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Effects Of Chemical Dispersant (Corexit® Ec9500A) On Gill Structure And Function, And The Associated Crustacean Hyperglycemic Hormone Mediated Stress Response In The Blue Crab (Callinectus Sapidus)

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BIOLOGY

ABSTRACT

Chemical dispersants are fast becoming the most widely used tool in the remediation of spilled oil. The sheer magnitude of the Deepwater Horizon oil spill necessitated the use of multiple forms of remediation at scales never attempted. As a result, there is a critical need to understand how the spill and ensuing cleanup efforts impacted the surrounding ecosystem and its inhabitants. The goal of this research is to better understand the impact of the chemical dispersant (Corexit®EC9500A) on a key ecological and economic species, the blue crab (*Callinectes sapidus*), in the Gulf of Mexico. The first part of my dissertation focuses on determining if exposure to Corexit® EC9500A affects the structure and function of gill epithelia. A 24-hour lethal concentration 50 (LC_{50}) for Corexit®EC9500A was determined to be 210ppm. Histological analysis of gills exposed for 24-h to a sub-lethal concentration (125ppm) of Corexit®EC9500A revealed cellular degradation, cuticle loss, and fluid buildup in the hemolymph spaces. Quantitative image analysis revealed the area/length ratio of gill lamellae in exposed crabs was greater than that for control crabs. Quantitative PCR was utilized to measure the relative transcript abundance of three critical ion transport proteins (Na^+/K^+ ATPase, plasma membrane Ca^{2+} ATPase (PMCA), and sarcoplasmic reticulum/endoplasmic reticulum Ca^{2+} ATPase (SERCA)) in gills from Corexit-exposed

and control crabs. The transcript abundance encoding each ion transport protein was significantly lower in gills from dispersant-exposed crabs than in gills from control crabs. The second part of my research focuses on elucidating the cellular mechanisms underlying the cytotoxic effects of Corexit® EC9500A. Results of immunohistochemical studies indicate that exposure to Corexit® EC9500A (125 ppm for 24 h) increased NADPH oxidase (NOX4), C-reactive protein (CRP), and cleaved (active) caspase-3 (CC3) immunoreactivity in gill lamellae. The final part of my research focuses on ascertaining if exposure to Corexit® EC9500A elicits a physiological stress response characterized by crustacean hyperglycemic hormone (CHH) mediated hyperglycemia. Exposure to a sub-lethal concentration of Corexit® EC9500A produced a transient (60 min) increase in hemolymphatic glucose, and a transient increase in abundance of the CHH transcript in eyestalk neural ganglia.

Keywords: chemical dispersant, crustacean hyperglycemic hormone, SERCA, PMCA, oil spil

DEDICATION

"If you have built castles in the air, your work need not be lost; that is where they should be. Now put the foundations under them."

- *Henry David Thoreau*

First, I would, like to dedicate this work to my parents Mack and Linda. Your sacrifices…the hours of my childhood you spent helping me study, going over my work after 16-hour shifts in the ER, being both tough and loving in the way that only parents can…instilled in me a sense of drive and perseverance that has guided me in my roles as a student, wife, and mother.

Second…to my amazing, headstrong, tenacious, perfect children Jordan and Noah. On the dark days…the times where nothing in the lab works right and the world in general seems bleak…you are my guiding light…the inspiration I fiercely hold onto to get back up and keep going. Always remember that with hard work, determination, love, and a little bit of luck anything is possible.

Lastly…Mitch, my husband, partner, and dearest friend. It feels near impossible for me to find adequate words to express the enormity of the impact your love, support

and sacrifice has had on my success over the years. Quite simply, without all that you have done and given up for me, I would never have completed this journey.

So, sometimes…when you cannot find the right words to express the depth of your emotion…you borrow from others-

…" Between me and the world

You are a calendar, a compass

A ray of light that slips through the gloom

You are a biographical sketch, a bookmark

A preface that comes at the end.

Between me and the world

You are a gauze curtain, a mist

A lamp shining into my dreams…" – Bei Dao

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Dr. Tollefsbol, Dr. Morris, Dr. Biga, Dr. Wibbels, Dr. Bej, and the Drs. Mukhtar. It takes a village, and all of you have offered materials, lab equipment and advice to me over the years. While I was alone in the lab…I never felt like I was without help or resources. Thank you all for your generosity!!

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CALCIUM SIGNALING AND REGULATION OF ECDYSTEROIDOGENESIS IN CRUSTACEAN Y-ORGANS

LIST OF ABBREVIATIONS

A23187	Antibiotic A 23187, Calimycin
ANOVA	One-way analysis of variance
ATPase	Adenosine triphosphatase
BEAS-2B	Human bronchial epithelium, normal
BP	British Petroleum
Ca^{2+}	Calcium
CADPS/CAPS	Calcium dependent secretion activator
cAMP	Cyclic adenosine monophosphate
Cas-PMCA	*Callinectes sapidus* plasma membrane Ca^{2+} ATPase
CC3	Cleaved Caspase-3
cDNA	Complementary deoxyribose nucleic acid
cGMP	Cyclic guanosine monophosphate
CHH	Crustacean hyperglycemic hormone
Cl^-	Chloride
CRP	C-reactive protein
Ct	Cycle threshold
DAPI	4’,6-diamidino-2-phenylindole
DEG	Differentially expressed genes
DOSS	Dioctyl sodium sulfosuccinate
DPBS	Dulbecco’s phosphate-buffered saline
DWH	Deepwater Horizon
EDTA	Ethylenediaminetetraacetic acid

Fluo-4	Fluorescent calcium indicator
GPCR	G-protein coupled receptor
H^+	Hydrogen
HSD	Tukey's honestly significant difference test
IBMX	3-isobutyl-1-methylxanthine
IP^3	Inositol triphosphate
ITP	Ion transport peptide
K^+	Potassium
kDa	kiloDalton
KEGG	Kyoto Encyclopedia of Genes and Genomes
La^{3+}	Lanthanum
LC_{50}	Lethal Concentration 50
MC252	Mississippi Canyon Block 252
MIH	Molt-inhibiting hormone
M-MLV	Murine leukemia virus
MOIH	Mandibular organ inhibiting hormone
mTOR	Mechanistic target of rapamycin
Na^+	Sodium
Na^+/K^+ ATPase	Sodium potassium ATPase
NADPH	Nicotinamide adenine dinucleotide phosphate
NCX	Sodium calcium exchanger
NOX4	NADPH oxidase 4
O.C.T.	Optimal cutting temperature compound

PBST	1x Dulbecco's phosphate-buffered saline + 0.1% Tween
PCR	Polymerase chain reaction
PDE	Phosphodiesterase
PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein kinase G
PMCA	Plasma membrane Ca^{2+} ATPase
ppm	Parts per million
ppt	Parts per thousand
QPCR	Quantitative polymerase chain reaction
rGC	Receptor guanylyl cyclase
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RQ1	RNA qualified
RT	Room temperature
SERCA	Sarco/endoplasmic reticulum ATPase
SNARE	SNAP Receptor proteins
SSDI	Subsurface direct injection
VIH	Vitellogenesis inhibiting hormone
WAF	Water-accommodated fraction
XO/SG	X-organ/sinus gland complex
Y-organ	Crustacean molting gland

INTRODUCTION

"We have not inherited an easy world. If developments like the Industrial Revolution…and the gifts of science and technology have made life much easier for us, they have also made it more dangerous."

-Ronald Reagan

Deepwater Horizon Oil Spill

The Gulf of Mexico is a major site of offshore drilling for crude oil, with an average of 1.5 million barrels of oil extracted daily. The regular extraction and movement of crude oil, coupled with the proximity of offshore drilling platforms to the shoreline, leaves the Gulf States particularly susceptible to the effects of potential oil spills [1]. The Deepwater Horizon (DWH) was an offshore drilling rig located at British Petroleum (BP) operated Macondo Prospect (MC252), in the east Mississippi canyon area (N 28.73667 W 88.38694) in the northern portion of the Gulf of Mexico, 66 km off the coast of Louisiana, operating at a depth of 1552 meters. On 20, April 2010, there was a catastrophic explosion on the DWH which resulted in the sinking of the rig and the death of 11 people [2]. As a result

of the explosion, crude oil from a broken well head on the sea floor leaked into the Gulf of Mexico at an estimated rate of 53,000 barrels per day, culminating in total of five million barrels of crude oil by the time the well was capped on 15 July 2010 [3]. The magnitude of the DWH oil spill necessitated a remediation response of equal enormity.

Methods of Oil Spill Remediation

There are three predominant methods of oil spill remediation. Mechanical remediation is the collection and physical removal of the surface oil slick with equipment such as skimmers and booms. Skimmers are specialized oleophilic absorbents fashioned into ropes, belts, drums, etc., that attract the oil to the sorbent surfaces. The attached oil is then removed with wipers or pressure rollers and stored in drums, barges, or other appropriate containers. Recovered oil is disposed of, or in some cases, can be recycled, which can lessen the waste management challenges. Mechanical remediation is the preferred recovery option as it is the safest for the environment. It is also the only form of remediation allowing for the removal of weathered oil. Unfortunately, the success rate of mechanical remediation is dependent upon weather conditions, wave height (≤ 2 meters), time-constraints, and the availability of support vessels and equipment [4, 5, 6].

In situ burning (ISB) is the collection, via skimmers and fireproof booms, of surface oil and its subsequent removal via fire. It is a historically well-established, inexpensive method of remediation similar to mechanical recovery in that it needs the use of a boom-base system. To be effective, ISB requires relatively thick slicks (≥ 3mm), daylight to allow for easy management, and wave heights of less than 1.2 meters, to

prevent emulsification, which can counteract ignition. The advantages of ISB include preventing the need for storage and disposal of collected oil, and as such, reduces resource and time constraints during a spill response. The negative effects of ISB's disadvantages range from black plume smoke detrimental to people and the atmosphere to dense burn residues which can sink into the water rendering them unrecoverable [6, 7].

Chemical dispersants are a proprietary blend of anionic and ionic surfactants in hydrocarbon solvents (ethylene glycol, glycol ethers, etc.) that function as a detergent [5, 8]. The surfactants have a hydrophilic and lipophilic end. When applied to an oil slick, such dispersants diffuse down into the oil layer and rest at the oil/water interface. There they act to lower surface tension and break the oil into small droplets, facilitating its dispersion into the water [8, 9, 10]. Use of chemical dispersants is cost-effective and is said to enhances bacterial biodegradation of the crude oil by hydrocarbon-degrading bacteria. The effectiveness of the dispersant, like the other forms of remediation, relies on weather conditions, water conditions, type of oil, and the level of oil weathering [4, 6].

Remediation of the Deepwater Horizon Oil Spill

In response to the sheer magnitude of the DWH oil spill, several response technologies were employed at various levels and to varying degrees of success. Mechanical remediation made up most of the clean-up efforts in marshes, bays, beaches, and protected waters [11]. Record keeping during this time was sparse, so it was estimated that skimming was responsible for removing 147,000 barrels, about 2-4% of the total spilled oil [7], but there have been estimates as high as 300,000 barrels of oil

[12]. Factors contributing to the low recovery rate could have been the sizable expanse of the oil spill, time-constraints, and dilution of the oil from subsea dispersant injection (SSDI) [12]. ISB was utilized to supplement the skimming operations. A total of 411 controlled oil burns (376 successful) were attempted between April and July, resulting in the burning of an estimated 310,000 barrels of oil, about 5-6% of the total crude oil released [13]. The bulk of the spilled oil was removed via chemical dispersant application. An estimated 1.84 million gallons of Corexit® brand dispersants were applied in remediation efforts [14]. Surface application of chemical dispersants consisted of 1.07 million gallons of COREXIT® 9527A and COREXIT® EC9500A. Subsea dispersant injection directly into the wellhead, at a depth of 1544 km, consisted of 772,000 gallons of COREXIT® EC9500A (CE) [15, 16].

COREXIT® EC9500A (NALCO)

COREXIT® 9527A and COREXIT® EC9500A contain two non-ionic surfactants: Tween 80 (ethoxylated sorbitan monooleate) and Span 80 (sorbitan monooleate), and the anionic surfactant dioctyl sodium sulfosuccinate (DOSS) in differing organic solvents. The chemical constituents of both dispersants were released in July 2010 in response to public outcry against the amounts of dispersant being used and the novel application method SSDI [17].

The use of chemical dispersant during the Deepwater Horizon spill generated concern for several reasons: First, the amount of dispersant applied during the spill was the largest application to date. Second, it was the first large-scale application of chemical dispersant into deep water, and there was little information on the fate of the dispersant or

possible harmful effects of subsea application. Third, as dispersants break the oil up into droplets that spread throughout the water column there is evidence of a temporary increase in the concentration of hydrocarbons, particularly toxic polycyclic aromatic hydrocarbons (PAH's). Finally, it is hypothesized that dispersion of droplets may increase the exposure to benthic marine life [16, 18, 19].

Effects on Marine Organisms

Effects of Corexit® on multiple invertebrate and vertebrate species have been reported. The following are representative examples. Goodbody-Gringley, et al. observed that Corexit® 9500 (25-100ppm) reduced settlement ability and survival among larvae of the corals *Porites asteroids* and *Montastraea faveolata* [20]. In addition, Corexit® EC9500A exposure was associated with a decrease in growth rates among larvae of the barnacle (*Amphibalanus improvisus*) and the acorn worm (*Schizocardium sp.*) [10], and with reduced swimming activity and motility among larvae of the blue crab (*Callinectes sapidus*) [21]. Finally, Adeyemo, et al. reported that exposure of silverside fish (*Menidia beryllina*) embryos to Corexit® EC9500A lowered heart rates, increased developmental abnormalities, and induced atypical expression of genes responsible for sexual differentiation, growth regulation and stress response [18]. The effects of Corexit® are not restricted to invertebrate and vertebrate animals. Exposure to Corexit® inhibited growth rates and motility in *Isochrysis galbana* and *Chaetoceros sp.*, phytoplankton that are critical food sources for larval mollusks, fish, and crustaceans [22].

It has long been hypothesized that dispersed oil, with a smaller surface to volume ratio, would become more bioavailable to hydrogen-degrading bacteria, catalyzing its biodegradation [23]. In typical environments, hydrogen-degrading bacteria comprise less than 1% of the total populace, existing off hydrocarbons produced by other living organisms [24]. Environments inundated with an excess of hydrocarbons experience a rapid increase in hydrogen-degrading microorganism populations, commonly exceeding 10% of the total bacterial population [25]. Corexit® exposure was associated with significant mortality of *Acinetobacter* and *Marinobacter*, hydrocarbon-degrading bacteria that occur naturally in seawater and act in bioremediation of spilled oil [26]. Although not a focus of this research, cytotoxic effects of the combination of Corexit® and crude oil (dispersed oil) have also been reported [27, 28, 29, 30].

Within two years of the Horizon oil spill, the diversity of seaweed communities in the northern Gulf of Mexico had dramatically declined from an excess of 60 species to less than 10 [31]. Felder, et al. reported the presence of severe manganous lesions penetrating the joints and gills of multiple crustacean species and an increase in lipoclastic microbes leading to the development of *shell disease*, an extensively studied disease with costly effects on commercial crab and shrimp fisheries [31].

The Blue Crab (*Callinectes sapidus,* Rathburn*)*

The documented effects of chemical dispersants amongst a vast range of marine species vary widely in the literature. To properly elucidate the effects of chemical dispersants in a controlled setting, the ideal animal model would have: (1) high ecological and commercial value; (2) an ecological distribution that overlaps significantly with areas

known to harbor many drilling platforms or potential exposure to oil spills; (3) a well-documented respiratory system analogous to what is found in humans; and, (4) an easily testable stress responses that could be managed in a controlled, reproduceable laboratory setting to reduce variation. The blue crab (*Callinectes sapidus)* was chosen for this dissertation research as it satisfied all four conditions and is a common model organism with well-known care and maintenance requirements.

In blue crabs, fertilized eggs are extruded from the oviduct and carried beneath the female abdomen. The eggs hatch within two to three weeks of extrusion in high salinity waters (20% or greater). The resulting larvae develop through an average of seven zoeal stages before maturing into a megalopa with a predominately planktonic existence floating at the surface of open waters near continental shelves (Figure 1). Between each stage, the animal sheds (molts) its old exoskeleton and replaces it with a new and larger version. As juvenile crabs grow and mature through successive molt cycles, they migrate towards low salinity nurseries in bays and estuaries with where they reside until adulthood. Males gain maturity after an average of 18 post-larval molts. Females gain maturity at the pubertal molt which occurs after 18 to 20 post-larval molts. Following the pubertal molt, while the female exoskeleton is soft, she will mate with a male. Generally, mating occurs

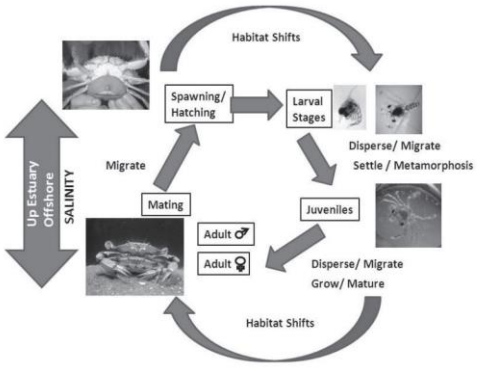

Figure 1. Schematic of blue crab life cycle showing life history stages and events, emphasizing habitat shifts and migration (Hines, et al, 2010)

in low-salinity waters and once complete, the inseminated female will migrate, sometimes up to 500km, to high-salinity waters for spawning. While males will mate multiple times, female crabs' mate only once in their lifetime, but have the ability to store sperm and will typically produce multiple broods of fertilized eggs [32, 33, 34, 35].

Blue crabs have a broad range spanning from Nova Scotia, down to Maine and northern Massachusetts, and continuing to Northern Argentina, Bermuda and the Antilles [36]. Specifically, in the United States the blue crab is commonly found on the Atlantic Coast from Massachusetts to the southernmost point of Texas. Mature crabs tend to remain in deep waters, but the young, developing zoeal can be found inshore where the water is shallow [32].

Blue crabs have long supported commercially important fisheries in three main areas: the Chesapeake Bay, Southeast Atlantic Coast, and the Gulf of Mexico [33, 37, 38, 35]. As of the 2018 NOAA Fisheries Report, the second most valuable fisheries in the United States are the crab fisheries with an estimated value of $645M [39]. Over the past decade, populations of blue crabs in these areas are seriously declining, due to a combination of commercial fishing expansion, enhanced environmental stressors, and an increase in major hurricanes influencing the water quality [35].

In addition to their economic importance, blue crabs are a keystone species playing ecologically important roles in the environments they inhabit. As examples, larval stages are eaten by shellfish, fish, and a variety of other planktivores [40], while the juvenile crabs are important prey for fish and birds [41]. Blue crab zoeae consume phytoplankton, dinoflagellates, and copepod nauplii, while megalops consume fish larvae, small shellfish, and aquatic plants [42, 43]. Predation of the infaunal

macrobenthos by juvenile and adult crabs plays a crucial role in determining species diversity and population density [44, 35].

Structure and Function of Blue Crab Gills

Crustacean gills have multiple functions, including respiration and ion transport (the basis for osmoregulation) [45]. The gills are located in lateral gill chambers, one on each side. A ventilatory flow of water is generated in each chamber by scaphognathites (gill bailers), which push a stream of water anteriorly across the gill surfaces. Because gills have multiple functions and are in continuous contact with water, they are logical candidate for assessing possible effects of chemical dispersant. Blue crabs have eight phyllobranchiate gills on each side. Each consist of a medial dumbbell-shaped stem with a dorsal afferent and a ventral efferent hemal channel. On both sides of the stem are paired parallel lamellae. The central stem consists of fibrous connective tissue extending into the lamellae along with clusters of podocytes suspended by thin connective tissue in irregular rows (Fig. 2). The podocytes appear similar in structure to cells in vertebrates and invertebrates known for ultrafiltration. Located distal to the afferent and efferent vessels are closely grouped tegmental glands that extend to the gill apex (Fig. 3). Within these, are clusters of what Phyliss Johnson termed “effete” glands, large glands devoid of secretions or granules. Water influx across the lamellae occurs in a countercurrent fashion to hemolymph flow which cycles through the lamella [46].

Each lamella is thick at the base and tapers progressively, moving towards the tip. The lamella consists of a single layer of cuticle-covered epithelium in a sack-like formation with a hemolymph space between the two epithelial layers. The cuticle is thin

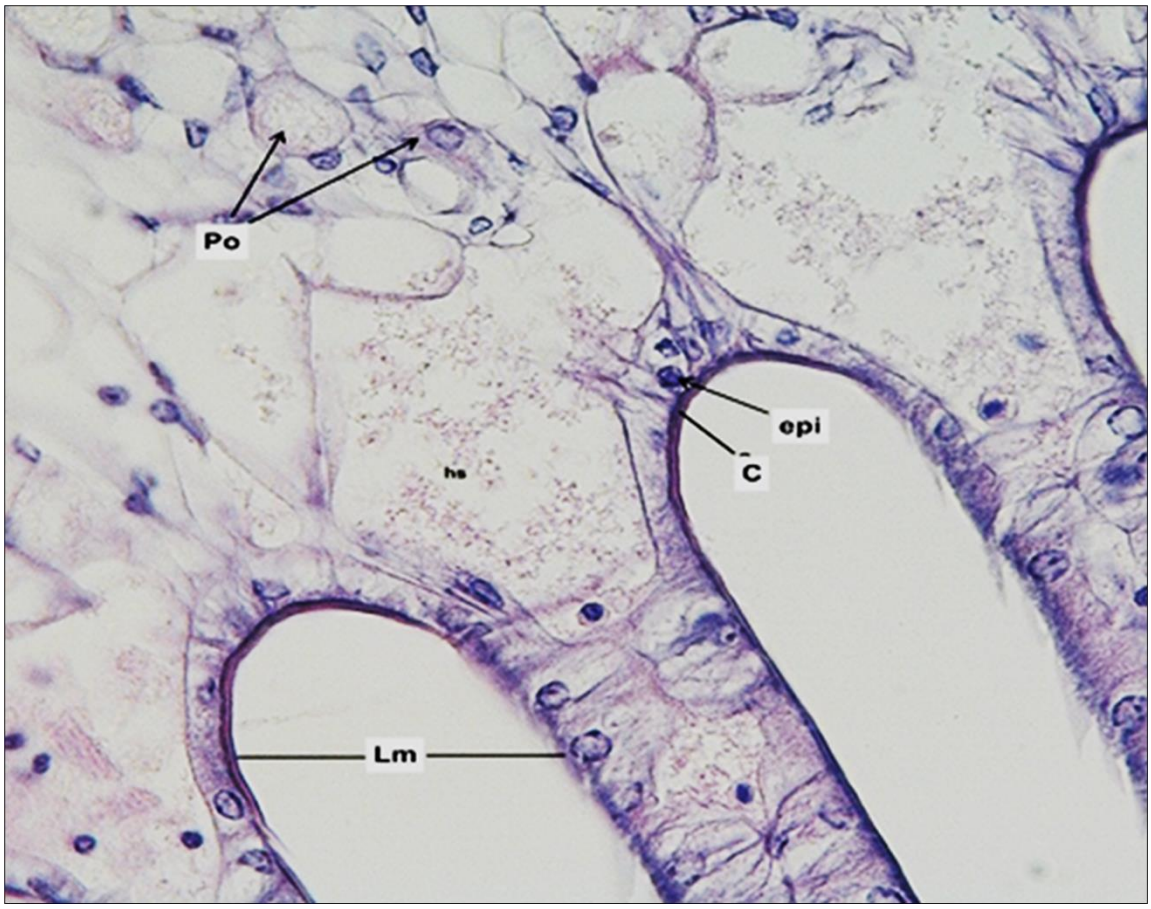

Figure 2. Central stem and posterior gill lamellae base. Sections were fixed in Davidson's fixative, processed, stained using hematoxylin and eosin, and viewed using a Nikon Eclipse 80i microscope. 400X magnification. Abbreviations: podocyte (Po), cuticle (c), lamellae (Lm), epithelial cell (epi).

