1 (1) MKLVIIAAFVAVAFAAPDRTYGPPNSG---SSEEAEILQ-HDFVLEDDGRYNLDVKTSNGISVSQHGSPDGPEGAVVKS
CsEarlyCP3 (1) MKLVIIASLVAVAFAAPDRTYGPPNSG---SSEEAEILQ-HDFVLEDDGRYNLDVKTSNGISVAQHGSPDGPDGAVVKS
CsEarlyCP4 (1) MKLVIIAAFVAVAFAAPDRTYGPPNSG---SSEEAEILQ-HDFVLEDDGRYNLDVKTSNGISVAQHGSPDGPEGAVVKS
CsEarlyCP5 (1) MKYLVLVTLAAAAAALP--TYD--------STKVVKTVR-DDRDHQGDGNYHLDVETENGIVLAQSGAPSGPSGTVVKA
CsEarlyCP6 (1) MQIVVLAALAVVAAAAP--QYD--------SREVVPILK-DERQQDEYGRYNLDVQTGNGIVLAQSGSPDGPEGSVIKA
CsCP14.1 (1) MHFVIIALLASVALAAP--TYDSS------SEKEAPILK-YDRIQEDDGRYNYEFETGNGISHSQSGSPGDETGAINKA
Uca EST (1) MKYLVLVALAAAATALP--TD---------STEVVKILR-DERNHQGDGNYNLDVETGNGIILAQSGSAAGPAGTVIKA
Procambarus CASP2 (1) MKLIVLVALVAVAVADKLPLN----------TPPVAILRSQQVNPDELGAHSSDFEAENGIKVQFSGSETATGGARL-V
GammarusEST (1) MKFILSVLVALAAARPQSYTAPGTRSATRDANEIPILRSDVVAPSADGEYSFDTETGDGILRSESGVGSGPDGAVESQ
EarlyCP Motif dsRNA
CsEarlyCP2 (76) GVYSYTAPDGTPVEVKFVAN-EHGYQPESDLLPVAPEFPHPIPQFVLDQIAKAAEEDRNRSPERRYTYA------(143)
CsEarlyCP1 (76) GVYSYTAPDGTSVEVKFVAN-EHGYQPESDLLPVAPEFPHPIPQFVLDQIAKAAEEDRNRSPERRYTYA------(143)
CsEarlyCP3 (76) GVFSYTAPDGTPVEVKFVAN-EHGYQPESDLLPVAPEFPHPIPQFVLDQIAKAAEEDRNRSPEDRYTYA------(143)
CsEarlyCP4 (76) GVYSYTAPDGTPVEVKFVAN-EHGYQPESDLLPVAPEFPHPIPQFVLDQIAKAAEEDRNRSPETRYTYA------(143)
CsEarlyCP5 (69) GQYSYTAPDGTPVVVKFVAD-ENGFQPQSDVLPVAPAFPHPIPQFVLDQIAFAAEEDARRAEGEKSSSREYI---(139)
CsEarlyCP6 (69) GQYSYTAPDGSPVVVKFVAD-EGGYQPQSDLLPVAPAFPHPIPQFVLDQIAKAAEEDRDRSREDRYD--------(134)
CsCP14.1 (71) GHMTYTAPDGTVVEMKFVAN-ENGFQPESPLLPVAPEFPHPIPQFVLDQIAFAAEQDAARAEGELSSEEGSYSYN(144)
Uca EST (68) GQYSYTAPDGTPVVVKFIAN-ENGYQPESDLLPVPAFPHPIPQFVLDQIAKAAEEDAARATNLERYSS------(135)
Procambarus CASP2 (69) GSYSYPQEDGSLATVEFVAD-ENGYQPQSDLLPVAPEFPHPIPQFVLDQIAFAEEERKRKEREGN----------(132)
Gammarus EST (80) GTVAFTFPDGQAFNLKFVATGDGGYQPESSWLPVAPAFPHPIPQFVLDQIAFAAEEDAAAARAGSTRSTQGGYAR(154)
GxxxxxxxxGxxxxxxYxAx-ExGYxxxxxxxPxxP
EarlyCP1 dsRNA