(< 1μm) with three-layers that are only evident at the lamella tip, where they thicken slightly to reveal an epicuticle, endocuticle, and the membranous layer [46, 47, 48]. The gill cuticle is narrower, more conductive, and permeable than the cuticle covering the carapace and hindgut. The epicuticle specifically, lacks the waxy waterproof layer found in most cuticles and it has been shown to be ion permeable. A high gill cuticle

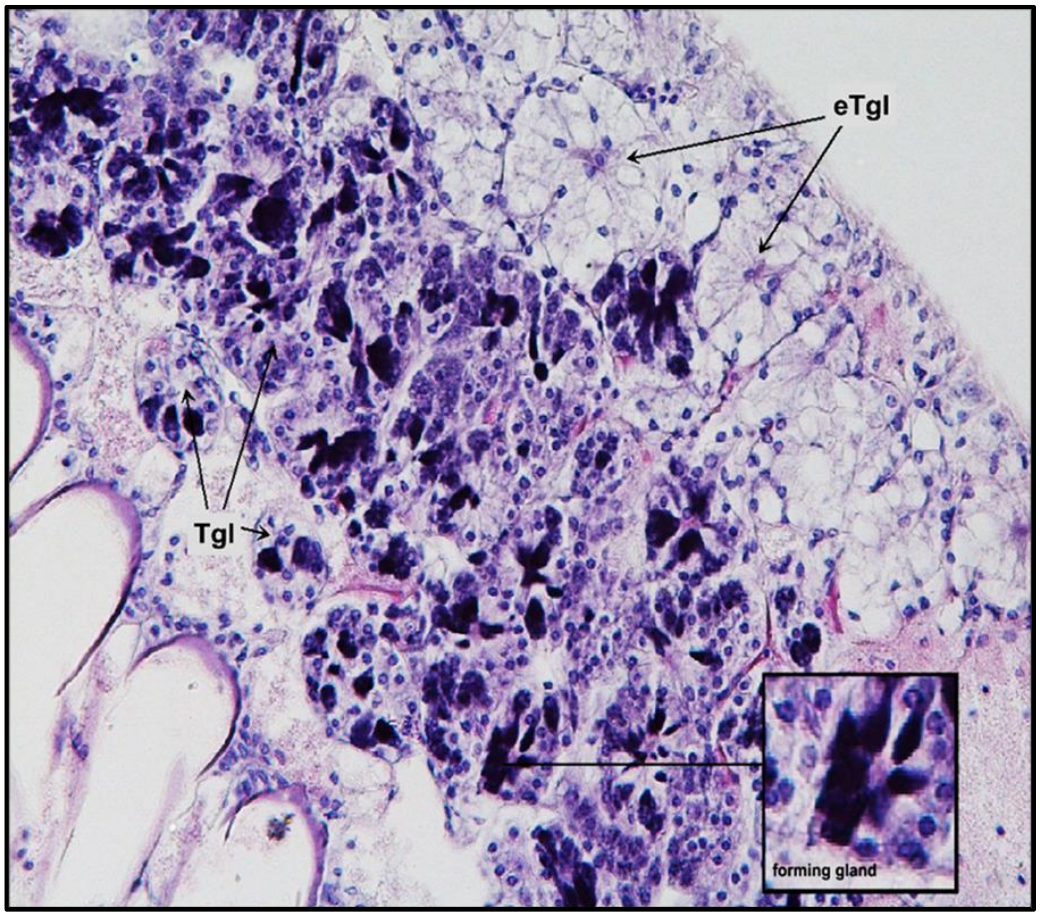

Figure 3. "Effete" glands and tegmental glands in the basal stem of an anterior gill. Gills were stained using hematoxylin and eosin and viewed using a Nikon Eclipse 80i microscope. *400X magnification.* Abbreviations: *effete tegmental gland (eTgl), tegmental gland (Tgl).*

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conductance compared to the epithelium is common in hyperosmoregulators and is thought to reduce passive salt loss [47]. Spanning the hemolymph space are pilaster cells that function to prevent shape distortions due to changes in hydrostatic pressure. In blue crabs, the four anterior gill pairs differ structurally and functionally from the four posterior gill pairs [46, 49].

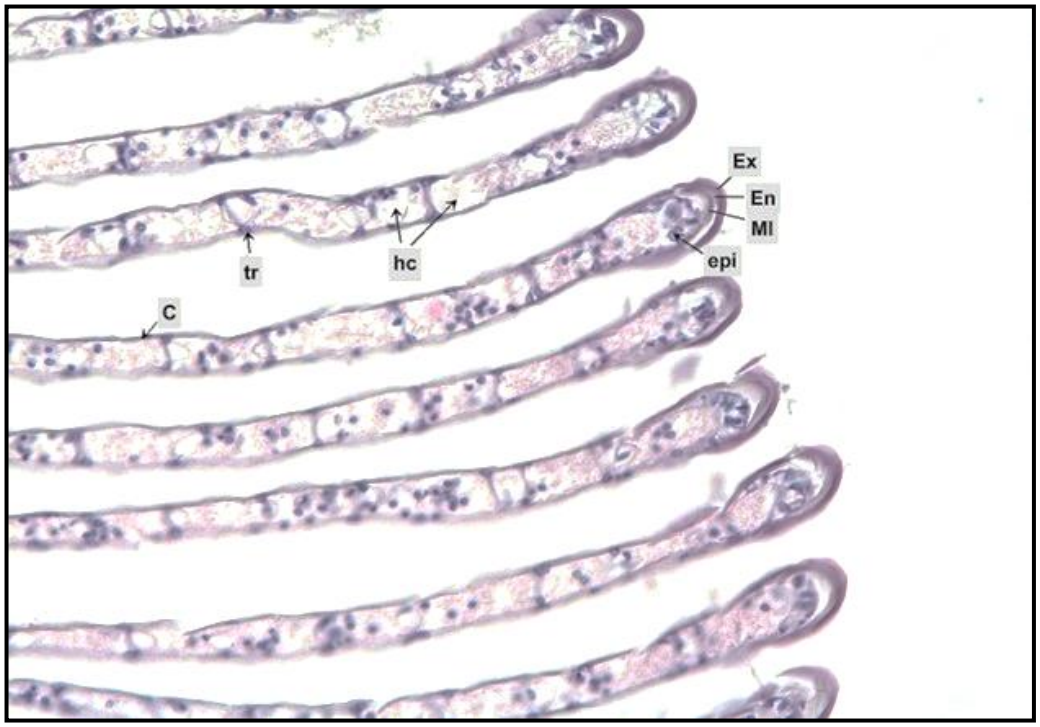

Figure 4. Anterior gill section with apex of respiratory lamellae. Gills were stained using hematoxylin and eosin and viewed using Nikon Eclipse 80i microscope. *400X magnification.* Abbreviations: *c,* cuticle; *hc*, hemolymph space; *tr*, trabecular cell; *epi,* epithelium; *Ex,* exocuticle; *En,* endocuticle; *Ml,* Membranous layer.

The lamellae of anterior gills are defined morphologically by thin epithelium, 1-5μm in height [46, 50]. Thin epithelium is composed of squamous epithelium, although this can thicken slightly to cuboidal epithelium at the base, near the gill stem. The four anterior gill pairs consist entirely of thin epithelium (Fig. 4) [46, 50]. The mechanisms of respiratory gas (O_2 and CO_2) exchange in *C. sapidus* have been well studied [51, 52, 53]. It is generally agreed that the anterior gill pairs function primarily in gas exchange [45, 49].

Blue crabs are considered to be osmoconformers at salinities above 27 and hyperosmoregulators below this threshold [54, 55]. The capacity for hyperosmoregulation, active uptake of ions from the environment, is attributed largely to

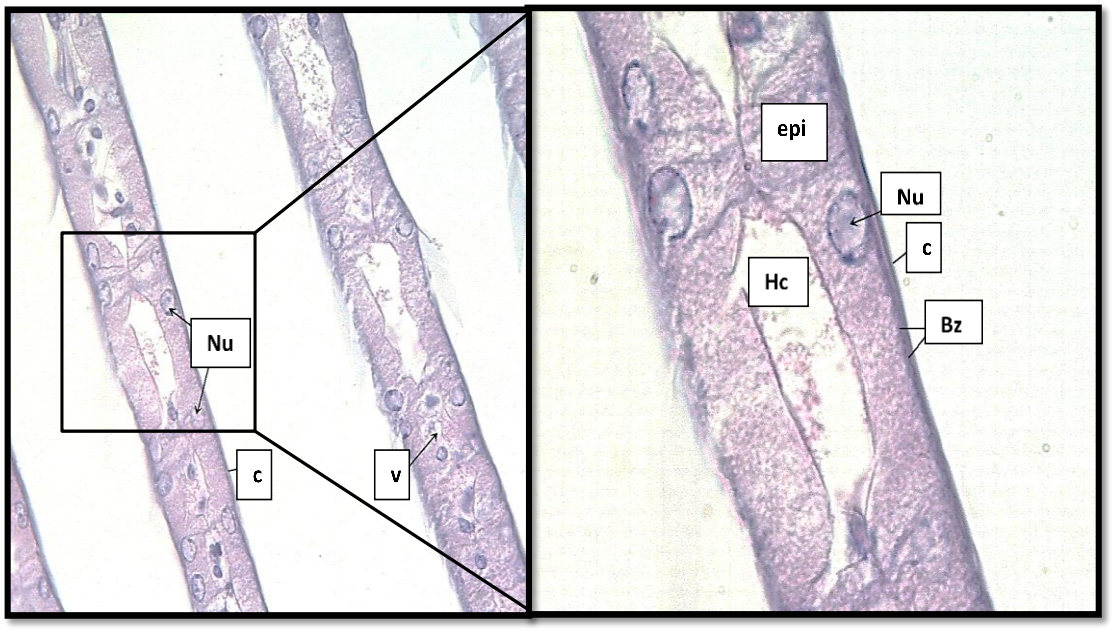

Figure 5. Posterior gill section with salt-absorbing lamellae. Gills were stained using hematoxylin and eosin and viewed using a Nikon Eclipse 80i microscope. *400X (right panel) and 1000X (right panel).* Abbreviations: *c,* cuticle; *Hc*, hemolymph space; *epi,* epithelium; *Bz,* basal zone; *Nu,* nucleus; *v,* vein.

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the four posterior gill pairs. The posterior gill lamellae are defined morphologically by large patches of thick epithelium (10µm to 20µm in height) surrounded by thin epithelium (Fig. 5). Thick epithelium has distinct apical and basal membranes, the latter has extensive membrane-infoldings and increased numbers of mitochondria, characteristics of ion-transporting epithelium. The lamellae are composed of cuboidal and columnar epithelium, which is noticeably thicker at the base and thinner at the tips. These patches of thick epithelium reside along the lateral portions of the gill lamellae [46, 47, 48]. The size and occurrence of thick patches is inversely proportional to the level of salinity in the environment [56, 48]. Blue crabs have developed a novel approach to acclimation to low salinity environments in which the thick patches play a key role. The

initial response involves an overall reduction in body surface permeability, which passively minimizes Na^+ loss and water influx [57]. Following this, acclimation occurs within a five-to-seven-day period during which there is a substantial increase in thick patch size attributed to a form of epithelial metaplasia; squamous epithelial cells of the thin epithelium reversibly differentiated into the cuboidal and columnar epithelial cells of the thick epithelium. This was observed in concordance with an increase in RNA/DNA content, absence of mitotic activity (cell proliferation), absence of hypertrophy among existing osmoregulatory cells [48], and a directly proportional increase in Na^+/K^+ ATPase activity [58, 56]. The differentiation of respiratory to osmoregulatory epithelium would allow for the creation of extensive infoldings where newly synthesized Na^+/K^+ ATPase could be inserted [48].

The principal facilitator for osmoregulatory uptake of Na^+ and Cl^- ions by blue crab gills is the Na^+/K^+ ATPase (or Na^+/K^+ pump). Na^+/K^+ ATPase is a highly conserved transmembrane protein found in the plasma membrane of all eukaryotic cells. First described in crustaceans by Skou in 1957 [59], Na^+/K^+ ATPase is restricted to the basolateral membrane of hyper-regulating gill epithelial cells where it has access to the cytosol of the epithelial cells and the hemolymph [56, 60]. Hydrolysis of ATP by Na^+/K^+ ATPase is coupled to the ejection of three Na^+ from cytosol to hemolymph in exchange for two K^+. The resulting electrochemical gradient is used as a driving force for other transporters located on the apical membrane of the epithelial cells, including a Na^+/H^+ exchanger [61] and a $Na^+/K^+/2\ Cl^-$ cotransporter [62]. Because of its central role in the process of osmoregulation, Na^+/K^+ ATPase has been the focus of multiple investigations of ion transport by gill epithelia [63].

Although not necessarily linked to osmoregulation, intracellular Ca^{2+} homeostasis is critical to the functioning of gill cells. In eukaryotic cells, cytosolic Ca^{2+} is maintained at a concentration roughly four orders of magnitude less than the concentration of extracellular Ca^{2+}. This is critical to Ca^{2+} signaling and to averting cytotoxicity [64]. Ca^{2+} homeostasis is particularly dicey in crustaceans, as there are dramatic changes in hemolymphatic Ca^{2+} levels associated with mineralization and demineralization of the exoskeleton as part of the molting cycle. Thus, gill epithelial cells must maintain low intracellular Ca^{2+} levels while being able to effect large transepithelial fluxes of Ca^{2+} during specific stages of the molt cycle. The concentration of Ca^{2+} in cytosol is controlled mainly by transport proteins intrinsic to the plasma membrane and the membranes of organelles [65, 66, 67, 68]. Several families of transporters are involved, including Ca^{2+} pumps (ATPases) and Ca^{2+} exchangers. The family of Ca^{2+} pumps include a plasma membrane Ca^{2+} ATPase (PMCA), and a sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA). Na^{+}/Ca^{2+} exchanges (NCX) are bidirectional transporters that mediate the exchange of Ca^{2+} for Na^{+} using the energy of the Na^{+} gradient generated by Na^{+}/K^{+} ATPase.

SERCA is a member of the P-type ATPase superfamily responsible for the re-uptake of Ca^{2+} ions into the endoplasmic reticulum (ER). SERCA was originally discovered and cloned in mammals who have five isoforms of the gene (SERCA1, SERCA2a, SERCA2b, SERCA3, SERCA4, and SERCA5) encoded by three homologous genes [69, 70]. Two isoforms of SERCA, homologous to vertebrate SERCA2a and SERCA2b, have been identified in a number of invertebrates, including insects and crustaceans [71, 72]. Our lab has previously cloned cDNAs encoding SERCA from blue

crab Y-organs [73]. The transcript of *Cas-SERCA* was widely detected across tissue, including muscle, gill, hepatopancreas, hypodermis, thoracic ganglion, and the Y-organs [74]. When assessed by stages of the natural molting cycle, SERCA showed stage-specific changes in each tissue, indicative of a role for Ca^{2+} signaling in molt cycle regulation [73].

PMCA is a member of the P-type ATPase superfamily, it is a high affinity, low-capacity Ca^{2+} pump that extrudes Ca^{2+} outside the cell to reduce intracellular Ca^{2+} levels [75, 76]. In non-excitable cells PMCA plays a predominant role in fine tuning Ca^{2+} homeostasis [77]. In mammals there are four genes encoding four different PMCA isoforms (PMCA1 to PMCA4). Our lab has previously cloned cDNAs encoding a putative PMCA gene (*Cas-PMCA)* from the blue crab [78]. The transcript of *Cas-PMCA* was universally detected in neural and non-neural tissue with an expression pattern similar to human *PMCA1 and PMCA4* [79]. The measurements of the transcript abundance of *Cas-PMCA* in Y-organs during a molt cycle revealed stage-specific changes. Generally speaking, the stage-specific variation of *Cas-PMCA* mRNA expression might be part of the mechanism by which Ca^{2+} homeostasis is maintained in *C. sapidus* Y-organ cells [78].

Crustacean Hyperglycemic Hormone

Crustacean hyperglycemic hormone (CHH), first discovered by Abramowitz et al. (1944) when injection of eyestalk extracts into blue crabs elicited a potent hyperglycemic effect, is a polypeptide neurohormone member of the CHH superfamily [80, 81]. The CHH precursor includes a signal peptide, a CHH related precursor peptide and the mature

peptide. The mature peptide is composed mainly of four alpha helices and has six conserved cysteine reside which create three disulfide bridges, a hallmark feature of CHH superfamily members [82, 83]. The majority of *CHH* genes have four exons and three introns [84] and are subject to alternative splicing resulting in two genetic variants, CHH-like peptide (CHH-L) and CHH [85]. Both variants undergo posttranslational modifications and have pyroglutamated N-terminals. Only the CHH variant has an amidated C-terminal which accounts for its hyperglycemic activity [85, 86]. CHH-L is expressed in extra-eyestalk tissues and as yet, no functional role for this variant has been identified [87].

Our lab has previously cloned cDNAs encoding a putative CHH preprohormone, *CsCHH-1*from blue crab eyestalks, which harbored all the structural features previously reported in other species. The transcript of *CsCHH-1* was only detected in the eyestalk ganglia. RT-PCR also revealed the presence of a CHH-like preprohormone in the thoracic ganglion, ventral nerve cord and the brain [88].

CHH is produced in a cluster of neurosecretory cell soma (the X-organ) and released from their associated axon terminals in the neurohemal sinus gland [89, 90]. Release of CHH occurs in response to both internal cues, including an endogenous circadian rhythm entrained to the light/dark cycle [91], and external cues, including environmental stressors such as emersion-induced hypoxia [92], thermal shock [93], parasite infection [94], and environmental pollutants [95, 96]. CHH release rapidly induces hyperglycemia by promoting glycogenolysis and inhibiting glycogen synthesis in the hepatopancreas and muscle. This initial response occurs within 15 to 20 minutes, generally lasts one to three hours, and is regulated by a negative feedback loop involving

depolarization of the CHH producing cells by D-glucose in the hemolymph [97, 98, 99]. In recent years, studies have shed light on other potential physiological roles for CHH such as regulation of the molting cycle through suppression of ecdysteroidogenesis (albeit at higher levels than MIH) [100, 101], regulation of ion transport and water influx. Swelling of the somatic tissue during and after ecdysis is accomplished by substantial water influx driven by a dramatic release of gut derived CHH during pre-molt [102]. Spanning-Pierrot et al. (2000) demonstrated that perfusion with sinus gland extract increased Na^+/K^+ATPase activity in the posterior gills of the crab *P. marmoratus* by 50% [103]. In accordance with this, Serrano et al. observed a significant decrease in hemolymph osmolality and Na^+ concentration after eyestalk ablation in the crayfish *Astacus leptodactylus* that was relieved by purified CHH [104].

EFFECT OF A CHEMICAL DISPERSANT (COREXIT® EC9500A) ON THE STRUCTURE AND ION TRANSPORT FUNCTION OF BLUE CRAB (CALLINECTES SAPIDUS) GILLS

by

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Abstract

Chemical dispersants are commercially available mixtures of surfactants and solvents that have become important tools in the remediation of spilled oil. Given the importance of oil to the world economy, the recurring nature of spills, and the prevalence of dispersant use in remediation, there is a critical need to understand potential toxic impacts of dispersants on invertebrate and vertebrate animals. Blue crabs (*Callinectes sapidus*) play ecologically important roles in the environments they inhabit and support economically important fisheries along the Atlantic Coast and in the Gulf of Mexico. In studies reported here, we assessed the impact of a chemical dispersant, Corexit® EC9500A, on the structure and ion transport function of blue crab gills. Exposure of blue crabs to Corexit® EC9500A for 24 hours (0-300 ppm in artificial seawater under static conditions) revealed a 24-h lethal concentration 50 (LC_{50}) value of 210 ppm. A histological analysis of gills from crabs exposed for 24 h to a sub-lethal concentration of Corexit® EC9500A (125 ppm) revealed evidence of loss or disruption of cuticle, and an increase in stained amorphous material in the hemolymph spaces of gill lamellae. Quantitative image analysis of stained gill sections revealed the area/length ratio of gill lamellae in crabs exposed to Corexit® EC9500A (24 h, 125 ppm), was greater than that in gill lamellae from control crabs; the results are consistent with the presence of edematous swelling in gill lamellae from dispersant-exposed crabs. Quantitative PCR was used to measure the relative abundance of transcripts encoding three ion transport proteins (Na^+/K^+ ATPase, plasma membrane Ca^{2+} ATPase (PMCA), and sarcoplasmic reticulum/endoplasmic reticulum Ca^{2+} ATPase (SERCA)) in gills from Corexit®-

exposed and control crabs. In general, the abundance of transcripts encoding each ion transport protein was lower in gills from dispersant-exposed crabs than in gills from control crabs. The combined results are consistent with the hypothesis that 24-h exposure of blue crabs to a sublethal concentration of Corexit® EC9500A impacts both the structure and ion transport function of gills.

Keywords: oil spill, Corexit® EC9500A, crab gills, Na^+/K^+ ATPase, PMCA, SERCA

1. Introduction

Spilled oil can have profound impacts on marine ecosystems (Fisher et al., 2014; Hallare et al., 2011; Montagna et al., 2013), the economy (Cano-Urbina et al., 2019; Ritchie et al., 1014; Sumaila et al., 2012;), and public health (Campbell et al., 1994; Gratten et al., 2011; Zock et al., 2012). Main anthropogenic sources of spilled oil include tanker spills and wellhead blowouts. For example, grounding of the Amoco Cadiz off the coast of Brittany, France in 1978 resulted in the release of 68.7 million gallons of crude oil into the Atlantic Ocean (Curl et al., 1992). And, as a result of an explosion on the Deepwater Horizon (BP MC252) offshore drilling platform in 2010, an estimated 205 million gallons of crude oil were ejected from the wellhead into the Gulf of Mexico (Crone and Tolstoy, 2010).

The primary means of spill remediation involves mechanical recovery of oil using skimmers and booms. However, the effectiveness of mechanical recovery is constrained by the size of the slick, equipment availability, and weather conditions. Secondary means of remediation include controlled burns, and the use of chemical dispersants (Broje and Keller, 2006; Fingas, 2013; Lessard and Demarco, 2000).

Chemical dispersants are a proprietary blend of anionic and ionic surfactants in one or more hydrocarbon solvents. When applied to spilled oil, the dispersant acts as a detergent, decreasing the interfacial surface tension between oil and water. The treated oil then enters the water column as fine droplets and is subjected to degradation by natural processes (Lessard and Demarco, 2000; National Research Council, 1989). Surface application of dispersant decreases the amount of oil on the sea surface (National Research Council, 2005). Subsurface application at the wellhead decreases the ascension

of oil to the surface and lowers the emission of volatile organic compounds into the atmosphere (Gros et al., 2017). An estimated 2.1 million gallons of chemical dispersant, predominately Corexit® EC9500A, were applied in remediation efforts following the sinking of the Deepwater Horizon. Of that total, approximately 1.4 million gallons of dispersant were applied to the surface slick using small boats and planes; another 0.77 million gallons were applied subsurface, directly at the wellhead, at a depth of 1544 meters (Kujawinski et al., 2011).

Although Corexit® EC9500A is effective in dispersing spilled oil, collateral effects of the dispersant on multiple invertebrate and vertebrate species have been reported. Examples include suppression of burying behavior in the marine sand snail (*Polinices conicus*) (Gulec et al., 1997), reduced settlement ability and survival among larvae of the corals *Porites asteroids* and *Montastraea faveolata* (Goodbody-Gringley et al., 2013), decreased growth rates among larvae of the barnacle (*Amphibalanus improvisus*) and the acorn worm (*Schizocardium sp.*) (Almeda et al., 2014), reduced swimming activity and motility among larvae of the blue crab (*Callinectes sapidus*) (Pie and Mitchelmore, 2015), and lowered heart rates, increased developmental abnormalities, and induced atypical expression of genes responsible for sexual differentiation, growth regulation and stress response in silverside fish (*Menidia beryllina*) embryos (Adeyemo et al., 2015). The effects of Corexit® EC9500A are not restricted to invertebrate and vertebrate animals, as exposure was associated with significant mortality of *Acinetobacter* and *Marinobacter,* hydrocarbon-degrading bacteria that occur naturally in seawater and act in bioremediation of spilled oil (Hamdan and Fulmer, 2011). In addition, exposure to Corexit® EC9500A inhibited growth rates and motility in *Isochrysis galbana*

and *Chaetoceros sp.,* phytoplankton that are critical food sources for larval mollusks, fish and crustaceans (Garr et al., 2014).

Blue crabs, the experimental animal used in studies reported here, are common in the western Atlantic from Cape Cod to northern Argentina (Williams, 1974). They support economically important fisheries in the Chesapeake Bay, along the Southeast Atlantic Coast, and in the Gulf of Mexico (Guillory et al., 2001; Steel and Perry, 1990; Zohar et al., 2008), and play ecologically important roles in the environments they inhabit (Fontenot and Rogillo, 1970; Van Engel, 1958; Virnstein, 1977). The life history of blue crabs encompasses multiple stages, locations, and environments. Juvenile crabs progress through multiple zoeal larval stages into a megalopa larval stage in high salinity open water near continental shelves. After successive molt cycles, the now adult crabs migrate towards bays and estuaries with brackish water where mating occurs. Following a successful mating, females migrate back to high-salinity water for spawning (Churchill, 1919; Milikin and Williams, 1984; Zohar et al., 2008).

Crustacean gills have multiple functions, including respiration, and ion transport (the basis for osmoregulation) (Henry et al., 2012). In addition, gill epithelia provide a protective barrier to environmental agents present in seawater (Henry et al., 2012). Blue crabs have eight paired phyllobranchiate gills located in lateral gill chambers. A ventilatory flow of water is generated in each chamber by scaphognathites (gill bailers), which push a stream of water anteriorly across the gill surfaces. Each gill consists of a medial dumbbell-shaped stem with a dorsal afferent and a ventral efferent vessel; on both sides of the stem are paired parallel lamellae (Fig. 1). In blue crabs, the four anterior gill pairs differ structurally and functionally from the four posterior gill pairs (Johnson, 1980;

Towle and Burnett, 2007). It is generally agreed that the anterior gill pairs function primarily in gas exchange (Henry et al., 2012; Towle and Burnett, 2007). Blue crabs are considered to be osmoconformers at salinities above 27 and hyperosmoregulators below this threshold (Mantell, 1967; Ballard and Abbott, 1969). The capacity for hyperosmoregulation, active uptake of ions from the environment, is attributed largely to the four posterior gill pairs.

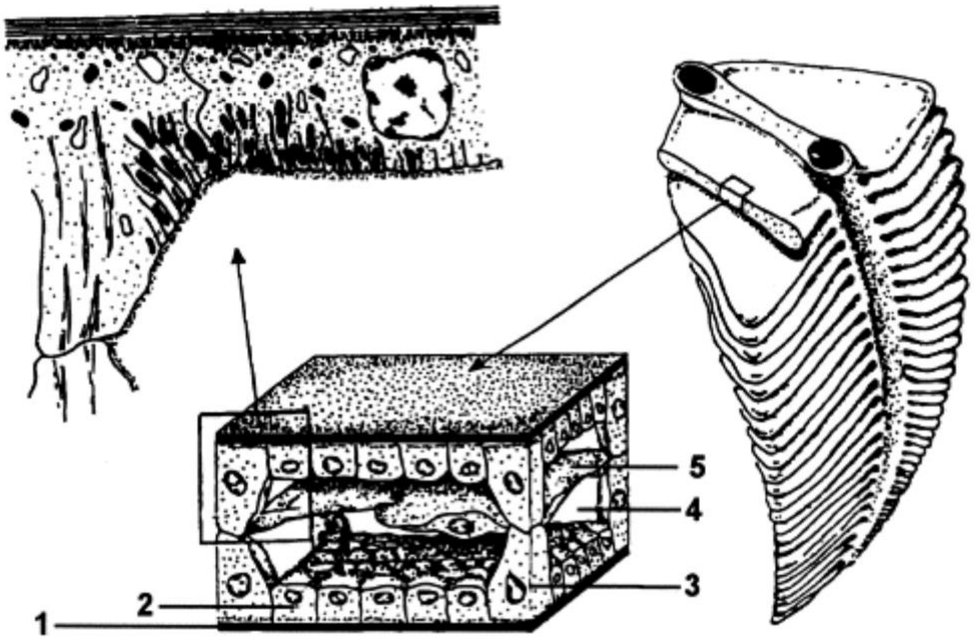

Figure 1. Representation of the morphology and fine structure of crab gills, including cuticle (1), epithelial cell (2), pilaster cell (3), hemolymph space (4), and interlamellar septum (5). Note: Adapted from "NaCl absorption across split gill lamellae of hyperregulating crabs: Transport mechanisms and their regulation" by H. Onken and S. Riestenpatt,1998, *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *119*, p. 883. Copyright 1998 by Elsevier. Adapted with permission.

Critical to the osmoregulatory uptake of Na^+ and Cl^- ions by blue crab gills is the Na^+/K^+ ATPase (or Na^+/K^+ pump). This transmembrane protein is restricted to the

basolateral membrane of gill epithelial cells (Towle and Kays, 1986). There it has access to the cytosol of the epithelial cells and to hemolymph. Hydrolysis of ATP by Na^+/K^+ ATPase is coupled to the ejection of three Na^+ from cytosol to hemolymph in exchange for two K^+. The resulting electrochemical gradient is used as a driving force for other transporters located on the apical membrane of the epithelial cells, including a Na^+/H^+ exchanger (Orlowski and Grinstein, 1997) and a $Na^+/K^+/2\ Cl^-$ cotransporter (Riestenpatt et al., 1996).

Although not necessarily linked to hyperosmoregulation, intracellular Ca^{2+} homeostasis is critical to the functioning of gill cells. In eukaryotic cells, cytosolic Ca^{2+} is maintained at a concentration that is roughly four orders of magnitude less than the concentration of extracellular Ca^{2+}. This is critical to Ca^{2+} signaling and to averting cytotoxicity (Kretsinger, 1976). Ca^{2+} homeostasis is particularly challenging in crustaceans, as there are dramatic changes in hemolymphatic Ca^{2+} levels associated with mineralization and demineralization of the exoskeleton as part of the molting cycle. Thus, gill epithelial cells must maintain low intracellular Ca^{2+} levels while being able to effect large transepithelial fluxes of Ca^{2+} during specific stages of the molt cycle. The concentration of Ca^{2+} in cytosol is controlled mainly by transport proteins intrinsic to the plasma membrane and the membranes of organelles (Berridge et al., 2000; Clapham, 2002; Guerini et al., 2005; Philipson and Nicholl, 2000). Several families of transporters are involved, including Ca^{2+} pumps (ATPases) and Ca^{2+} exchangers. The family of Ca^{2+} pumps include a plasma membrane Ca^{2+} ATPase (PMCA), and a sarcoplasmic reticulum/endoplasmic reticulum Ca^{2+} ATPase (SERCA). The former transports Ca^{2+} out

of the cell, while the latter transports Ca^{2+} into the sarcoplasmic reticulum/endoplasmic reticulum.

Na^{+}/Ca^{2+} exchanges (NCX) are bidirectional transporters that mediate the exchange of Ca^{2+} for Na^{+} using the energy of the Na^{+} gradient generated by Na^{+}/K^{+} ATPase. Our lab has previously cloned from blue crabs cDNAs encoding PMCA (Chen et al., 2013) and SERCA (Roegner et al., 2018).

The several life-cycle stages and broad geographic distribution of blue crabs increase the likelihood of their exposure to chemical dispersants used in remediation of spilled oil. That crab gills have multiple functions and are in continuous contact with seawater, suggests they may be useful targets for assessing possible effects of chemical dispersant. In studies reported here we describe effects of Corexit® EC9500A on the histology and ion transport function of blue crab gills.

2. Materials and Methods

2.1 Experimental Animals

Adult blue crabs (C. *sapidus)* were purchased from local markets in Birmingham, AL, or from Gulf Specimen Marine Laboratories, Inc. (Panacea, FL). Crabs were maintained individually under a photoperiod of 12L:12D in compartmented tanks containing artificial sea water (23°C, 25 ppt) recirculated through a filter of oyster shells and activated charcoal, fed pieces of shrimp or fish every other day, and staged according to accepted criteria (Drach and Tchernigovtzeff, 1967; Stevenson, 1972).

2.2 Determination of lethal concentration 50 (LC_{50}) value for Corexit® EC9500A

To determine a 24-hour LC_{50} value, crabs were exposed to Corexit® EC9500A (0-300 ppm) under static conditions in glass aquaria containing artificial sea water aerated using air stones. Corexit® EC9500A was kindly provided by NALCO Company (Naperville, IL). The 24-hour LC_{50} value was determined by the graphical method in accordance with guidelines described by the United States Environmental Protection Agency (United States Environmental Protection
Agency, 2002).

2.3 Histological Methods

To assess the effect of Corexit® EC9500A on blue crab gill histology, crabs were exposed to dispersant (0, 62.5 or 125 ppm) for 24 h as described above. After treatment, crabs were anesthetized on ice, and a window cut into the dorsal carapace using a Dremel instrument. Gills were excised, cut laterally into several pieces, and fixed in Davidson's fixative (Moore and Barr, 1954) for 24 h. Fixed tissue was processed utilizing a Tissue-Tek VIP 5 Vacuum Infiltration Processor, placed in warm paraffin for 72 h, and imbedded into paraffin blocks. Blocks were sectioned and tissue sections heat-fixed onto slides. Sections were stained using hematoxylin and eosin (Johnson, 1980), and viewed using a Nikon Eclipse 80i microscope. Images were captured at total magnifications of 40X, 100X, 200X and 400X using a Nikon CoolPix camera, and annotated using Nikon NIS Elements AR imaging software. To assess the possibility of edema in Corexit® EC9500A-exposed gills, the ratio of gill lamellae area/gill lamellae length was determined as previously described (Li et al., 2015). Briefly, slide, section and lamella

number were chosen using a random number generator (Random.org). Area and length measurements were done using Nikon NIS Elements AR imaging software at a total magnification of 40X.

2.4 Isolation of RNA

Tissues were dissected from control and dispersant-exposed crabs, transferred to RNAlater (Life Technologies/Ambion, Grand Island, NY, USA) and stored at −20ºC. Total RNA was extracted using TRIzol® Reagent (ThermoFisher Scientific, Waltham, MA). Tissues (10-100 mg) were disrupted and homogenized in 1 ml TRIzol using a Tissue Tearor (BioSpec Products, Inc. Bartlesville, OK), phase separated by the addition of chloroform, and RNA precipitated using 0.5 ml isopropyl alcohol. The RNA pellet was then washed with 75% ethanol and resuspended in 50 µl of RNase-free water. Concentration and purity of the RNA were determined using a Nanodrop spectrophotometer (ThermoFisher, Waltham, MA, USA).

2.5 Determination of Na^+/K^+ATPase, PMCA, SERCA and transcript abundance in gill tissue

Extracted RNA was treated with RQ1 RNase-free DNase and reverse transcribed using random hexamer primers (0.5 µg) and M-MLV reverse transcriptase (200 units) (all reagents from Promega, Madison, WI, USA). The steady-state level of the Na^+/K^+ATPase transcript was determined by quantitative real-time PCR using a Mini Opticon™ Real-Time PCR Thermal Cycler. Primers NAKq2F and NAKq2R (Table 1) were designed to amplify an 80-bp fragment of the *Na^+/K^+ATPase* target gene. Primers 18Sreal-F2 and 18Sreal-R2 (Table 1) were used to amplify a 201-bp fragment of the 18s

rRNA reference gene (Chen et al., 2013). To validate the quantitative PCR assay, PCR efficiency was assessed for both target and reference genes. cDNA was pooled from replicate samples, serially diluted (10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}), and amplified using the gene-specific primers for each amplicon. The slope of each titration curve (log dilution factor verses threshold cycle number, Ct) was used to calculate the amplification efficiency (Ginziner, 2002).

For QPCR, Perfecta SYBR® Green FastMix from QuantaBio (Beverly, MA, USA) was used, and the PCR reaction set up as follows: 10 µl SYBR Green Fast Mix, 0.5 µl forward primer, 0.5 µl reverse primer, 2.5 µl cDNA template, and 6.5µl RNase free H_2O. PCR parameters for both target and reference genes were 95 ºC for 15 sec followed by 40 cycles of amplification (denaturation at 95 ºC for 5 s, annealing for 55 ºC for 15 s, and extension at 72 ºC for 10 s). After amplification, melt curve analysis was conducted from 53 ºC to 95ºC. The relative expression of Na^+/K^+ATPase between tissue specific control and treatment groups was determined by the comparative threshold cycle method ($2^{-\Delta\Delta CT}$) (Wong and Medrano, 2005; Livak and Schmittgen, 2001). The mean ΔCt value from the 0 ppm group was used as calibrator. For each replicate sample, the real-time PCR assay was performed at least 3 times.

Steady-state levels of the PMCA transcript were determined by quantitative real-time PCR as described by Chen et al. (2013); steady-state levels of the SERCA transcript were determined by quantitative real-time PCR as described by Roegner et al. (2018). Primer sequences are shown in Table 1.

2.6 Statistical analyses

The statistical significance of differences in gill lamellae area/length ratios between control and Corexit-exposed crabs was determined by one-way analysis of variance (ANOVA), followed by Tukey's honestly significant difference (HSD) test. The robustness of the real-time PCR assays was determined by assessing the homogeneity of linear regression lines for the titration curve of the gene of interest and the titration curve of the 18s rRNA reference gene (Ginzinger, 2002). The statistical significance of differences in *Cas-PMCA, Cas-SERCA* and Na^+/K^+*ATPase* transcript abundance between control and Corexit-exposed crabs was determined by ANOVA. All statistical analyses were performed in R Commander (Fox and Bouchet-Valat, 2019).

3. Results

3.1. 24-Hour lethal concentration 50 (LC_{50}) value for Corexit® EC9500A

One measure of acute aquatic toxicity is an LC_{50} value, the concentration of a test agent that causes mortality in 50% of exposed individuals. In our initial studies, crabs were exposed to various concentrations of Corexit® EC9500A for 24 hours to determine a 24-hour LC_{50} value. Concentrations of Corexit® EC9500A at or below 125 ppm had no apparent effect on 24-hour survival; the 24-hour LC_{50} value was determined to be 210 ppm (Fig. 2). Based on the results, Corexit® EC9500A concentrations of 125 ppm (60% of LC_{50}) and 62.5 ppm (30% of LC_{50}) were selected for subsequent exposure experiments to assess possible effects of sub-lethal concentrations of Corexit® EC9500A on blue crab gill structure and ion transport function.

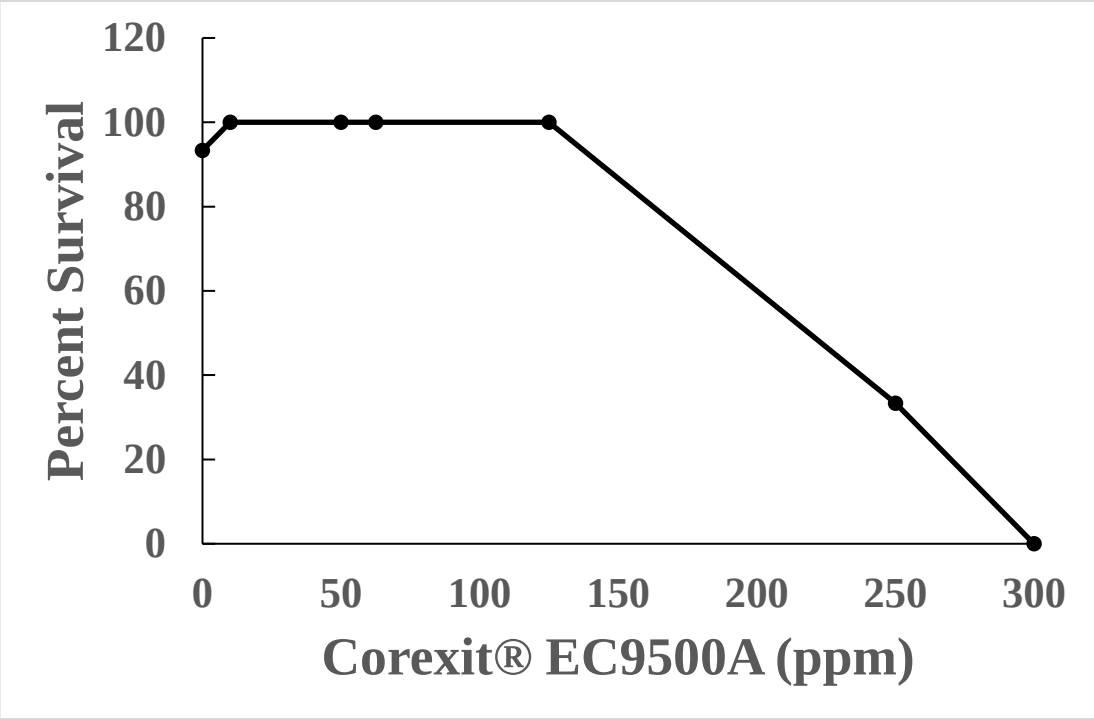

Figure 2. Effect of Corexit 9500A on the survival of adult blue crabs. Adult blue crabs were exposed to a range of Corexit 9500A concentrations (0-300 ppm) for 24 hours under static conditions in glass aquaria containing artificial sea water aerated using air stones. For each concentration of dispersant, N = 3-15.

3.2. Effect of Corexit® EC9500A on blue crab gill histology

To determine whether exposure to Corexit® EC9500A has effects on gill structure, we examined the histology of gills from control (untreated) and Corexit-exposed blue crabs. The histology of gills from control crabs (Fig. 3A) was consistent with that previously reported for the species (Johnson, 1980). Specifically, individual gill lamellae from both anterior gills (left panel) and posterior gills (right panel) were composed of two epithelial layers covered on their outer surfaces by cuticle and separated by a hemolymph space structurally stabilized by trabecular cells (Fig. 3A). Anterior gills

(left panel) and posterior gills (right panel) from crabs exposed to Corexit® EC9500A (125 ppm, 24 h; Fig. 3B) showed evidence of loss or disruption of cuticle, and an increase in stained amorphous material in the hemolymph spaces. In addition, there appeared to be edematous swelling of the lamellae. Gills from the 62.5 ppm treatment group showed damage consistent with that seen in gills from the 125 ppm treatment group (data not shown).

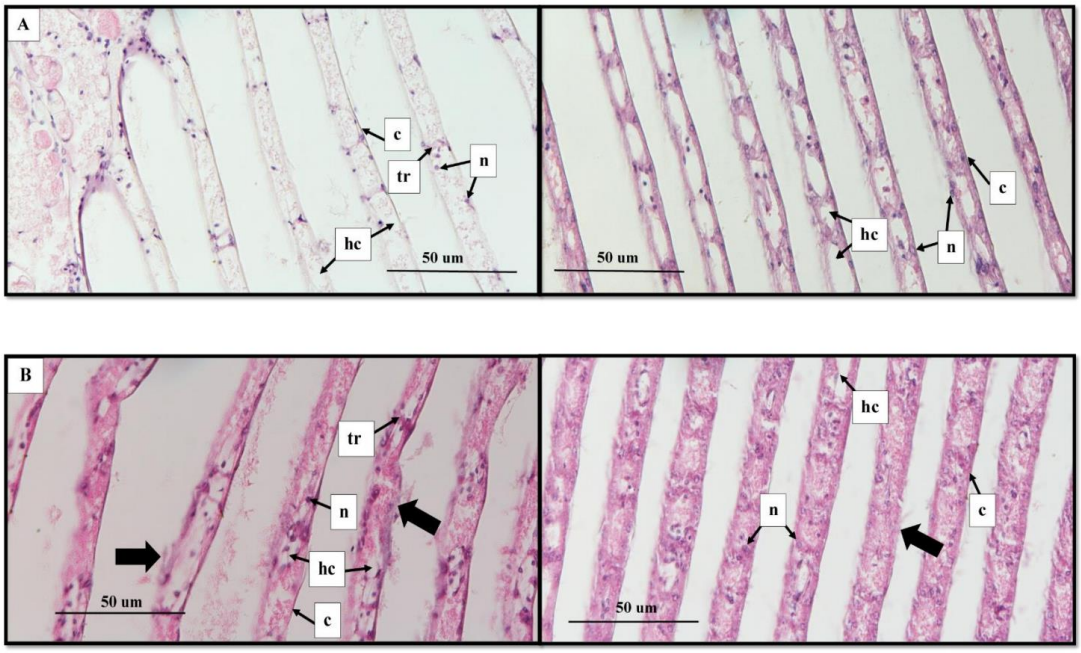

Figure 3. Effect of Corexit 9500A on blue crab gill histology. Crabs were maintained for 24 hours in glass aquaria containing artificial seawater without Corexit 9500A (**Panel A**) or with Corexit 9500A, 125 ppm (**Panel B**). After 24-h, gills were excised, fixed in Davidson's fixative, processed, stained using hematoxylin and eosin, and viewed using a Nikon Eclipse 80i microscope. For each panel, the image at left is an anterior gill and the image at right is a posterior gill, 200X. Abbreviations: hemolymph space (hc), nucleus (n), cuticle (c), trabecular cell (tr). Bold arrows indicate disruption of cuticle.

3.3. Effect of Corexit® EC9500A on area/length ratios of gill lamellae

To assess the possibility of Corexit-induced edema, area/length ratios of gill lamellae were determined by quantitative image analyses. For anterior gill lamellae, exposure to Corexit® EC9500A resulted in a concentration-dependent increase in the gill lamellae area/length ratio (Fig, 4A). For posterior gill lamellae, exposure to Corexit® EC9500A at a concentration of 62.5 ppm had no detectable effect on the gill lamellae area/length ratio, while exposure to Corexit® EC9500A at 125 ppm produced a significant increase in the gill lamellae area/length ratio (Fig. 4B).

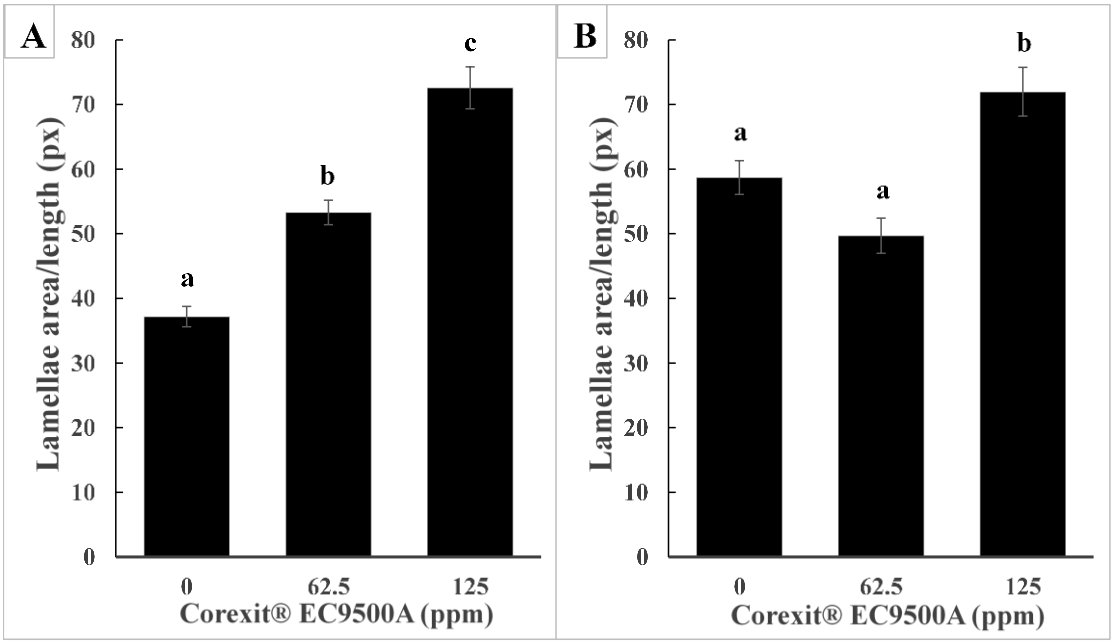

Figure 4. Area/length ratios of gill lamellae from control and Corexit 9500A-exposed blue crabs. Area and length measurements of gill lamellae in anterior gill sections (**Panel A**) and posterior gill sections (**Panel B**) from control (0 ppm) and Corexit 9500A-exposed crabs were done using Nikon NIS Elements AR imaging software at a total magnification of 40X. Bars represent mean± SD. The statistical significance of differences among means was determined by ANOVA followed by Tukey's HSD test. Bars shown with a different lowercase letter are significantly different ($p < 0.05$).

3.4. Effect of Corexit® EC9500A on SERCA, PMCA and Na^+/K^+ ATPase transcript abundance in blue crab gills

To assess possible effects of Corexit® EC9500A on the ion transport function of blue crab gills, we determined the relative abundance of transcripts encoding Na^+/K^+ ATPase, PMCA, and SERCA in anterior and posterior gills from control and Corexit-exposed crabs (Fig. 5). For posterior gills (striped bars), the relative abundance of transcripts encoding Na^+/K^+ ATPase (Fig. 5A), PMCA (Fig. 5B), and SERCA (Fig. 5C) was lower in crabs exposed to Corexit® EC9500A (125 ppm) than it was in control (0 ppm) crabs. For anterior gills (solid bars), the relative abundance of transcripts encoding Na^+/K^+ ATPase (Fig. 5A) and SERCA (Fig. 5C) was likewise lower in Corexit-exposed crabs than in control crabs. We did not detect an effect of Corexit® EC9500A on the relative abundance of the PMCA transcript in anterior gills (Fig. 5B).

4. Discussion

Results reported here show a 24-hour LC_{50} value of 210 ppm Corexit® EC9500A for adult blue crabs (Fig. 2). By comparison, the 24-hour LC_{50} value for exposure of zebrafish (*Danio rerio*) to Corexit® EC9500A was >400 ppm (see George-Ares and Clark, 2000). George-Ares and Clark (2000) reviewed 48- and 96-hour LC_{50} findings for Corexit® EC9500A exposure across a variety of crustacean, mollusk, and fish species. The reported LC_{50} values ranged from 3.5 to >1055 ppm. Interpretation of the data is

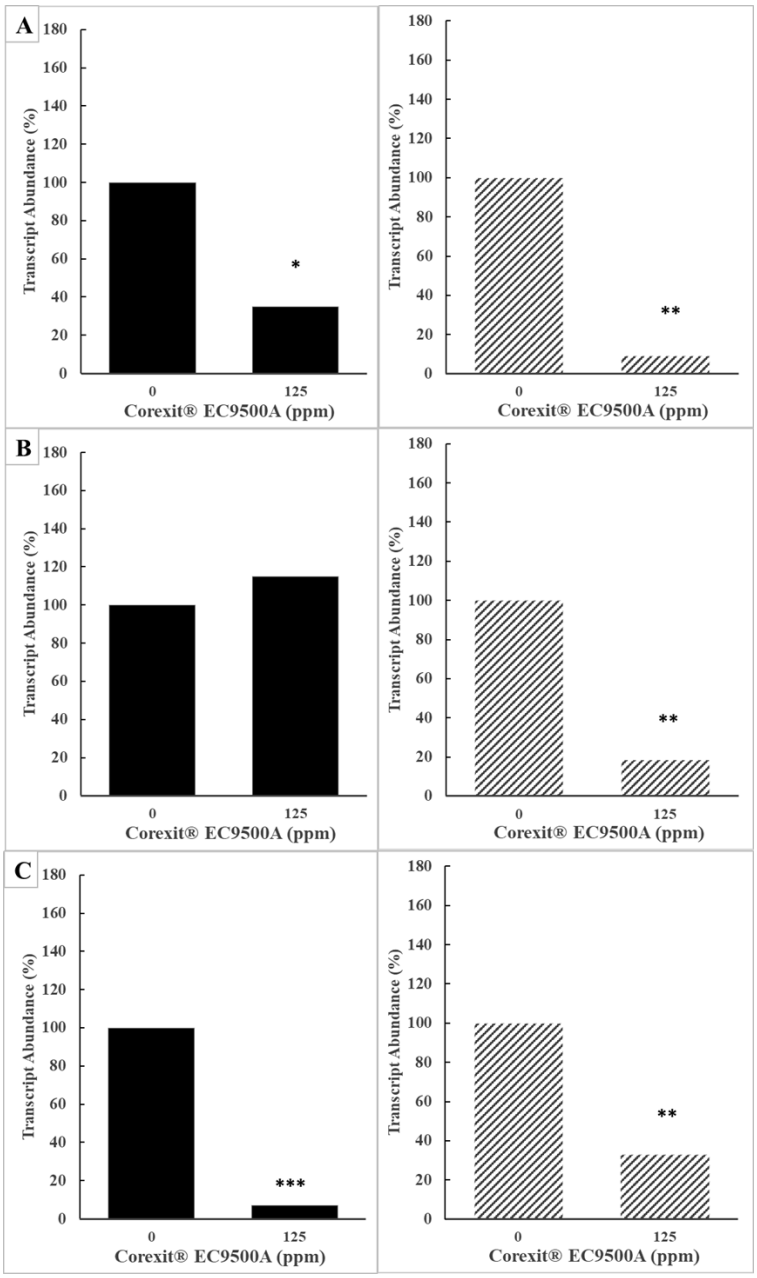

Figure 5. Abundance of the transcripts encoding Na^+/K^+ ATPase (**Panel A**), PMCA (**Panel B**) and SERCA (**Panel C**) in gills from control (0 ppm) and Corexit® 9500A-exposed crabs. For each panel, transcript abundance in anterior gills is shown in solid bars and transcript abundance in posterior gills is shown in striped bars. Relative transcript abundance was determined by quantitative PCR using the mean ΔCt value from the 0 ppm group as calibrator. Bars represent mean (N = 9-15). The statistical significance of differences between means was determined by ANOVA. A statistically significant difference between control (0 ppm) and Corexit-treated groups is indicated by an asterisk ($p < 0.05$).

complicated by the fact that some of the studies were done using spiked (declining exposure) conditions, while others were done using continuous exposure conditions. In general, however, the combined LC_{50} data confirm that increasing exposure duration results in increased dispersant toxicity and suggest that fish are less sensitive to Corexit® EC9500A than are crustaceans. The data also indicate that sensitivity may be influenced by age and life cycle stage. Thus, larval and juvenile fish are generally more sensitive to Corexit® EC9500A than are adult fish (Georg-Ares and Clark, 2000), and in crustaceans, sensitivity appears to change with molting and life cycle stage (Fucik et al., 1995).

There have been a limited number of field studies assessing the concentration of dispersant in seawater. Lewis and Aurand (1997) estimated a dispersant concentration of 5 ppm at 1 m depth based on a standard surface application rate. Bocard et al. (1984) measured a maximum dispersant concentration of 13 ppm at 0.6 m depth. Once released, dispersants are subject to dilution, dissolution and degradation, resulting in a decrease in dispersant concentration to <1 ppm within hours (National Research Council, 1989). Considered together, the LC_{50} data and the measured concentrations of dispersant in seawater indicate that Corexit® EC9500A alone is not predicted to have acutely toxic effects producing mortality in species for which 24-96 hour LC_{50} data are available. However, two additional points are worth emphasizing. First, the LC_{50} data cited above provide information on the toxicity of dispersant alone. Although the matter is not entirely settled, several lines of evidence indicate that dispersant-treated (dispersed) oil may be more toxic than oil itself. As examples, Gulec et al. (1997) reported that a water-accommodated fraction (WAF) of crude oil was less toxic to amphipods (*Allorchestes compressa*) and snails (*Polinices conicus*) than was a mixture of WAF and Corexit®

EC9500A. Bhattacharyya et al. (2003) found that Corexit® EC9500A enhanced South Louisiana crude oil toxicity in freshwater microcosms containing insects (*Chironomus tentans*), crustaceans (*Daphnia pulex*), and fish (*Oryzias latipes*). And Rico-Martinez et al. (2013) reported that a mixture of Corexit® EC9500A and Macondo crude oil was up to 52 times more toxic to a marine rotifer (*Brachionus plicatilis*) than was crude oil alone. Second, although the concentration of dispersant in seawater is reported to decrease to <1 ppm within hours of application (National Research Council, 1989), individual constituents of the dispersant may persist in the environment. Thus, dioctyl sodium sulfosuccinate (DOSS), an anionic surfactant in Corexit® EC9500A, was detected in seawater sampled from the Gulf of Mexico 64 days after subsurface application of the dispersant ceased, suggesting biodegradation of DOSS was slow or negligible (Kujawinski et al., 2011).

Multiple impacts of sub-lethal concentrations of Corexit® EC9500A have been reported for both invertebrate and vertebrate species (Gulec et al., 1997; Goodbody-Gringley et al., 2013; Almeda et al., 2014; Pie and Mitchelmore, 2015; Adeyemo et al., 2015). In the current studies, we observed that exposing blue crabs to sub-lethal concentrations of Corexit® EC9500A produced changes in gill histology (Fig. 3), and induced edema in gill lamellae (Fig. 4). The findings are consistent with a previous report by Li et al. (2015) of histological changes and edematous swelling in gill lamellae of zebrafish exposed to a sublethal concentration (150 ppm) of Corexit® EC9500A. In the same report, Li et al. (2015) found that exposing monolayers of a human bronchial epithelial cell line (BEAS-2B) to 100 ppm Corexit® EC9500A for 1 h resulted in disruption of intercellular junctions and the actin cytoskeleton as adjudged by

immunohistochemistry. The combined results suggest that exposure to sub-lethal concentrations of Corexit® EC9500A can impact the structural integrity of respiratory tissue in both invertebrate and vertebrate animals.

To assess whether dispersant-induced changes in blue crab gill structure are associated with changes in gill function, we measured the relative abundance of transcripts encoding three ion transport proteins (Na^+/K^+ ATPase, PMCA and SERCA) in anterior and posterior gills from control (untreated) and Corexit-exposed crabs (Fig. 5). For posterior gills, the relative abundance of each transcript was lower in dispersant-exposed gills than in control gills. Similarly, for anterior gills, the abundance of transcripts encoding Na^+/K^+ ATPase and SERCA was lower in dispersant exposed gills than in control gills. The reason for the lack of an observed effect on PMCA transcript abundance in anterior gills is unknown. It should be pointed out that transcript abundance is a function of both transcription and RNA stability; our methods do not discriminate between the two. It is also worth emphasizing that the differences reported in Figure 5 apply only to steady state transcript levels. Assessment of protein levels, enzyme activities, or both would provide additional insight. Nevertheless, the most straightforward interpretation of the results is that exposure of blue crabs to Corexit® EC9500A negatively impacts the ion transport function of gills. Suppression of Na^+/K^+ ATPase activity would be expected to disrupt the osmoregulatory function of crab gills (Towle and Kays, 1986; Riestenpatt et al., 1996; Orlowski and Grinstein, 1997). Because the blue crab life cycle involves migration from high salinity open seawater to brackish bays and estuaries, and then back to open seawater, disruption of the osmoregulatory function of gills may be of particular concern. Ca^{2+} homeostasis is critical to the

functioning of all cells (Kretsinger, 1976). Suppression of SERCA and PMCA activity in gills would be expected to have widespread effects on gill function, including impacts on transepithelial fluxes of Ca^{2+} associated with the molt cycle, and on gill cell viability. Structural changes observed in BEAS-2B human bronchial epithelial cells after Corexit® EC9500A exposure were associated with an increase in epithelial permeability and disruption of barrier function (Li et al., 2015). This is an important consideration for dispersant application modalities that may expose workers involved in spill remediation, coastal residents, or both (Krajnak et al., 2011; Roberts et al., 2011; Laffon et al., 2016). Based on the effects of Corexit® EC9500A on blue crab gill histology, and the associated edematous swelling, we hypothesize that the dispersant is likely to disrupt the barrier function of gill epithelia. Further studies to test this hypothesis are needed. Also needed are studies to test the hypothesis that dispersant disrupts the respiratory function of crustacean gills.

To summarize, the 24-h LC_{50} data reported here suggest that concentrations of Corexit® EC9500A in seawater during spill remediation are not likely to have acutely lethal effects on adult blue crabs. However, we observed that 24-h exposure of crabs to a sub-lethal concentration of Corexit® EC9500A (125 ppm) impacted gill histology, produced edematous swelling of gill lamellae, and suppressed the abundance in gill tissue of transcripts encoding three ion transport proteins (Na^+/K^+ ATPase, PMCA, and SERCA). The combined results are consistent with the hypothesis that 24-h exposure of blue crabs to a sublethal concentration of Corexit® EC9500A impacts both the structure and ion transport function of gills.

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Supplementary Information

Table 1. Primer sequences for quantitative real-time PCR determination of the relative abundance of transcripts encoding three ion transport proteins in *C. sapidus*.

Primer	Sequence
PMCAreal-F1	5′-GCGGGGTTGAGGGAGTAGAG-3′
PMCAreal-R1	5′-AGGTCTTC GGGGGCTTAGGA-3′
SERCAQf1	5’-GACTGAGACGGGTCTTACCGAAGG-3’
SERCAQr1	5’- GGTCTCTTCTCCTTCTTCAAAGCACG-3’
NAKq2F	5’-GAATGGTGATGCTTCTGAGG-3’
NAKq2R	5’-TTGTTGGTGGAGTTGAAAGG-3’
18Sreal-F2	5′-TCAAGTGTCTGCCTT ATCAGCT-3′
18Sreal-R2	5′-TCGGATGAGTCTCGCATCGT-3′

EXPOSURE OF BLUE CRABS (*CALLINECTES SAPIDUS)* TO A CHEMICAL DISPERSANT (COREXIT® 9500A) INDUCES IN GILLS CELLULAR EVENTS LINKED TO CYTOTOXICITY, AND ELICITS AN ENDOCRINE-MEDIATED PHYSIOLOGICAL STRESS RESPONSE

by

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In preparation for (*Comparative Biochemistry and Physiology, Part C*)

Format adapted for dissertation

Abstract

Although use of chemical dispersant has become an important tool in oil spill response strategies, there remain concerns about possible effects of dispersant on marine life and public health. We have previously reported that exposure of adult blue crabs (*Callinectes sapidus*) to sub-lethal concentrations of the dispersant Corexit® EC9500A disrupts both the structure and ion transport function of gills. Follow-up studies reported here were designed to investigate the cellular mechanisms that underlie the cytotoxic effects of dispersant on gills; specifically, to determine whether Corexit® EC9500A promotes creation of reactive oxygen species (ROS) leading to caspase-3-dependent apoptosis. Results of immunohistochemical studies indicated that exposing blue crabs to Corexit® EC9500A (125 ppm for 24 h) increased NADPH oxidase (NOX4) immunoreactivity, C-reactive protein (CRP) immunoreactivity, and cleaved (active) caspase-3 (CC3) immunoreactivity in lamellae of anterior and posterior blue crab gills. The results are consistent with the hypothesis that exposure of blue crabs to a sublethal concentration of Corexit® EC9500A triggers in gills oxidative stress, NOX4- and CRP-mediated production of ROS, and subsequent caspase-3-dependent apoptosis. Another aim of the current studies was to determine whether exposure of blue crabs to Corexit® EC9500A elicits a physiological stress response characterized by hyperglycemia. In Crustacea, a physiological stress response is mediated by an eyestalk neurohormone, crustacean hyperglycemic hormone (CHH). We found that exposing blue crabs to Corexit® EC9500A (125 ppm for 4 h) produced a transient (60 min) increase in hemolymphatic glucose, and a transient increase in abundance of the CHH transcript in eyestalk neural ganglia. The results are consistent with the hypothesis that exposure of

blue crabs to Corexit® EC9500A promotes a CHH-driven physiological stress response that includes an increase in hemolymphatic glucose.

Keywords: oil spill remediation, Corexit® EC9500A, crab gills, CHH, NOX4, CRP, CC3, apoptosis

1. Introduction

The use of chemical dispersants has become a well-established approach to oil spill remediation. Chemical dispersants are a proprietary blend of anionic and ionic surfactants in one or more hydrocarbon solvents. When applied to spilled oil, the dispersant acts as a detergent, decreasing the interfacial surface tension between oil and water. The treated oil then enters the water column as fine droplets and is subjected to degradation by natural processes (Lessard and Demarco, 2000; National Research Council, 1989). Surface application of dispersant decreases the amount of oil on the sea surface (National Research Council, 2005). Subsurface application at the wellhead decreases the ascension of oil to the surface and lowers the emission of volatile organic compounds into the atmosphere (Gros et al., 2017).

As a result of an explosion on the Deepwater Horizon (BP MC252) offshore drilling platform in 2010, an estimated 205 million gallons of crude oil were ejected from the wellhead into waters of the Gulf of Mexico (Crone and Tolstoy, 2010). In response, an estimated 2.1 million gallons of chemical dispersant, predominately Corexit® EC9500A, were applied in remediation efforts. Of that total, approximately 1.4 million gallons of dispersant were applied to the surface slick using small boats and planes; another 0.77 million gallons were applied directly at the wellhead, at a depth of 1,544 meters (Kujawinski et al., 2011).

Although Corexit® EC9500A has proven effective in dispersing spilled oil, detrimental effects of the dispersant on multiple invertebrate and vertebrate species have been reported. Examples include reduced settlement ability and survival among larvae of the corals *Porites asteroids* and *Montastraea faveolata* (Goodbody-Gringley et al., 2013),

decreased growth rates among larvae of the barnacle (*Amphibalanus improvisus*) and the acorn worm (*Schizocardium sp.*) (Almeda et al., 2014), and lowered heart rates, increased developmental abnormalities, and atypical expression of genes responsible for sexual differentiation, growth regulation and stress response in silverside fish (*Menidia beryllina*) embryos (Adeyemo et al., 2015). The experimental animal used in studies reported here is the blue crab, *Callinectes sapidus.* Pie and Mitchelmore (2015) found that exposure to Corexit® EC9500A reduced swimming activity and motility among blue crab larvae. More recently, our laboratory observed that exposing adult blue crabs to Corexit® EC9500A disrupted the structural and functional integrity of gills (Weiner et al., unpublished).

Concerns also exist about the potential impacts of chemical dispersants on human health (Goldstein et al., 2011), particularly on the health of workers involved in spill remediation efforts (Anderson et al., 2011; Roberts et al., 2014; Sriram et al., 2011). To assess possible impacts of chemical dispersant on human respiratory tissue, Li et al. (2015) assessed the effects Corexit® EC9500A on a human epithelial cell line (BEAS-2B) and found that exposure resulted in an increase in BEAS-2B monolayer permeability (an indication of acute injury) and disruption of barrier function. Cytotoxic effects of Corexit® EC9500A on several additional mammalian cell types, including neuronal cells and kidney cells, have also been reported (Zheng et al., 2014).

In an effort to understand the mechanisms that underlie the cytotoxic effects of Corexit® EC9500A, Zheng et al. (2014) exposed cultured mammalian cells to the dispersant. They found that Corexit® EC9500A increased production of reactive oxygen species (ROS) and pro-apoptotic factors, including cleaved (active) caspase-3 (CC3).

Various enzyme systems produce ROS, including the mitochondrial electron transport chain, cytochrome P450, lipoxygenase, and the NADPH oxidase (NOX4) complex (Inoue et al., 2003). ROS production is also induced by C-reactive protein (CRP) (Kobayashi et al., 2003; Ryu et al., 2007). Li et al. (2015) reported that exposure of BEAS-2B cells to Corexit® EC9500A resulted in induction of NOX4 and CRP, increased production of ROS, caspase-3 activation, and apoptosis. The results are consistent with the hypothesis that Corexit® EC9500A exposure triggers oxidative stress, NOX4- and CRP-mediated production of ROS, and subsequent caspase-3-dependent apoptosis (Fig. 1).

In companion experiments, preliminary results showed CC3 immunoreactivity in gills of blue crabs exposed to Corexit® EC9500A (Li et al., 2015). Thus, we hypothesize that the pathway shown in Figure 1 may likewise mediate negative impacts of Corexit® EC9500A on the structural and functional

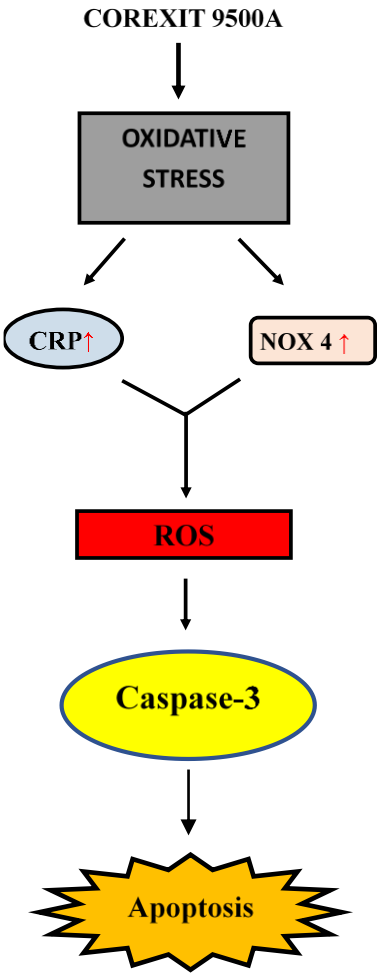

Figure 1. Cellular events hypothesized to mediate Corexit® EC9500A-induced cytotoxicity in respiratory epithelia. Exposure to Corexit® EC9500A is hypothesized to trigger oxidative stress, NOX4- and CRP-mediated production of ROS, and subsequent caspase-3-dependent apoptosis. Adapted from "Heme Oxygenase-1 Protects Corexit 9500A-Induced Respiratory Epithelial Injury across Species" by F. Li, et al., 2015, *PLoS ONE*, *10,* p. 1. Copyright 2015 by Li et al. Adapted with permission.

integrity of *C. sapidus* gills. Blue crabs have eight paired gills. Each gill consists of a double row of lamellae extending laterally from a central stem. The anterior gill pairs (pairs 1-4) function primarily in gas exchange; the osmoregulatory function of gills is attributed largely to the four posterior gill pairs (pairs 5-8) (Johnson, 1980; Towle and Burnett, 2007).

Several lines of evidence indicate that a physiological stress response in Crustacea is mediated by crustacean hyperglycemic hormone (CHH) (Fanjul-Moles, M.L., 2006), a polypeptide neurohormone produced by neurosecretory cells located on neural ganglia present in the eyestalks, and released from their associated axon terminals in the neurohemal sinus gland (Carlisle and Passano, 1953; Kleinholz, 1976). The response is characterized, at least in part, by an increase in the concentration of glucose in hemolymph. CHH increases hemolymphatic glucose through mobilization of glucose from glycogen deposits (Santos and Keller, 1993). Release of CHH, occurs in response to an endogenous circadian rhythm entrained to the light/dark cycle (Kallen et al., 1988, 1990), and to environmental stressors, including emersion-induced hypoxia (Chang et al., 1998; Webster, 1996; Chung and Webster, 2006) thermal shock (Santos et al., 1997; Chang et al., 1998; Kuo and Yang 1999; Chung and Webster, 2006), exercise (Morris et al., 2010; Turner et al., 2013), bacterial or parasitic infection (Lorenzon et al., 1997; Stentiford et al., 2001), and environmental pollutants (Reddy et al., 1996; Lorenzon et al., 2000, 2004). Considering the cytotoxic effects of Corexit® EC9500A on gill epithelia, we hypothesize that exposure of *C. sapidus* to dispersant will elicit a CHH-mediated physiological stress response.

Although the first function ascribed to CHH was regulation of hemolymphatic glucose (review: Santos and Keller, 1993), subsequent findings have led to an emerging consensus that CHH is a regulator of diverse physiological functions, including not only glucose homeostasis, but also reproduction (De Kleijn and Van Herp, 1998; Khayat et al., 1998), salt and water balance (Spanings-Pierrot et al., 2000; Serrano et al., 2003), lipid metabolism (Santos et al., 1997), production of ecdysteroid molting hormones by molting glands (Webster, 1993), and ecdysis proper (Chung et al., 1999).

Studies reported here are a step towards determining whether exposure of blue crabs to Corexit® EC9500A: (a) triggers NOX4- and CRP-mediated production of ROS and subsequent caspase-3-dependent apoptosis in gill tissue, and (b) elicits a CHH-mediated physiological stress response.

2. Materials and Methods

2.1. Experimental animals

Adult blue crabs (C. sapidus) were purchased from local markets in Birmingham, AL. Crabs were maintained individually under a photoperiod of 12L:12D in compartmented tanks containing artificial sea water (23°C, 25 ppt) recirculated through a filter of oyster shells and activated charcoal, fed pieces of shrimp or fish every other day, and staged according to accepted criteria (Drach and Tchernigovtzeff, 1967).

2.2. Histological methods

The general histology of blue crab gills was assessed as previously described (Weiner et al., unpublished). Briefly, gills were excised from ice-anesthetized crabs, cut laterally into several pieces, and fixed in Davidson's fixative (Moore and Barr, 1954) for 24 h. Fixed tissue was processed utilizing a Tissue-Tek VIP 5 Vacuum Infiltration Processor, placed in warm paraffin for 72 h, and imbedded into paraffin blocks. Blocks were sectioned and tissue sections heat-fixed onto slides. Sections were stained using hematoxylin and eosin (Johnson, 1980), and viewed using a Nikon Eclipse 80i microscope. Images were captured at total magnifications of 40X, 100X, 200X and 400X using a Nikon CoolPix camera, and annotated using Nikon NIS Elements AR imaging software.

2.3. Corexit® EC9500A exposure protocol

Crabs were exposed to Corexit® EC9500A (125 ppm) under static conditions in glass aquaria containing artificial sea water aerated using air stones. Corexit® EC9500A was kindly provided by NALCO Company (Naperville, IL).

2.3. Assessment of NOX4, CRP and CC3 immunoreactivity in gill tissue

For immunohistochemistry, gills 3,4,6 and 7 were excised from ice-anesthetized crabs, cut laterally into several pieces, and fixed in 4% formaldehyde/1x Dulbecco's phosphate-buffered saline (DPBS) (Fisher, Waltham, MA) for 1 h at 4ºC. After fixation, samples were washed overnight at 4ºC with 1x DPBS, followed by cryoprotection in 10% sucrose/1x DPBS solution overnight at 4ºC. Tissue samples were then transferred to a small mold, embedded in optimal cutting temperature (OCT) compound (Fisher, Waltham, MA), and rapidly frozen using an ethanol/dry ice bath. Frozen samples were sectioned to a thickness of 8 μm using a ThermoFisher NX50 cryostat and allowed to dry

at room temperature (RT) overnight prior to staining. Sample slides were washed at RT in a 0.1% Tween-20/1x DPBS (PBST) (Fisher, Waltham, MA) solution for 10 mins in a staining dish on a rocker to remove excess OCT. Permeabilization of the tissue samples was done by washing the slides in a 1% Triton-X/1x DPBS solution (Fisher, Waltham, MA) for 15 min at RT. Primary antibodies: (1) polyclonal goat/anti-human CRP (Bethyl Laboratories, Montgomery, TX), (2) polyclonal rabbit/anti-human NOX4 (Invitrogen, PA5-53304), and (3) polyclonal rabbit/anti-mouse CC3 (Invitrogen, Carlsbad, CA) were applied to the slides at a concentration of 1:100 and allowed to incubate in a humidity chamber overnight at 4℃. Slides were then washed in PBST solution for 2 x 20 min, followed by a 2-hour RT incubation protected from light in a 1:2000 dilution of the appropriate secondary antibodies, anti-rabbit Alexa Fluor 488 (Invitrogen, Carlsbad, CA), or anti-goat Texas Red (Invitrogen, Carlsbad, CA). The slides where then washed 2 x 20 min in PBST to remove any remaining unbound antibodies and reduce background fluorescence before applying Invitrogen™ProLong™Gold Antifade Mountant with 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen, Carlsbad, CA), and adding coverslip. Fluorescence microscopy and imaging was performed either on an EVOS FL Cell Imagining System (Fisher, Waltham, MA), or a Nikon Eclipse 80i microscope. Images were post-processed using Nikon NIS Elements (AR) imaging software.

2.4. Determination of hemolymphatic glucose levels

Hemolymph was drawn through the arthrodial membrane at the base of the walking legs using a needle and syringe. Draws were done between 10:00-11:00 am in order to reduce the potentially confounding influence of circadian changes in hemolymphatic glucose concentration (Kallen et al., 1990). Hemolymph samples (500

µl) were immediately diluted with 125 µl Pantin's saline containing 0.01M EDTA to prevent clotting, centrifuged (2,000g, 20 min, 4°C) and stored at −80°C (Lee et al., 2000). The glucose concentration in the supernatant was determined using a Glucose Assay Kit (Abcam, Cambridge, MA) according to the manufacturer's protocol. Briefly, the diluted hemolymph preparation (50 µl) and assay reagent (500 µl) were added to 1.5-ml centrifuge tubes and mixed by vortex. Samples were heated (100°C, 8 min), and then cooled on ice for 4 min. The optical density was read using a BioTek Synergy H1 Microplate Reader (Winooski, VT, USA). A standard curve was generated for each microplate read using the kit glucose standard. Glucose concentrations in the samples were determined from the standard curve and corrected for dilution according to manufacturer's guidelines. Statistical analysis was performed using a one-way analysis of variance (ANOVA) with Tukey's honestly significant difference (HSD) test.

2.5. Isolation of RNA

Eyestalks were clipped from ice-anesthetized control and dispersant-exposed crabs. Neural ganglia were dissected from eyestalks under Pantin's saline (Pantin, 1934), transferred to RNAlater (Life Technologies/Ambion, Grand Island, NY, USA), and stored at −20ºC. Total RNA was extracted using TRIzol® Reagent (ThermoFisher Scientific, Waltham, MA). Tissues (10-100 mg) were disrupted and homogenized in 1 ml TRIzol using a Tissue Tearor (BioSpec Products, Inc. Bartlesville, OK), phase separated by the addition of chloroform, and RNA precipitated using 0.5 ml isopropyl alcohol. The RNA pellet was washed with 75% ethanol and resuspended in 50 µl of RNase-free water. Concentration and purity of the RNA were determined using a Nanodrop spectrophotometer (ThermoFisher, Waltham, MA, USA).

2.6. Determination of CHH transcript abundance in C. sapidus eyestalks

Extracted RNA was treated with RQ1 RNase-free DNase and reverse transcribed using random hexamer primers (0.5 µg) and M-MLV reverse transcriptase (200 units) (all reagents from Promega, Madison, WI, USA). The steady-state level of the CHH transcript (Cs-CHH1) was determined by quantitative real-time PCR using a Mini Opticon™ Real-Time PCR Thermal Cycler as previously described (Zheng et al., 2010). Briefly, forward (CHHrtimeF1: 5' CTGTATGATGGCCACGATCTCA 3') and reverse (CHHrtimeR1: 5' CAGCTCGTTGAAGATGGCTCTGT 3') primers were designed to amplify a 205-bp fragment of a CHH gene (CsCHH-1) previously cloned from eyestalk ganglia of C. sapidus (Choi et al., 2006). Control primers (18Sreal-F2: 5' TCAAGTGTCTGCCTTATCAGCT 3' and 18Sreal-R2: 5' TCGGATGAGTCTCGCATCGT 3') were designed to amplify a 201-bp fragment of the 18s rRNA reference gene (Zheng et al., 2010). To validate the quantitative PCR assay, PCR efficiency was assessed for both target and reference genes. cDNA was pooled from replicate samples, serially diluted (10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}), and amplified using the gene-specific primers for each amplicon. The slope of each titration curve (log dilution factor verses threshold cycle number, Ct) was used to calculate the amplification efficiency (Ginzinger, 2002).

For QPCR, Perfecta SYBR® Green FastMix from QuantaBio (Beverly, MA, USA) was used, and the PCR reaction set up as follows: 10 µl SYBR Green Fast Mix, 0.4 µl forward primer, 0.4 µl reverse primer, 1 µl cDNA template, and 8.2 µl RNase free H2O. PCR parameters for the target gene were 94 ºC for 3 min followed by 45 cycles of amplification (denaturation at 94 ºC for 15 s, annealing at 58.5 ºC for 15 s, and extension

at 72 ºC for 30 s). PCR parameters for the reference gene were the same as described for the target gene except the annealing temperature was 56 ºC. The relative expression of CHH between tissue specific control and treatment groups was determined by the comparative threshold cycle method (2-ΔΔCT) (Wong and Medrano, 2005; Livak and Schmittgen, 2001). The mean ΔCt value from the 0 ppm group was used as calibrator. For each replicate sample, the real-time PCR assay was performed at least 3 times.

2.7. Determination of the hemolymphatic ecdysteroid titer

Hemolymph (1 ml) was drawn from crabs as described above, diluted 1:3 with methanol, centrifuged (15 min, 1500g), and the supernatant saved. Pellets were extracted two additional times with 2 ml methanol, and the supernatants combined with the first. The combined supernatants were dried under a gentle stream of air, and the dried residue dissolved in 1 ml methanol. Aliquots were removed, dried using a SpeedVac Concentrator (Savant), and their ecdysteroid content determined by radioimmunoassay as previously described (Watson et al., 1996). Briefly, [23,24-^{3}H] ecdysone (PerkinElmer Waltham, MA; specific activity: 69.1Ci/mmol) was used as the labeled ligand, and ecdysone (standard range: 0.05-4.0 ng) was used as the unlabeled ligand. The antiserum (HA-22E) was generated in rabbits against an ecdysone-22-succinylthyroglobin conjugate synthesized by Dr. D.H.S. Horn (CSIRO, Melbourne, Australia). Assays were terminated using protein A (Warren et al., 1984).

2.8. Statistical analysis

Statistical significance of variations in hemolymph ecdysteroid levels: (1) between treatment and control group at each time mark, (2) and within the control and the treatment groups across all time points, were determined separately by one-way analysis

of variance (ANOVA) and followed by Tukey's HSD test, performed in R commander (Fox, 2005).

3. Results

3.1. Histology of blue crab gills

Histological features of crustacean gill lamellae are shown in Figure 2. Each gill consists of a double row of lamellae radiating from a central stem (not shown). Individual gill lamellae from the anterior gills (Fig. 2, left panel) and posterior gills (Fig. 2, right panel) are composed of two epithelial layers creating a sac-like structure and an

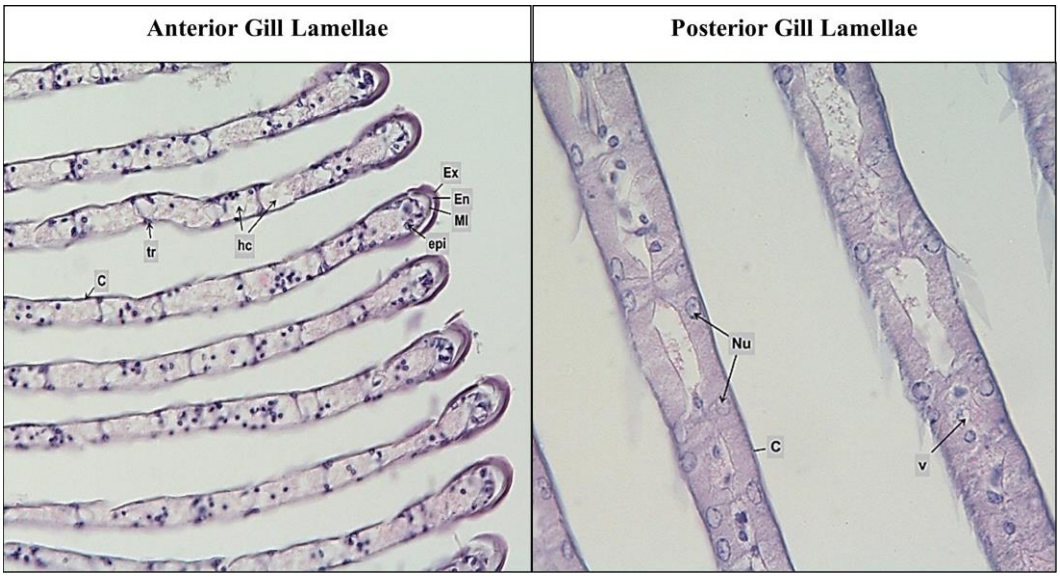

Figure 2. Histology of blue crab gills. Gills were fixed in Davidson's fixative, processed, stained using hematoxylin and eosin, and viewed using a Nikon Eclipse 80i microscope. Anterior gill lamellae are shown at left, *200X*; posterior gill lamellae are shown at right, *400X*. Abbreviations are as follows: *c,* cuticle; *epi,* epithelium; *Ex,* exocuticle; *En,* endocuticle; *Ml,* Membranous layer*; hs*, hemolymph space; *Nu,* nucleus; *tr*, trabecular cell; *v,* vein.

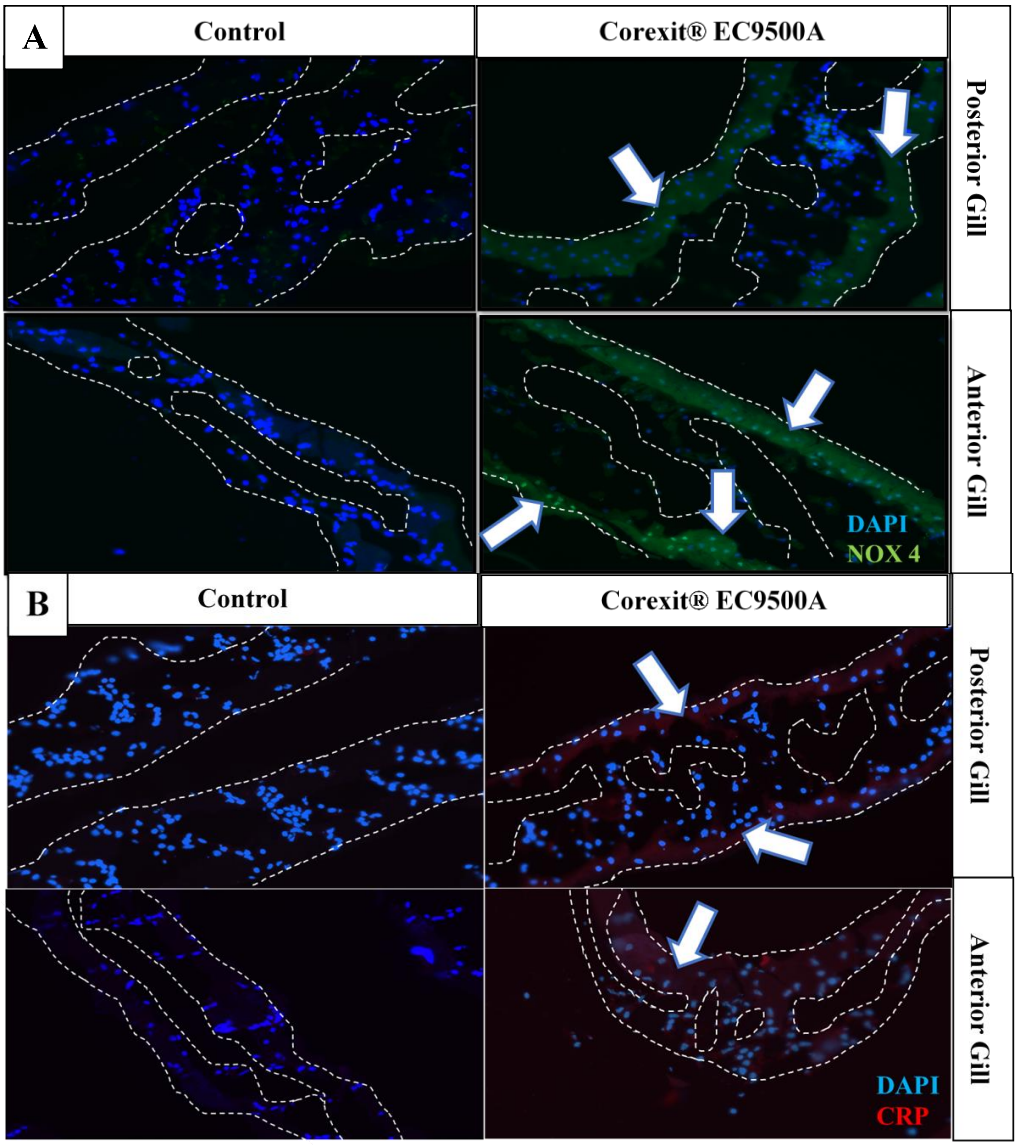

Figure 3. Exposure to Corexit 9500A increases NOX4 and CRP immunoreactivity in blue crab gill lamellae. Crabs were maintained for 24 h in artificial seawater (Control) or artificial seawater containing 125ppm Corexit 9500A. Gills were fixed and processed for immunohistochemistry. Representative images of posterior and anterior gill sections are shown. **Panel A:** For assessment of NOX4 immunoreactivity (green immunofluorescence**),** the primary antibody was polyclonal rabbit/anti-human NOX4, and the secondary antibody was anti-rabbit Alexa Fluor 488. **Panel B:** For assessment of CRP immunoreactivity (red immunofluorescence**),** the primary antibody was polyclonal goat/anti-human CRP, and the secondary antibody was anti-goat Texas Red. For both panels, the coverslip mountant contained DAPI nuclear stain (blue). White arrows indicate areas of immunoreactivity.

internal hemolymph space. The epithelial layers are covered by a tri-layered cuticle and supported internally by structurally stabilizing trabecular cells.

3.2. Effect of Corexit® EC9500A on NOX4, CRP and CC3 immunoreactivity in gill tissue

Immunohistochemistry was used to assess the possible involvement of NOX4, CRP, and CC3 in mediating the cytotoxic effects of Corexit® EC9500A on blue crab gills. NOX4 immunoreactivity (Fig. 3A) and CRP immunoreactivity (Fig. 3B) were observed in epithelia of both posterior and anterior gills from crabs exposed to 125 ppm Corexit® EC9500A for 24 hours. Neither NOX4 immunoreactivity (Fig. 3A) nor CRP immunoreactivity (Fig. 3B) was apparent in gills of control crabs. CC3, an active form of the proenzyme caspase-3, is a generally accepted cellular marker for apoptosis activation (Cohen, 1997; Earnshaw et al., 1999). Strong CC3 immunofluorescence was observed in epithelia of posterior gills from exposed crabs (Fig. 4); CC3 was not apparent in anterior or posterior gills from control crabs (Fig. 4). CC3 immunoreactivity was detected in anterior gill epithelia from Corexit-exposed crabs, but the signal was considerably weaker than that observed in epithelia of posterior gills from exposed crabs (Fig. 4).

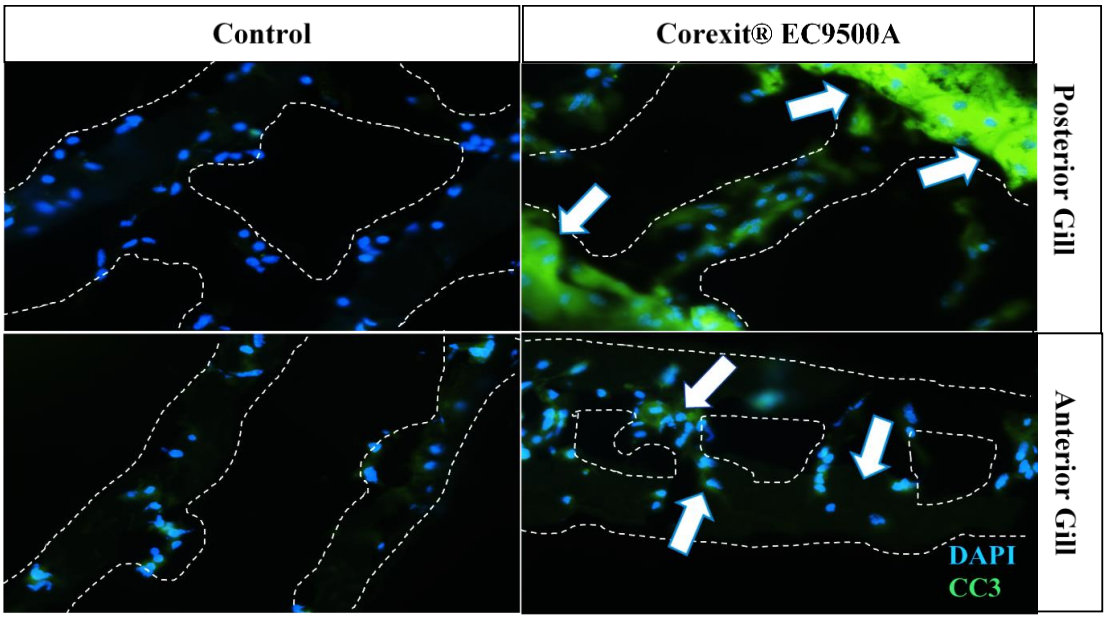

Figure 4. Exposure to Corexit® EC9500A increases CC3 immunoreactivity in blue crab gill lamellae. Crabs were maintained for 24 h in artificial seawater (Control) or artificial seawater containing 125ppm Corexit® EC9500A. Gills were fixed and processed for immunohistochemistry. Representative images of posterior and anterior gill sections are shown. For assessment of CC3 immunoreactivity (green immunofluorescence**),** the primary antibody was polyclonal rabbit/anti-mouse CC3, and the secondary antibody was anti-rabbit Alexa Fluor 488. The coverslip mountant contained DAPI nuclear stain (blue). White arrows indicate areas of immunoreactivity.

3.2. Effect of Corexit® EC9500A on hemolymphatic glucose levels

To assess the possibility that exposure to Corexit® EC9500A elicits a physiological stress response in *C. sapidus*, the concentration of glucose in hemolymph was determined in Corexit-exposed (125 ppm) and control (0 ppm) crabs over a time course of four hours. For control crabs, the concentration of glucose in hemolymph did not change significantly during the 4-hour exposure ($p > 0.05$). For crabs exposed to Corexit® EC9500A (125 ppm), the level of glucose in blood increased from 31 mg/dl at 0 h to 229 mg/dl at 1 h ($p < 0.05$), and then dropped to a basal level for the remainder of

the 4-h time course (Fig. 5). The concentration of glucose in hemolymph of Corexit-exposed crabs was greater than that in control crabs at 1 h ($p < 0.05$); hemolymphatic glucose levels did not differ between Corexit-exposed and unexposed crabs at any of the other time points ($p > 0.05$).

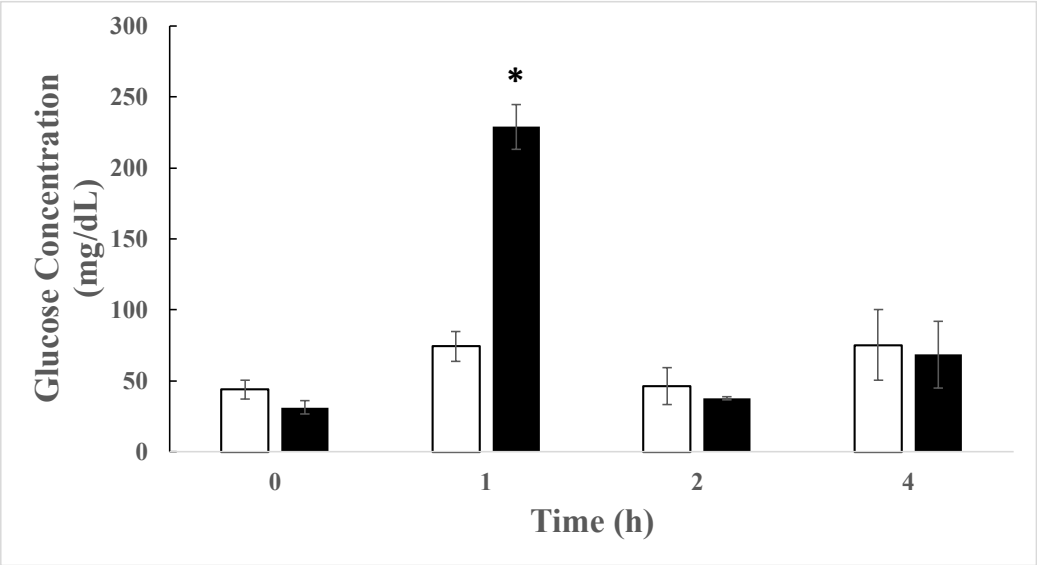

Figure 5. Effect of Corexit® EC9500A on the concentration of glucose in blue crab hemolymph. Crabs were maintained in artificial seawater (open bars) or artificial seawater containing Corexit® EC9500A, 125ppm (solid bars). Hemolymph was drawn at the timepoints indicated, and glucose content determined using a commercially available kit. Each bar represents a mean ± SEM (N = 3-5). A statistically significant difference in hemolymphatic glucose concentration between control and Corexit-exposed crabs is indicted by an asterisk ($p < 0.05$).

3.3. Effect of Corexit® EC9500A on CHH transcript abundance in blue crab eyestalk ganglia

As a step toward determining whether the increase in hemolymphatic glucose observed in dispersant-exposed crabs was driven by CHH, we measured the relative abundance of the *Cs-CHH1* transcript in eyestalk neural ganglia of Corexit-exposed (125

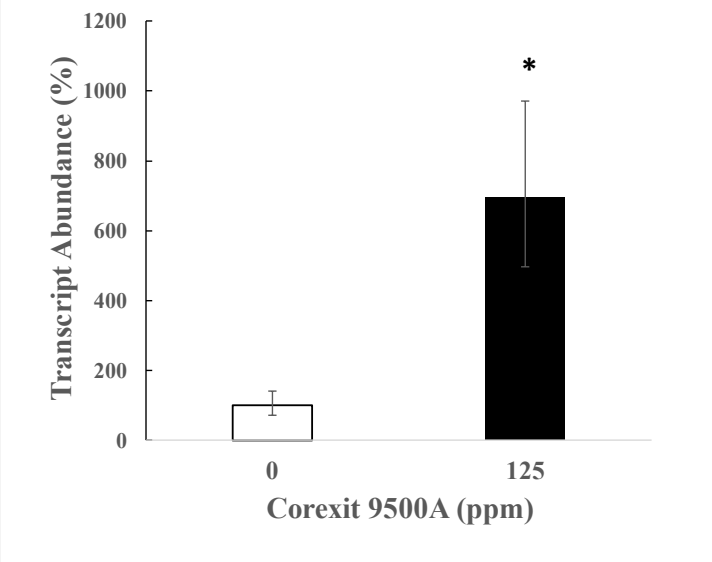

Figure 6. Abundance of transcripts encoding Cs-CHH1 in eyestalk ganglia from control (0 ppm) and 125ppm Corexit-exposed crabs. Relative transcript abundance was determined by quantitative PCR using the mean ΔCt value from the 0 ppm group as calibrator. Each bar represents a mean ± SEM (N = 15). The statistical significance of differences between means was determined by ANOVA. A statistically significant difference between control (0 ppm) and Corexit-exposed (125 ppm) groups is indicated by an asterisk ($p < 0.05$).

ppm, 1 h) and control (0 ppm, 1 h) crabs. As shown in Figure 6, the relative abundance of the *Cs-CHH1* transcript was 7% higher in eyestalk ganglia of Corexit-exposed crabs than it was in eyestalk ganglia of control crabs ($p < 0.05$).

3.4. Effect of Corexit® EC9500A on the hemolymphatic ecdysteroid titer

The concentration of ecdysteroids in hemolymph of Corexit-exposed and control crabs was determined by radioimmunoassay. For Corexit-exposed crabs, the concentration of ecdysteroids in hemolymph ranged from 3.95 – 4.90 ng/ml (Fig. 7); ecdysteroid content of hemolymph did not change significantly during the 4-hour exposure ($p > 0.05$). For control crabs, the ecdysteroid content of hemolymph ranged from 3.23 – 4.03 ng/ml (Fig. 7); differences between time points were not statistically significant ($p > 0.05$). The concentration of ecdysteroids in hemolymph of

Corexit-exposed crabs was not significantly different from that in hemolymph of control crabs at any time point ($p > 0.05$).

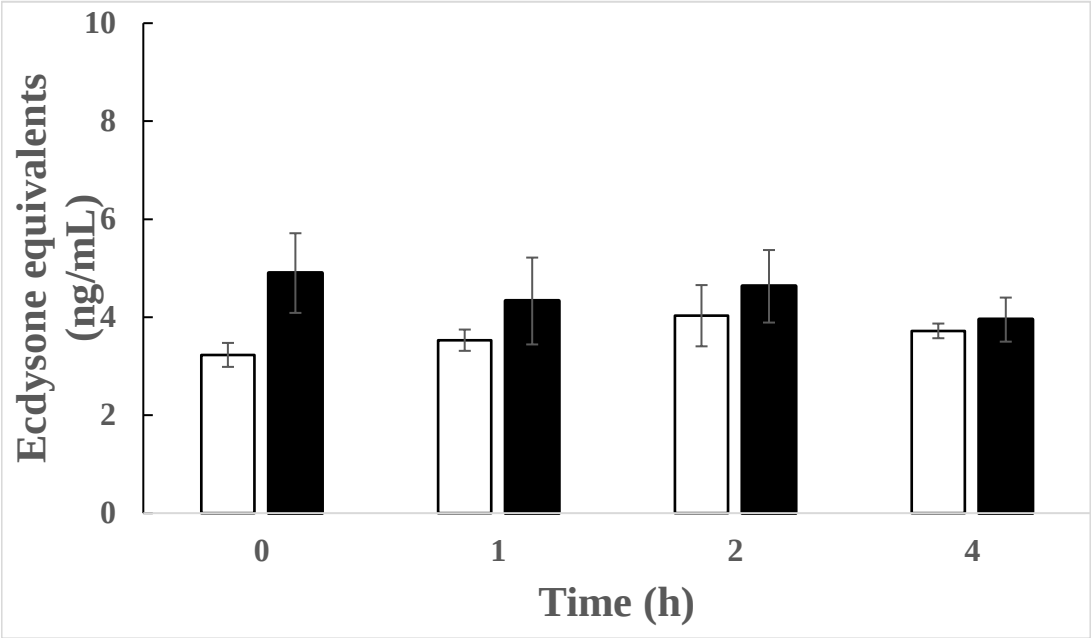

Figure 7. Effect of Corexit® EC9500A on the hemolymphatic ecdysteroid titer in *C. sapidus*. Crabs were maintained in artificial seawater (open bars) or artificial seawater containing Corexit® EC9500A, 125ppm (solid bars). Hemolymph was drawn at the timepoints indicated, and ecdysteroid content determined by radioimmunoassay. Each bar represents a mean ± SEM (N = 5-6). There were no statistically significant differences between means as determined by ANOVA.

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4. Discussion

We have previously reported that exposure of adult blue crabs to sub-lethal concentrations of Corexit® EC9500A negatively impacts both the structure and ion transport function of gills. More specifically, a histological assessment of gills from crabs exposed for 24 h to Corexit® EC9500A (125 ppm) revealed loss or disruption of cuticle, and edematous swelling of lamellae (Weiner et al., unpublished). In addition, the relative abundance of transcripts encoding three ion transport proteins (Na^+/K^+ ATPase, plasma

membrane Ca^{2+} ATPase (PMCA), and sarcoplasmic reticulum/endoplasmic reticulum Ca^{2+} ATPase (SERCA)) was lower in gills from Corexit-exposed crabs than it was in gills from control crabs (Weiner et al., unpublished). Suppression of Na^{+}/K^{+} ATPase activity would be expected to disrupt the osmoregulatory function of crab gills (Towle and Kays, 1986; Riestenpatt et al., 1996; Orlowski and Grinstein, 1997). Suppression of SERCA and PMCA activity in gills would be expected to have widespread effects on gill function, including impacts on transepithelial fluxes of Ca^{2+} associated with the molt cycle, and on gill cell viability.

The above findings are consistent with a previous report by Li et al. (2015) of histological changes and edematous swelling in gill lamellae of zebrafish exposed to a sublethal concentration (150 ppm) of Corexit® EC9500A. In that same report, Li et al. (2015) found that exposing monolayers of a human bronchial epithelial cell line (BEAS-2B) to 100 ppm Corexit® EC9500A for 1 h resulted in disruption of intercellular junctions and the actin cytoskeleton as adjudged by immunohistochemistry. Structural changes observed in BEAS-2B human bronchial epithelial cells after Corexit® EC9500A exposure were associated with an increase in epithelial permeability and disruption of barrier function (Li et al., 2015). Shi et al. (2014) also observed cytotoxic effects of Corexit® EC9500A on BEAS-2B cells. The combined results indicate that exposure to Corexit® EC9500A negatively impacts respiratory epithelia across taxa, including terrestrial, freshwater, and marine species.

In studies designed to determine the mechanism by which dispersant elicits cytotoxic effects in respiratory epithelia, Li et al. (2015) found that exposure of human airway epithelial cells and mice lungs to Corexit® EC9500A triggers oxidative stress,

NOX4- and CRP-mediated production of ROS, and subsequent caspase-3-dependent apoptosis. In comparative experiments, exposure to Corexit® EC9500A resulted in an increase in NOX4 immunoreactivity and CRP transcript abundance in zebrafish gills, and induction of CC3 immunoreactivity in blue crab gills (Li et al., 2015). In the current studies, we observed NOX4 immunoreactivity and CRP immunoreactivity in gills of Corexit-exposed blue crabs. Confirming our previous observation, we also observed CC3 immunoreactivity in gills of Corexit-exposed blue crabs. It's worth emphasizing that the methods used in the studies reported here provide a measure of immunoreactivity rather than bioactivity. Needed are experiments designed to assess dispersant-induced changes in the bioactivity of NOX4, CRP, and CC3, including measurements of ROS production and apoptosis. Nevertheless, considered together, the combined results are consistent with the hypothesis that Corexit® EC9500A exposure triggers oxidative stress, NOX4- and CRP-mediated production of ROS, and subsequent caspase-3-dependent apoptosis (see Fig. 1), and that the mechanisms underlying dispersant-induced cytotoxicity in respiratory epithelia are common across taxa.

To assess the possibility that exposure to Corexit® EC9500A induces a physiological stress response in blue crabs, we measured hemolymphatic glucose levels over a 4-h time course in dispersant-exposed and control crabs. We observed a statistically significant increase in hemolymphatic glucose at 1 h of exposure; the increase was transient as glucose fell to a basal level by 2 h and then remained low through 4 h of exposure. The result is consistent with a previous report that exposing blue crabs to emersion stress results in a significant but transient (60 min) increase in hemolymphatic glucose (Santos and Colares, 1989). Exposing the red swamp crayfish

(*Procambarus clarkii*) to thermal stress elicited an increase in hemolymphatic glucose beginning at 30 min, peaking at 1 h, and then dropping gradually to basal levels by 4 h of exposure (Zou et al., 2003). Other studies have found a more prolonged increase in hemolymphatic glucose after exposure to stressors. For example, exposure of rockpool shrimp (*Palaemon elegans*) to Hg^{2+} (0.5 mg/l) resulted in an increase in hemolymphatic glucose beginning at 30 min, peaking at 3 h and remaining elevated above basal levels through 5 h of exposure (Lorenzon, 2005). Additional aspects of the hyperglycemic stress response seen in crustaceans have been recently reviewed (Chen et al., 2020).

Multiple reports indicate that stress-induced hyperglycemia in Crustacea is stimulated by CHH (Chen et al., 2020). As examples, Zou et al. (2003) reported that exposing *P. clarkii* to thermal stress resulted in an increase in the hemolymphatic CHH titer, and that the pattern of change in hemolymphatic CHH was temporally and quantitatively similar to the pattern of change in hemolymphatic glucose. And Chung and Zmora (2008) found that injecting CHH into *C. sapidus* increased hemolymphatic glucose by five-fold, while exposing *C. sapidus* to hypoxic stress produced an increase in the concentration of CHH and glucose in hemolymph. In studies reported here, we found the relative abundance of the *Cs-CHH1* transcript was substantially higher in eyestalk ganglia of Corexit-exposed crabs than it was in eyestalk ganglia of control crabs. It should be pointed out that transcript abundance is a function of both transcription and RNA stability; our methods to not discriminate between the two. It is also worth emphasizing that the differences reported in Figure 6 apply only to steady state transcript levels. Assessment of CHH polypeptide levels in hemolymph would provide additional insight. Nevertheless, our findings are consistent with the hypothesis that exposure of

blue crabs to Corexit® EC9500A promotes a CHH-driven increase in hemolymphatic glucose as part of a physiological stress response.

Mattson and Spaziani (1985) found that daily 30-min intervals of handling stress caused a significant decrease in the hemolymphatic titer of ecdysteroid molting hormones in intact, but not eyestalk-ablated, Pacific rock crabs (*Cancer antennarius*). Because crab Y-organs, the glandular sources of ecdysteroids, possess CHH receptors (Webster, 1993), and CHH in high dose suppresses ecdysteroid production by Y-organs *in vitro* (Webster and Keller, 1986), we hypothesized that a CHH-driven stress response might include an effect on the hemolymphatic ecdysteroid titer. Our results (Fig. 7) showed no effect of the dispersant on the ecdysteroid titer in *C. sapidus*. Given the apparent transient surge in CHH, it is possible that either the magnitude or the duration of the CHH stimulus was not sufficient to influence ecdysteroidogenesis. It is also possible that the 4-hr time course of the experiment was not sufficient to observe an effect of stress on the ecdysteroid titer.

To summarize, results reported here are consistent with the hypothesis that exposure of blue crabs to a sublethal concentration of Corexit® EC9500A triggers oxidative stress, NOX4- and CRP-mediated production of ROS, and subsequent caspase-3-dependent apoptosis. The findings support the notion that the mechanisms underlying dispersant-induced cytotoxicity in respiratory epithelia are common across taxa. Results of the current study are also consistent with the hypothesis that exposure of blue crabs to Corexit® EC9500A promotes a CHH-driven physiological stress response that includes an increase in hemolymphatic glucose.

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CALCIUM SIGNALING AND REGULATION OF ECDYSTEROIDOGENESIS IN CRUSTACEAN Y-ORGANS

by

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Abstract

Crustacean Y-organs secrete ecdysteroid molting hormones. Ecdysteroids are released in increased amount during premolt, circulate in hemolymph, and stimulate the events in target cells that lead to molting. During much of the molting cycle, ecdysteroid production is suppressed by molt-inhibiting hormone (MIH), a peptide neurohormone produced in the eyestalks. The suppressive effect of MIH is mediated by a cyclic nucleotide second messenger. A decrease in circulating MIH is associated with an increase in the hemolymphatic ecdysteroid titer during pre-molt. Nevertheless, it has long been hypothesized that a positive regulatory signal or stimulus is also involved in promoting ecdysteroidogenesis during premolt. Data reviewed here are consistent with the hypothesis that an intracellular Ca^{2+} signal provides that stimulus. Pharmacological agents that increase intracellular Ca^{2+} in Y-organs promote ecdysteroidogenesis, while agents that lower intracellular Ca^{2+} or disrupt Ca^{2+} signaling suppress ecdysteroidogenesis. Further, an increase in the hemolymphatic ecdysteroid titer after eyestalk ablation or during natural premolt is associated with an increase in intracellular free Ca^{2+} in Y-organ cells. Several lines of evidence suggest elevated intracellular calcium is linked to enhanced ecdysteroidogenesis through activation of Ca^{2+}/calmodulin dependent cyclic nucleotide phosphodiesterase, thereby lowering intracellular cyclic nucleotide second messenger levels and promoting ecdysteroidogenesis. Results of transcriptomic studies show genes involved in Ca^{2+} signaling are well represented in Y-organs. Several recent studies have focused on Ca^{2+} transport proteins in Y-organs. Complementary DNAs encoding a plasma membrane Ca^{2+} ATPase (PMCA) and a sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) have been cloned from crab

Y-organs. The relative abundance of PMCA and SERCA transcripts in Y-organs is elevated during premolt, a time when Ca^{2+} levels in Y-organs are likewise elevated. The results are consistent with the notion that these transport proteins act to maintain the Ca^{2+} gradient across the cell membrane and re-set the cell for future Ca^{2+} signals.

Keywords: Ca^{2+} signaling, Y-organs, ecdysteroid, crustacean molting, molt-inhibiting hormone

1. Introduction

1.1 Calcium Signaling

The concentration of Ca^{2+} outside of eukaryotic cells is approximately four orders of magnitude greater than the concentration of Ca^{2+} in cytosol (~100 nM) (Kretsinger, 1976). In addition, eukaryotic cells sequester Ca^{2+} inside organelles, including the endoplasmic reticulum (ER), mitochondria, and Golgi apparatus (Wheatly et al., 2002a; Li et al., 2013). Cells use the resulting transmembrane Ca^{2+} gradients to drive intracellular Ca^{2+} signals. A Ca^{2+} signal is triggered by ligand-induced activation of a plasma membrane receptor, or by an electrical signal which causes the opening of Ca^{2+} channels in the plasma membrane and the membranes of organelles (Carafoli et al., 2001). Once released into the cytosol, Ca^{2+} triggers a downstream response by interacting with Ca^{2+} sensor proteins, the most common of which is calmodulin (Guerini et al., 2005). To preserve the functional integrity of the Ca^{2+} signal, and to prevent cellular toxicity, the intracellular Ca^{2+} concentration is tightly regulated. The Ca^{2+} concentration in cytosol is controlled mainly by proteins intrinsic to the plasma membrane and to the membranes of organelles. Two main families of proteins are involved, Ca^{2+} pumps (ATPases) and Ca^{2+} exchangers. The family of Ca^{2+} pumps include a plasma membrane Ca^{2+} ATPase (PMCA) and a sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA). The former transports Ca^{2+} out of the cell; the latter transports Ca^{2+} into the sarcoplasmic/endoplasmic reticulum (Carafoli et al., 2001; Guerini et al., 2005). Na^+/Ca^{2+} exchangers (NCX) are bidirectional transporters that mediate the exchange of Ca^{2+} for Na^+ using the energy of the Na^+ gradient generated by Na^+/K^+ ATPase (Philipson and

Nicoll, 2000). While NCX in the plasma membrane is primarily responsible for large scale removal of Ca^{2+}, more specific and localized regulation falls to the Ca^{2+} pumps (Flik et al., 1994; Philipson and Nicoll, 2000). In general, the pumps and exchangers re-set the cell for future Ca^{2+} signals and maintain the Ca^{2+} gradient across the cell membrane.

Calcium signaling is involved in the regulation of multiple developmental and physiological processes, including fertilization, embryonic pattern formation, programmed cell death, muscle contraction, and neurotransmitter release (Berridge, et al., 2000; Clapham, 2007; Kahl and Means, 2003; Slusarski and Pelegri, 2007; Whitaker, 2006). Data reviewed here are consistent with the hypothesis that calcium signaling is also involved in the regulation of steroid hormone synthesis by crustacean molting glands (Y-organs).

1.2. Endocrine regulation of crustacean molting

As background, incremental growth occurs in crustaceans when the calcified exoskeleton is shed (molted), and a pliant new exoskeleton, previously formed beneath the old one, is expanded by absorption of water and hardened by mineralization. The newly hardened and larger exoskeleton is then capable of accommodating additional soft tissue growth. The molting process is cyclic and can be divided into the following stages: intermolt (C), pre-molt (D_0-D_4), ecdysis (molting) (E), and post-molt (A-B) (Skinner, 1985). The cellular events that lead to molting are driven by C-27 steroid hormones (ecdysteroids) synthesized by Y-organs, paired endocrine glands located in the anterior cephalothorax (Echalier, 1954; Lachaise et al. 1993). Briefly, Y-organs take up

lipoprotein-bound cholesterol from the surrounding hemolymph and use it as a biosynthetic precursor for synthesis of ecdysteroids (Watson and Spaziani, 1985a, 1985b). Ecdysteroids are released in increased amount during premolt, circulate in hemolymph, bind to nuclear receptors, and stimulate the events in target cells that lead to molting (Lachaise et al., 1993; Riddiford et al., 2000).

During intermolt, synthesis of ecdysteroids by Y-organs is suppressed by molt-inhibiting hormone (MIH), a polypeptide neurohormone produced by neurosecretory cells located on neural ganglia present in the eyestalks, and released from their associated axon terminals in the neurohemal sinus gland (Lachaise et al., 1993; Nakatsuji et al., 2009; Schoettker and Gist, 1990). Thus, surgically ablating the eyestalks, or silencing the MIH gene through RNA interference, leads to enhanced ecdysteroid synthesis by Y-organs, an increase in the hemolymphatic ecdysteroid titer, and precocious molting (Keller and Schmid, 1979; Chang and Bruce, 1980; Hopkins, 1983; Watson and Spaziani, 1985b; Pamuru et al., 2012), while replacement therapy using eyestalk extract or synthetic MIH lowers the ecdysteroid titer and delays molting (Bruce and Chang, 1984; Chang et al., 1987; Nakatsuji and Sonobe, 2004). A decrease in circulating MIH is associated with an increase in the hemolymphatic ecdysteroid titer during pre-molt (Nakatsuji and Sonobe, 2004). Amino acid sequence data place MIH in the crustacean hyperglycemic hormone (CHH) family of neurohormones; the family includes (in addition to MIH and CHH) vitellogenesis inhibiting hormone (VIH), mandibular organ inhibiting hormone (MOIH), and ion transport peptide (ITP) (Webster et al., 2012). Identification of the cellular signaling pathways that link MIH receptor occupancy to suppression of ecdysteroidogenesis remains an area of active research (reviews:

Nakatsuji et al., 2009; Covi et al., 2009; Chen, et al., 2020). Several lines of evidence indicate cGMP signaling is linked to MIH action. As examples, addition of recombinant MIH to incubation medium significantly enhanced glandular cGMP accumulation in Y-organs of *C. sapidus* but had no effect on glandular cAMP (Nakatsuji et al., 2006a). And a cGMP analog (8-Br-cGMP) significantly suppressed ecdysteroid production by *C. sapidus* Y-organs, but neither cAMP analogs (db-cAMP or 8-Br-cAMP) nor an activator of adenylyl cyclase (forskolin) had a detectable effect on ecdysteroidogenesis (Nakatsuji et al., 2006a). These and related findings (Saïdi et al., 1994; Baghdassarian et al., 1996; Sedlmeier and Fenrich, 1993; Nakatsuji et al., 2006b; Zheng et al., 2006), along with persuasive data indicating the CHH receptor is a receptor guanylyl cyclase (rGC) (Goy, 1990), are consistent with the hypothesis that cGMP is the second messenger proximately linked to MIH action, and that the MIH receptor is an rGC. However, the matter is not entirely settled. An alternative hypothesis suggests the MIH receptor is a G protein-coupled receptor, and that MIH receptor occupancy is linked to cAMP signaling. Consistent with this hypothesis, Mattson and Spaziani (1985) reported that adding eyestalk extract to incubations of crab (*Cancer antennarius*) Y-organs resulted in a 4-fold increase in glandular cAMP; the increase was detectable 1 h after treatment and was sustained through 6 h of incubation. In addition, a cAMP analog, or pharmacological agents that increase cAMP, each mimicked the inhibitory action of MIH, while a cGMP analog did not. It's worth noting that the eyestalk extract used in incubations almost certainly contained bioactive factors, including neurohormones, other than MIH, and that intracellular cGMP levels were not measured. Nevertheless, Zmora et al. (2009) used radioligand binding assays (with [^{125}I]MIH as ligand) to demonstrate the existence of

putative MIH receptors on plasma membranes of hepatopancreas from *C. sapidus*. Incubating hepatopancreas fragments with MIH for 1h produced a modest (~1.5-fold) increase in tissue cAMP content, and an increase in vitellogenein transcript abundance, but had no effect on tissue cGMP content. Chemical cross-linking of [^{125}I]MIH to hepatopancreas membrane preparations revealed MIH binding to a ~51 kDa protein. The size of the putative MIH receptor in hepatopancreas is consistent with that of a G protein-coupled receptor. A putative MIH receptor of the same size but different [^{125}I]MIH-binding properties was detected in *C. sapidus* Y-organs (Zmora et al., 2009).

While a decrease in circulating MIH is associated with an increase in the hemolymphatic ecdysteroid titer during pre-molt (Nakatsuji and Sonobe, 2004), it has long been hypothesized that a positive regulatory signal or stimulus is also involved in promoting the surge in ecdysteroidogenesis that occurs during premolt (Skinner, 1985). Data reviewed here are consistent with the hypothesis that an intracellular Ca^{2+} signal provides that stimulus.

2. Effect on ecdysteroidogenesis of agents that perturb intracellular Ca2+ in Y-organs

Calcium signaling has been implicated in stimulation of steroidogenesis in vertebrate testes (Janszen et al., 1976; Sullivan and Cooke, 1984), ovaries (Veldhuis and Klase, 1982; Jayes et al., 2000) and adrenal cortices (Birmingham et al., 1953; Catt et al., 1987). Likewise, a Ca^{2+} signal promotes ecdysteroidogenesis in insect prothoracic glands, functional analogs of Y-organs (Smith et al., 1985). Key experiments designed to test the hypothesis that a Ca^{2+} signal promotes ecdysteroidogenesis in Y-organs involved use of pharmacological agents that increase intracellular Ca^{2+}. Mattson and Spaziani (1986)

observed that adding the calcium ionophore A23187 (Reed and Lardy, 1972), to incubations of crab (*C. antennarius*) Y-organs stimulated ecdysteroid production in a dose-dependent manner (Fig.1). Likewise, Sedlmeier and Seinsche (1998) reported that adding A23187 to incubations of crayfish (*Orconectes limosus*) Y-organ cells dose-dependently stimulated ecdysteroid production. This same group also found that *in vitro*

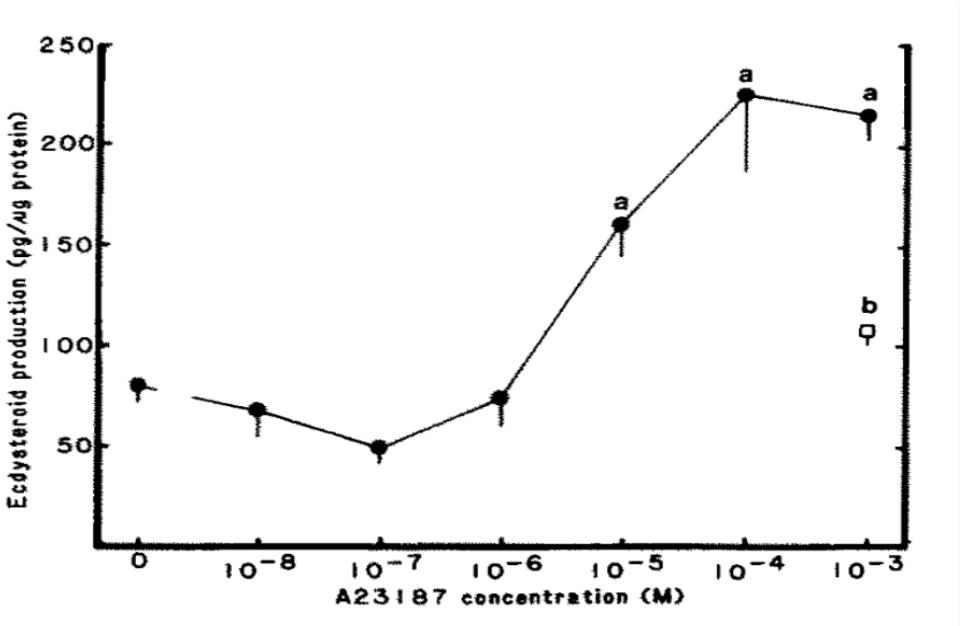

Figure 1. Concentration effects of calcium ionophore A23187 on ecdysteroid production. Y-organ gland quarters were incubated 6 h in saline containing 10 mM Ca^{2+} (solid circles) or 0 Ca^{2+} + 0.l mM EGTA (open square) plus the indicated concentrations of A23187, after which ecdysteroid levels in medium were quantified by radioimmunoassay. Points are the mean and SEM of four incubations. a, $P < 0.01$-0.001 vs 0 to 10^{-6}M values; b, $P<0.05$ vs 10^{-7}M A23187 value.

Note: From "Regulation of crab Y-organ steroidogenesis in vitro: evidence that ecdysteroid production increases through activation of cAMP phosphodiesterase by calcium-calmodulin." by M.P. Mattson and E. Spaziani, 1986, *Molecular and Cellular Endocrinology, 48,* p. 141. Copyright 1986 by Elsevier. Reprinted with permission.

ecdysteroid production by *O. limosus* Y-organ cells was increased dose-dependently by thapsigargin (Sedlmeier and Seinsche, 1998), a compound that increases cytosolic free Ca^{2+} by specifically inhibiting SERCA (Thastrup et al., 1990). Further, ecdysteroidogenesis in crab (*C. antennarius*) and crayfish (*O. rusticus* and *O. immunis*) Y-organs was diminished in Ca^{2+}-free incubation medium and enhanced in medium containing Ca^{2+} (Spaziani et al., 2001).

Complementary experiments tested the effect on ecdysteroidogenesis of agents that lower intracellular Ca^{2+} or disrupt Ca^{2+} signaling. Adding lanthanum (La^{3+}), a Ca^{2+} antagonist (Weiss, 1974), to incubations of of *C. antennarius* Y-organs suppressed ecdysteroid output (Mattson and Spaziani, 1986). Further, ruthenium red, a compound that blocks calcium signaling through interactions with several cellular proteins (Alnaes and Rahamimoff, 1975), suppressed ecdysteroid production by *C. antennarius* Y-organs *in vitro* (Mattson and Spaziani, 1986). In separate experiments, an intracellular calcium chelator (TMB-8), a calmodulin inhibitor (trifluoroperazine), or calcium channel blockers (verapamil, nifedipine, or nicarpidine) individually suppressed ecdysteroidogenesis by crab (*Menippe mercenaria*) Y-organs, crayfish (*Orconectes* sp.) Y-organs, or both (Spaziani et al., 2001). Likewise, Sedlmeier and Seinsche (1998) found that calcium channel blockers (pimozide, nimodipine, or flunarizide) individually suppressed ecdysteroid production by crayfish (*O. limosus*) Y-organs *in vitro*.

The combined results from experiments using agents that perturb intracellular calcium or disrupt calcium signaling are consistent with the hypothesis that a calcium signal promotes the surge in ecdysteroidogenesis that drives molting. The trigger for the hypothesized increase in intracellular calcium is unknown. One possibility is that it is

elicited by a specific, as yet unidentified, ligand. Another possibility is that it is a consequence of the general increase in hemolymphatic Ca^{2+} that occurs during premolt as Ca^{2+} is mobilized from the old exoskeleton (Greenaway, 1974, 1985).

3. Measurement of intracellular Ca^{2+} levels in Y-organs

The calcium signal hypothesis predicts that an increase in ecdysteroidogenesis will be associated with an increase in intracellular free Ca^{2+} in Y-organ cells. Chen and Watson (2011) used a fluorescent calcium indicator (Fluo-4) and fluorescence imaging methods to measure the level of Ca^{2+} in *C. sapidus* Y-organ cells after experimentally activating the glands by surgically removing the eyestalks. They found that eyestalk ablation (removing the eyestalk source of MIH) produced an increase in free Ca^{2+} in Y-organ cells, and that the increase in free Ca^{2+} was associated with an increase in the hemolymphatic ecdysteroid titer. However, although eyestalk ablation has historically been used as an experimental tool to activate Y-organs, the procedure removes the source of neurohormones other than MIH, and biochemical and physiological parameters of an experimentally accelerated (induced) molting cycle differ in several respects from those of a natural molting cycle (Spaziani et al., 1982). Therefore, in companion studies, Ca^{2+} levels in Y-organ cells and ecdysteroid levels in hemolymph were determined during stages of a natural molting cycle of *C. sapidus* (Chen et al., 2012). As shown in Figure 2a, there occurred stage-specific changes in total intracellular calcium fluorescence in Y-organ cells during the molting cycle. Mean calcium fluorescence increased by 5.8-fold between intermolt and stage D_3 of premolt, and then dropped abruptly, reaching a level in

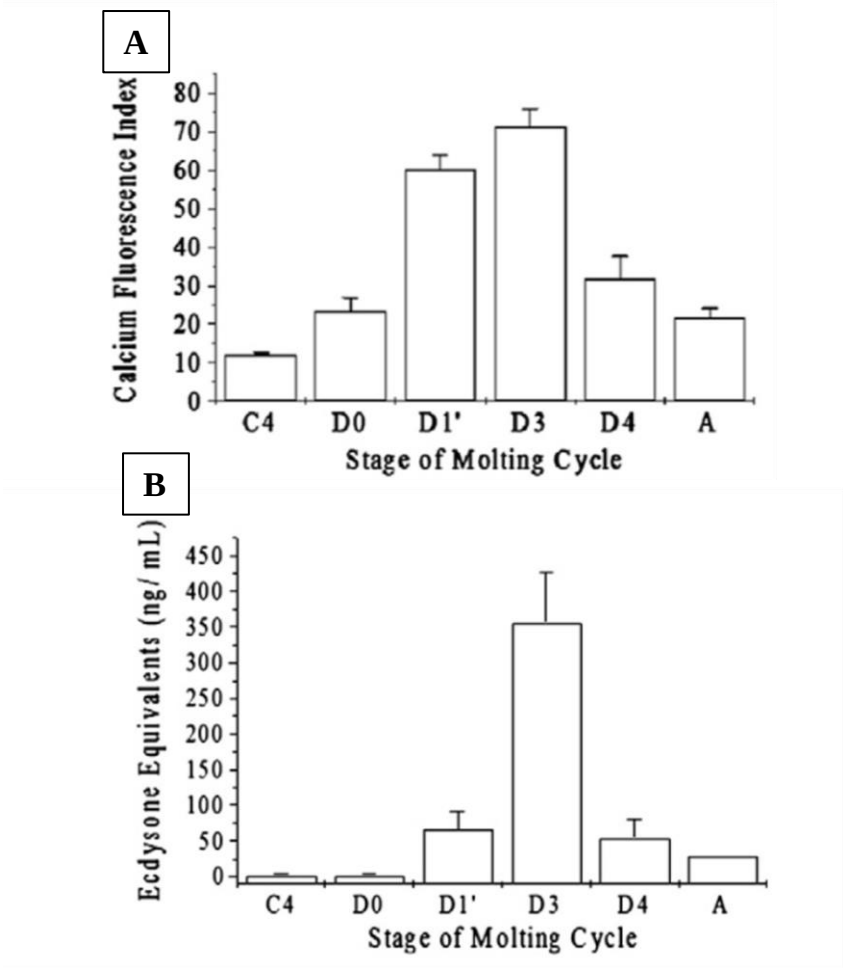

Figure 2. Stage-specific changes in calcium levels in Y-organs and ecdysteroid levels in hemolymph during a natural molting cycle of *C. sapidus*. Y-Organs and hemolymph samples were taken from crabs in the following stages of the molting cycle: intermolt (C4), premolt stages D0, D1', D3 and D4, and postmolt (A). **A)** Calcium-specific fluorescence in Y-organ cells. Bars represent mean calcium fluorescence + SE (N= 16 cells per stage). The statistical significance of differences among means was determined by one-way analysis of variance followed by Tukey's HSD test. The following pairwise comparisons to be significant at the 0.05 level: C4–D1', C4–D3, C4–D4, D0–D1', D0–D3, D1'–D4, D1'–A, D3–D4, D3–A. **B)** Hemolymphatic ecdysteroid titer. Ecdysteroid content of hemolymph was determined by radioimmunoassay. Bars represent mean + SE. Following one-way analysis of variance, Tukey's test showed the following pairwise comparisons to be significant at the 0.05 level: C4–D3, D0–D3, D1'–D3, D3–D4.

Note: From "Stage-specific changes in calcium concentration in crustacean (*Callinectes sapidus*) Y-organs during a natural molting cycle, and their relation to the hemolymphatic ecdysteroid titer." by H.-Y. Chen, R.M. Dillaman, R.D. Roer and R.D. Watson, 2012, *Comparative Biochemistry and Physiology, Part A, 163*, p. 172.

ecdysteroid titer in Y-organ donor crabs (Fig. 2b) rose from 2.9 ng/ml in intermolt to 357.1 ng/ml in D_3, and then dropped to 55.3 ng/ml in D_4. The results support the hypothesis that ecdysteroidogenesis is stimulated by an increase in intracellular Ca^{2+}.

4. Cellular mechanism of calcium action in Y-organs

The cellular mechanism by which Ca^{2+} stimulates ecdysteroidogenesis is not yet fully understood. Several lines of evidence suggest elevated intracellular calcium is linked to enhanced ecdysteroidogenesis through activation of cyclic nucleotide phosphodiesterase (PDE). PDE enzymes catalyze the degradation (inactivation) of cyclic nucleotide second messengers (Conti and Beavo, 2007). Mattson and Spaziani (1986) found that adding a nonselective PDE inhibitor (3-isobutyl-1-methylxanthine, IBMX) to incubations of crab Y-organs suppressed ecdysteroid secretion and blocked the stimulatory effect of A23187 on ecdysteroidogenesis. Further, in cell-free Y-organ preparations, PDE activity was dose-dependently increased by calcium (10^{-7}-10^{-4} M) and suppressed by a calmodulin inhibitor (Mattson and Spaziani, 1986). Nakatsuji et al. (2006b) reported the occurrence of stage-specific changes in PDE activity in Y-organs during a molt cycle of the crayfish, *Procambarus clarkii*: PDE activity was highest during middle premolt (Fig. 3), a time when the hemolymphatic ecdysteroid titer is likewise elevated (Nakatsuji et al., 2000). Based on their structure, biochemical properties, mode of regulation, and sensitivity to pharmacological agents, mammalian PDE enzymes are categorized into 11 isotype families (PDE1-PDE11) (Francis et al., 2001). Experiments using isotype-selective

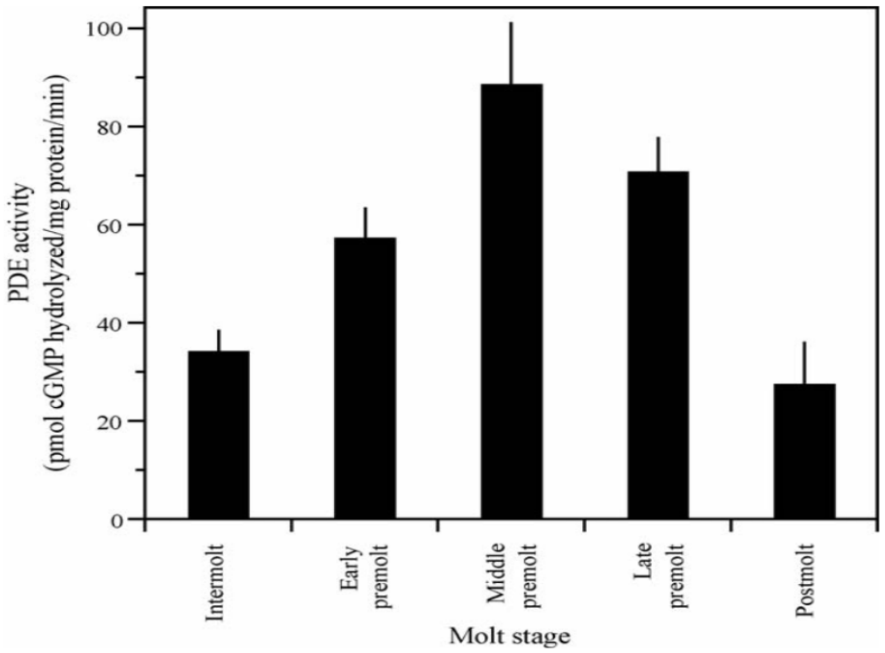

Figure 3. PDE activity in Y-organs during a molt cycle of *P. clarkii*. Y-Organs were removed from crayfish in various stages of the molt cycle and glandular PDE activity determined using [^{3}H]cGMP as substrate. Results are expressed as mean ± SE. Data were analyzed by ANOVA and Tukey's multiple comparisons. Among the multiple comparisons, middle premolt was significantly different from intermolt and postmolt ($p < 0.001$).

Note: From "Molt-inhibiting hormone-mediated regulation of ecdysteroid synthesis in Y-organs of the crayfish (Procambarus clarkii): involvement of cyclic GMP and cyclic nucleotide phosphodiesterase." by T. Nakatsuji, H. Sonobe and R.D Watson, 2006, *Molecular and Cellular Endocrinology*, 253, p. 79. Copyright 2006 by Elsevier. Reprinted with permission.

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inhibitors showed that PDE activity in Y-organs of *P. clarkii* was strongly inhibited by IBMX or 8MM-IBMX (a selective inhibitor of PDE1), findings consistent with the hypothesis that the PDE isotype in Y-organs of *P. clarkii* is a PDE1 family enzyme (Nakatsuji et al., 2006b) (Fig 4). PDE1 enzymes are Ca^{2+}/calmodulin dependent and

catalyze hydrolysis of both cAMP and cGMP (Francis et al., 2001). Considered together, the results are consistent with the notion that an increase in intracellular Ca^{2+} during premolt stimulates PDE1 activity, thereby lowering intracellular cyclic nucleotide levels, antagonizing MIH, and promoting ecdysteroidogenesis.

It has also been suggested that elevated intracellular calcium may be linked to enhanced ecdysteroidogenesis through activation of protein kinase C (PKC), but the results are mixed. Adding an activator of PKC (phorbol 12-myristate 13-acetate) to incubations of crab (*C. antennarius*) Y-organs dose-dependently stimulated ecdysteroid production (Mattson and Spaziani, 1987). By contrast, adding phorbol 12-myristate 13-acetate to incubations of crayfish (*O. immunis*) Y-organs suppressed ecdysteroid production (Spaziani et al., 2001). The reason for the difference between species is unknown.

5. Proteins that regulate intracellular Ca^{2+} in Y-organs

The proteins that regulate intracellular Ca^{2+} levels (principally PMCA, SERCA, and NCX) have been studied in crustacean gill, antennal gland and hepatopancreas, mainly with an eye to understanding their roles in the transcellular Ca^{2+} flux associated with demineralization and mineralization of the exoskeleton during the molting cycle (Wheatly et al., 2002a, 2002b, 2007). Because of the emerging consensus that a surge in intracellular Ca^{2+} promotes ecdysteroidogenesis, several recent studies have focused on Ca^{2+} transport proteins in Y-organs.

5.1. PMCA

PMCA, a member of P-type ATPase superfamily, is a high-affinity, low-capacity Ca^{2+} pump (Mangialavori et al., 2010). In mammals, there are four genes encoding four different PMCA isoforms (PMCA1-PMCA4). The complexity of the PMCA variants is further enhanced via the alternative splicing of each gene. The variants are expressed in a tissue-specific and developmental stage-dependent manner (Brini and Carafoli, 2011; Stafford et al., 2017). Chen et al., (2013) used a PCR-based strategy to clone from Y-organs of *C. sapidus* a cDNA (*Cas-PMCA*) encoding a putative PMCA protein. The 4292 base pair cDNA includes a 3510 bp open reading frame encoding a 1170-residue protein (Cas-PMCA). The conceptually translated protein has a relative molecular mass of 128.8 $x10^3$ and contains all signature domains of an authentic PMCA, including ten transmembrane domains and a calmodulin binding site. The predicted membrane topography of Cas-PMCA is as expected for an authentic PMCA protein. A phylogenetic analysis of amino acid sequences of PMCA proteins from different species showed Cas-PMCA clusters with other arthropod PMCA proteins. An assessment of tissue distribution showed the *Cas-PMCA* transcript to be broadly distributed in both neural and non-neural tissues. Studies using quantitative real-time PCR showed the level of the *Cas-PMCA* transcript in Y-organs was low during intermolt, increased significantly to a peak in premolt stage D2, then fell sharply, and remained low during postmolt (Fig. 5). The overall pattern of *Cas-PMCA* transcript abundance is generally similar to the pattern of stage-specific changes in intracellular free Ca^{2+} seen in Y-organ cells (Chen et al., 2012).

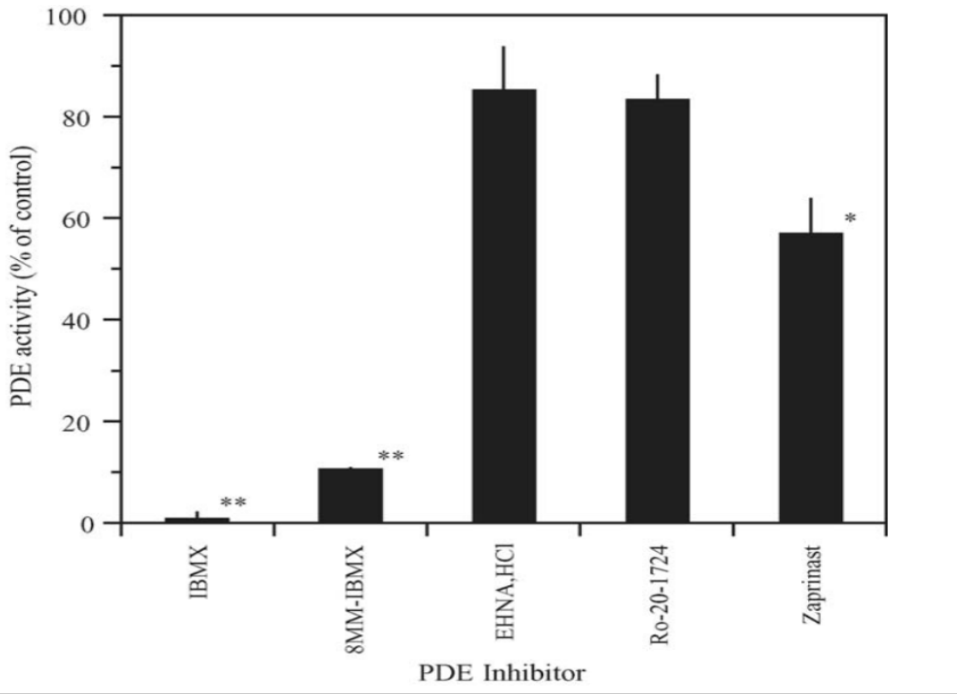

Figure 4. Effect of isozyme-selective PDE inhibitors on PDE activity in Y-organs of *P. clarkii*. A PDE preparation from pooled Y-organs at the middle premolt stage was used for individual inhibition assays. IBMX (non-selective inhibitor, 1 mM), 8MM-IBMX (PDE1 inhibitor, 0.1 mM), EHNA, HCl (PDE2 inhibitor, 0.05 mM), Ro-20-1724 (PDE4 inhibitor, 0.1 mM), and Zaprinast (PDE5 inhibitor, 0.01 mM) were added to the reaction mixture. The control reaction received an equal volume of vehicle. Results are expressed as percent of control. Bars represent mean ± SE. Data were analyzed by ANOVA and Tukey's multiple comparisons. Among the multiple comparisons, the following treatments were significantly different from control: IBMX (**$P < 0.0001$), 8MM-IBMX (**$P < 0.0001$), and Zaprinast (*$P < 0.0005$).

Note: From "Molt-inhibiting hormone-mediated regulation of ecdysteroid synthesis in Y-organs of the crayfish (Procambarus clarkii): involvement of cyclic GMP and cyclic nucleotide phosphodiesterase." by T. Nakatsuji, H. Sonobe and R.D Watson, 2006, *Molecular and Cellular Endocrinology*, 253, p. 79. Copyright 2006 by Elsevier. Reprinted with permission.

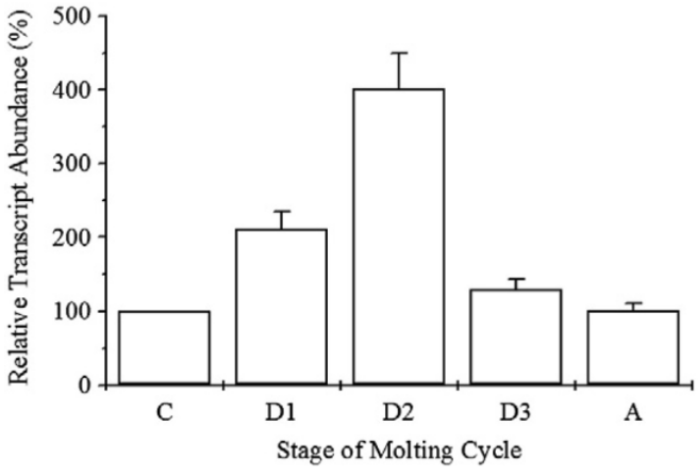

Figure 5. Relative abundance of the Cas-PMCA transcript in Y-organs during a molting cycle of *C. sapidus*. At various stages of the molting cycle, total RNA was extracted from Y-organs, and relative transcript abundance was determined by quantitative real-time PCR; a replicate from intermolt stage was used as calibrator. Bars represent mean + SD. Molt stages are intermolt (C), premolt (D1–D3), and postmolt (A). Tukey's test results showed the following pairwise comparisons to be significant at the 0.05 level: C–D1, C–D2, D1–D2, D1–A, D2–D3, D2–A.

Note: From "Molecular cloning of a plasma membrane Ca^{2+} ATPase (PMCA) from Y-organs of the blue crab (*Callinectes sapidus*), and determination of spatial and temporal patterns of PMCA gene expression." by H.Y. Chen, R.D. Roer and R.D. Watson, 2013, *Gene,522,* p. 16. Copyright 2013 by Elsevier. Reprinted with permission.

5.2. SERCA

Like PMCA, SERCA is an ATP-dependent ion pump (Palmgren and Nissen, 2011). Three homologous genes encode SERCA in mammals, and alternative splicing of

primary transcripts yield multiple isoforms, including SERCA1, SERCA2a, SERCA2b, SERCA3, SERCA4, and SERCA5 (Grover and Khan, 1992; Wu and Lytton, 1993). Complementary DNA encoding SERCA was first cloned in mammals (MacLennan et al., 1985; Brandl et al., 1986). SERCA genes have subsequently been cloned in a variety of vertebrates (Karin et al., 1989; Vilsen and Andersen, 1992; Tullis and Block, 1996), and in several invertebrates, including insects (Váradi et al., 1989; Lockyer et al., 1998) and crustaceans (Palmero and Sastre, 1989; Zhang et al., 2000; Mandal et al., 2009). Roegner et al. (2018) cloned from Y-organs of *C. sapidus* a cDNA (*Cas-SERCA*) encoding a putative SERCA protein. The cloned *Cas-SERCA* cDNA (3806 bp) includes a 3057-bp open reading frame that encodes a 1019-residue protein (Cas-SERCA). The conceptually translated protein has a predicted molecular mass of 111.42×10^3 and contains all signature domains of an authentic SERCA, including ten transmembrane domains and a phosphorylation site at aspartate 351. A homology model of Cas-SERCA closely resembles models of related SERCA proteins (Fig. 6).

Phylogenetic analysis shows Cas-SERCA clusters with SERCA proteins from other arthropods. An assessment of tissue distribution indicates the *Cas-SERCA* transcript is widely distributed across tissues. Quantitative real-time PCR was used to quantify *Cas-SERCA* transcript abundance in Y-organs during a molting cycle, and radioimmunoassay was used to quantify ecdysteroids in hemolymph: the abundance of the *Cas-SERCA* transcript in Y-organs increased gradually during pre-molt; similarly, the level of ecdysteroids in hemolymph increased during pre-molt (Roegner et al., 2019).
The pattern of changes in *Cas-PMCA* and *Cas-SERCA* transcript abundance during the molting cycle indicate that stage-specific changes in Ca^{2+} in Y-organs cells are not likely

to be a consequence of downregulation of the activity of the relevant enzymes. Rather, it appears that the increase in *Cas-PMCA* and *Cas-SERCA* expression in Y-organs during premolt may be a cellular response designed to maintain cytosolic Ca^{2+} homeostasis. This

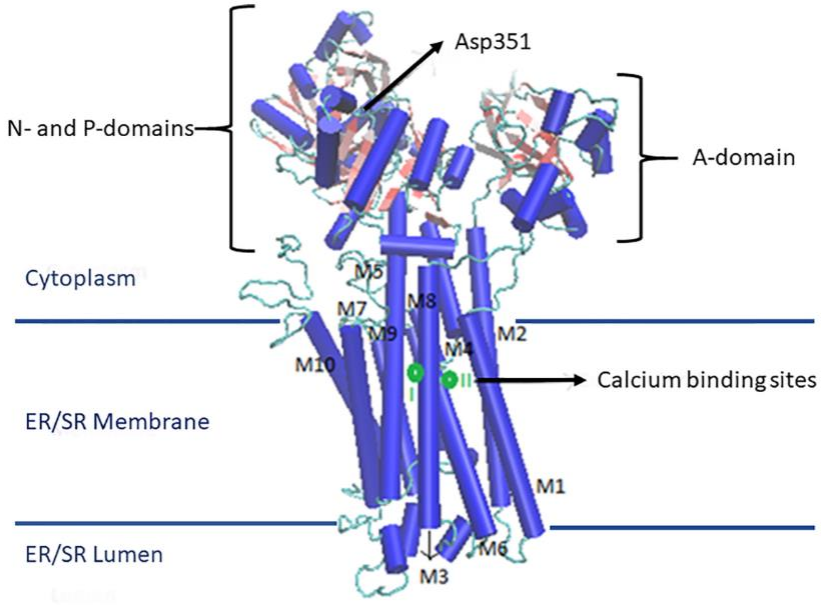

Figure 6. Homology model of the Cas-SERCA protein in its predicted membrane position. The model was built from the deduced amino acid sequence and modeled from a rabbit SERCA in E1 conformation (PDB 1SU4). Two putative Ca2+ binding sites (I and II, shown as green dots) and ten transmembrane domains (M1–10) are shown within the ER/SR membrane. The phosphorylation residue Asp351, and the actuator (A), nuclear binding (N), and phosphorylation (P) domains are labeled on the cytosolic side of the membrane.

Note: From "Molecular cloning and characterization of a sarco/endoplasmic reticulum Ca2+ ATPase (SERCA) from Y-organs of the blue crab (*Callinectes sapidus*)." by M.E. Roegner and R.D. Watson, 2018, *Gene*, 673, p. 17. Copyright 2018 by Elsevier. Reprinted with permission.

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interpretation is consistent with previous reports involving multiple cell types that

indicate increases in cytosolic Ca^{2+} are associated with upregulation of PMCA (Gao and Wheatly, 2004; Kuo, et al., 1997; Marian et al., 2007).

5.3. NCX

The Na^{+}/Ca^{2+} exchanger (NCX), a member of the Ca^{2+}/cation antiporter superfamily, is a low affinity, high-capacity transmembrane protein with an essential role in transepithelial calcium movement (Giladi et al., 2016). NCX was first cloned from canine cardiac muscle (Nicoll et al., 1990), and has since been identified in virtually all animal cells (Blaustein and Lederer, 1999). In mammals, there are three gene isoforms (NCX1- NCX3), with 17 different variants due to tissue-dependent alternative gene splicing (Blaustein and Lederer, 1999; Philipson and Nicoll, 2000).

NCX activity has been characterized in crustacean antennal glands, hepatopancreas, and gills of multiple taxa, including lobsters, crayfish and crabs (Flik et al., 1994; Flik and Haond, 2000; Ahearn et al., 2004; Wheatly et al., 2002a, 2007). NCX expression in antennal glands of *P. clarkii* is greater during premolt (when the exoskeleton is demineralized) and postmolt (when the exoskeleton is mineralized) than it is during intermolt (Wheatly et al., 2007). The data are consistent with the hypothesis that NCX plays a role in transepithelial Ca^{2+} transport associated with demineralization and mineralization of the exoskeleton during the molting cycle. A cDNA encoding a putative NCX protein was recently cloned from crayfish (*Astacus leptodactylus*) neural ganglion (Ergin and Purali, 2018). The transcript was widely distributed across tissues, including antennal gland, gill, and hepatopancreas. NCX has not been identified in Y-organs of any

crustacean species, and its role in regulating Ca^{2+} homeostasis in Y-organs has not been investigated.

6. Insights from Transcriptomics

Recent transcriptomic studies provide insight into cell signaling pathways in Y-organs and their possible link to regulation of the synthesis and secretion of ecdysteroids. Das et al. (2016) reported a transcriptomic analysis of Y-organs from intermolt black land crabs (*Gecarcinus lateralis*). Subsequently, a transcriptomic approach was used to assess cell signaling pathways involved in activation of *G. lateralis* Y-organs following eyestalk ablation (Shyamal et al., 2018) or multiple leg autotomy (Das et al., 2018). The results have been reviewed (Mykles and Chang, 2020). More recently, Roegner and Watson (2020) reported *de novo* transcriptome assembly for Y-organs of *C. sapidus*, and analysis of differentially expressed genes (DEG) between intermolt and natural (rather than experimentally induced) pre-molt.

In general, composite data from the several studies show genes involved in multiple cell signaling pathways, including Ca^{2+} signaling, cGMP signaling, cAMP signaling, and mTOR signaling are expressed in Y-organs (Das et al., 2016, 2018; Roegner et al., 2020). Mapping of Kyoto Encyclopedia of Genes and Genomes (KEGG) terms from the annotated reference transcriptome of *C. sapidus* Y-organs showed twenty-two genes involved in calcium signaling, including two Ca^{2+} ATPases, SERCA and PMCA, as well as G-protein coupled receptors tied to the IP_3 pathway (Fig. 7) (Roegner and Watson, 2020). Differential gene expression (DGE) analysis between Y-organs of intermolt and premolt *C. sapidus* revealed upregulation in premolt Y-organs of three genes involved in Ca^{2+} signaling (Roegner and Watson, 2020). This is consistent with a previous report that

several genes involved in Ca^{2+} signaling are upregulated in premolt Y-organs of *G. lateralis* (Das et al., 2018).

Because the stimulatory effect of Ca^{2+} on ecdysteroidogenesis appears to be mediated by PDE ((Mattson and Spaziani, 1986; Nakatsuji et al., 2006b), consideration here of transcriptomic data concerning cyclic nucleotide signaling in Y-organs is warranted. Mapping of KEGG terms from the transcriptome of *C. sapidus* revealed fifty genes in the cGMP-protein kinase G (PKG) pathway, including PKG, two PDE, and two rGC provisionally identified as atrial natriuretic peptide receptor A and atrial natriuretic peptide receptor B. (Roegner and Watson, 2020). DEG showed five of the genes involved in the cGMP-PKG signaling pathway were upregulated in premolt Y-organs; no genes involved in the cGMP-PKG signaling pathway were observed to be downregulated in premolt Y-organs (Roegner and Watson, 2020). Genes involved in cAMP signaling are also well represented in the Y-organ transcriptome. Multiple genes involved in cAMP-PKA signaling, including a large number of putative G-protein coupled receptors (GPCR), have been identified in Y-organs of *G. lateralis* (Das et al. 2016, 2018; Shyamal et al., 2018; Tran et al., 2019; Mykles and Chang, 2020). Likewise, 61 genes in the cAMP-PKA pathway have been identified in Y-organs of *C. sapidus* (Roegner and Watson, 2020). DEG analysis showed four downregulated and one upregulated GPCR in Y-organs from premolt *C. sapidus* (Roegner and Watson, 2020). Available transcriptomic data to not resolve the issue of whether cGMP signaling, cAMP signaling, or both are involved in MIH-induced suppression of ecdysteroidogenesis but provide a foundation

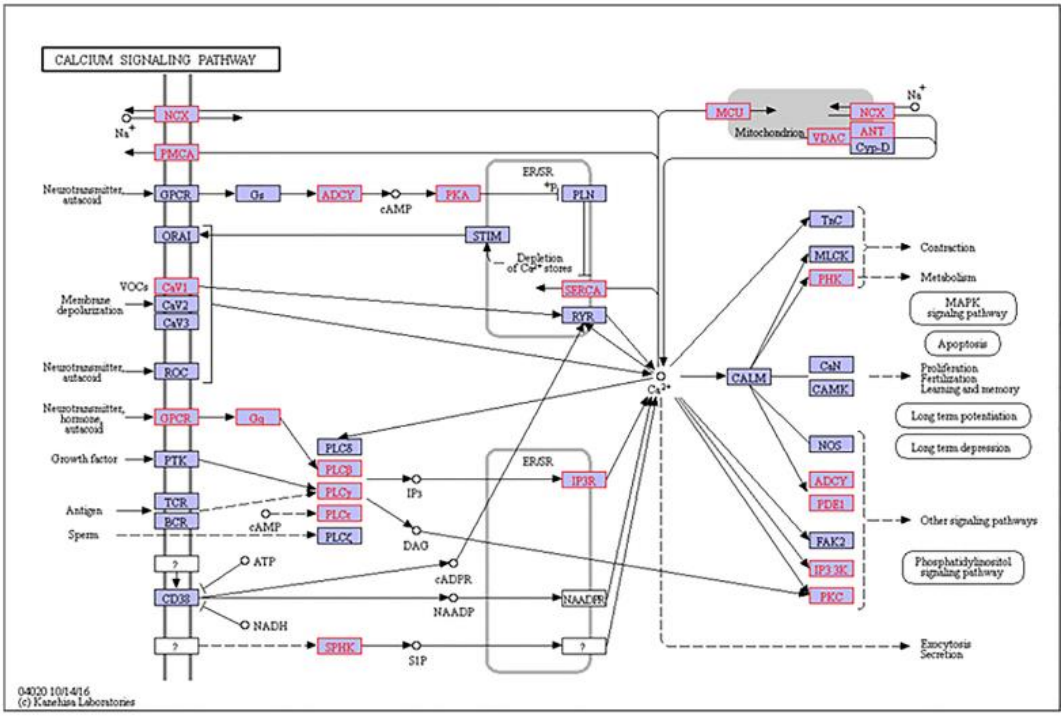

Figure 7. KEGG calcium signaling pathway. Shown is an analysis of KEGG terms using KEGG mapping to the Ca^{2+} signaling pathway. Highlighted in red are the genes with KEGG annotations in the *C. sapidus* Y-organ transcriptome.

Note: From "*De novo* transcriptome assembly and functional annotation for Y-organs of the blue crab (*Callinectes sapidus*), and analysis of differentially expressed genes during pre-molt." by M.E. Roegner and R.D. Watson, 2020, *General and Comparative Endocrinology*, p. 5. Copyright 2020 by Elsevier. Reprinted with permission.

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for development of hypotheses that may lead to resolution of the issue. For example, available cDNA sequence data for the two putative rGC in the *C. sapidus* transcriptome (Roegner and Watson, 2020) provide a base for further studies designed to assess the possibility that they may encode an MIH receptor, a CHH receptor, or both.

KEGG analysis of the *C. sapidus* Y-organ transcriptome also identified nineteen KEGG terms that mapped into pathways involving synaptic vesicle cycles and SNARE

interactions (Roegner and Watson, 2020). DEG analysis showed upregulation (log2FC=2.53) of a calcium-dependent secretion activator (CADPS or CAPS) (Roegner and Watson, 2020). The *CADPS* gene encodes a neural- or endocrine-specific protein required for Ca^{2+}-regulated exocytosis of secretory vesicles (Nishizaki et al., 1992; Speidel et al., 2004). It has been historically accepted that steroid hormones are released from steroidogenic endocrine glands by simple diffusion (Sibley et al., 1980). However, Birkenbeil and Eckert (1983) reported ecdysteroid-immunoreactive vesicles and exocytotic figures in Y-organs of the crayfish, *Orconectes limosus*, and hypothesized that ecdysteroids may be released by exocytosis (see also Lachaise et al., 1983). Ecdysteroid-immunoreactive vesicles and exocytotic figures have also been observed in prothoracic glands of the waxmoth, *Galleria mellonell*a (Birkenbeil and Eckert, 1982; Birkenbeil, 1983). More recently, Yamanaka et al. (2015) observed that inhibition of Ca^{2+} signaling in prothoracic glands of *Drosophila melanogaster* resulted in accumulation of unreleased ecdysteroids, and that knockdown of Ca^{2+}-mediated vesicle exocytosis produced developmental defects that could be rescued by feeding ecdysone. They concluded that ecdysteroids can be released from prothoracic glands by vesicle-mediated exocytosis and hypothesized that the mechanism may be involved in release of steroid hormones from vertebrate steroidogenic endocrine glands as well. The possibility of exocytotic release of steroid hormones from vertebrate steroidogenic glands is supported by data from multiple sources (reviewed in Nussdorfer, 1986). Findings from transcriptomic studies cited here are consistent with the notion that a Ca^{2+} signal, in addition to stimulating ecdysteroid biosynthesis, may also play a role in promoting the release (secretion) of ecdysteroids.

7. Summary

Since the first description of Y-organs (Gabe, 1953), their identification as molting glands (Echalier, 1954), and the isolation of ecdysteroids from insects and crustaceans (Butendandt and Karlson, 1954; Horn et al., 1966), much has been learned about the cell signaling pathways involved in regulation of ecdysteroidogenesis. Aspects of this information are summarized in a model shown in Figure 8. Y-Organs take up lipoprotein-bound cholesterol from the surrounding hemolymph and use it as a biosynthetic precursor for synthesis of ecdysteroids (Watson and Spaziani, 1985a, 1985b). MIH-driven suppression of both cholesterol uptake and ecdysteroidogenesis is mediated by a cyclic nucleotide second messenger (cGMP or cAMP) through activaton of protein kinase (PKG or PKA) (see Nakatsuji et al., 2009; Covi et al., 2009; Chen, et al., 2020). Data reviewed here are consistent with the hypothesis that a Ca^{2+} signal promotes ecdysteroidogenesis in Y-organs, and that elevated intracellular calcium is linked to enhanced ecdysteroidogenesis through activation of a calcium-calmodulin dependent PDE (PDE1), which in turn acts to lower intracellular cyclic nucleotide levels, thereby promoting ecdysteroidogenesis. Although not depicted in Figure 8, several lines of evidence indicate that a Ca^{2+} signal may also stimulate the secretion of ecdysteroids. Calcium transport proteins (PMCA, SERCA, and likely NCX) function to maintain the Ca^{2+} gradient across the cell membrane and re-set the cell for future Ca^{2+} signals.It should be emphasized that Figure 8 is working model intended to serve as a foundation for formulation of testable hypotheses. In addition, it's worth noting that the calcium hypothesis depicted in Figure 8 does not preclude the involvement of other cell signaling pathways in the stimulation of ecdysteroidogenesis. For example, persuasive data

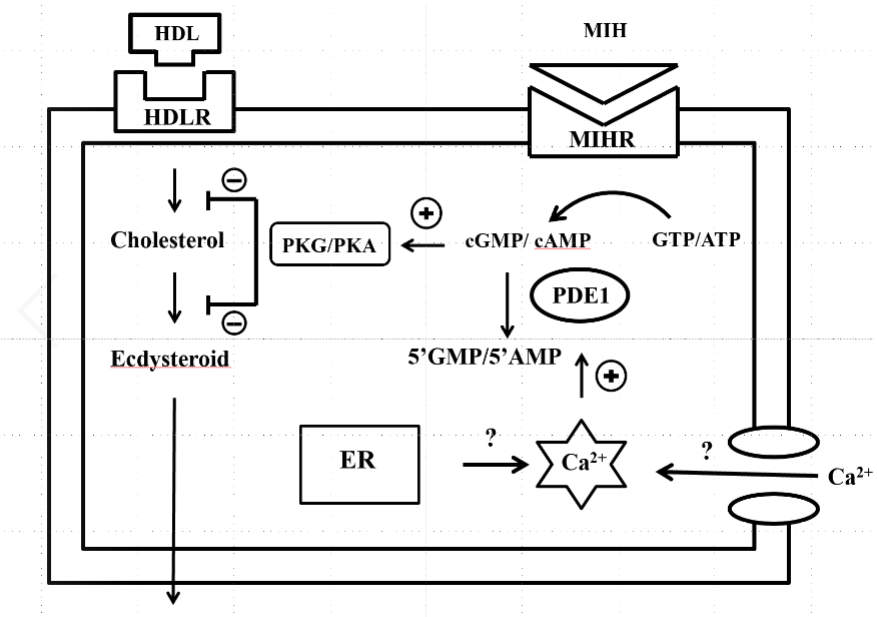

Figure 8. Cell signaling pathways and regulation of ecdysteroidogenesis in Y-organs. Y-organs take up high density lipoprotein (HDL)-bound cholesterol from hemolymph and use it as a biosynthetic precursor for synthesis of ecdysteroids. Binding of MIH to its receptor (MIHR) leads to an increase in the level of a cyclic nucleotide second messenger (cGMP or cAMP). Activated protein kinase (PKG or PKA) suppresses uptake of HDL-bound cholesterol and synthesis of ecdysteroid molting hormones. Flux of Ca^{2+} across the plasma membrane, from organelles (e.g., endoplasmic reticulum, ER), or both, activates a Ca^{2+}/calmodulin-dependent PDE (PDE1), thereby lowering intracellular cyclic nucleotide levels and promoting ecdysteroidogenesis.

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implicate the mTOR signaling pathway in stimulation of ecdysteroidogenesis during premolt (Abuhagr et al., 2014; Abuhagr et al., 2016). Needed are experiments designed to identify the stimulus that elicits the Ca^{2+} signal, and to determine if the signal results from the movement of Ca^{2+} across the plasma membrane, across the membranes of organelles, or both. Study of NCX in Y-organs is also warranted.

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CONCLUSION AND IMPACT

The magnitude of the Deepwater Horizon oil spill and the unprecedented use of chemical dispersants has spurred a multitude of published papers examining the effects of the crude oil, dispersant, and dispersed oil on the marine environment and its inhabitants. The novel dispersant application method, SSDI, subjected marine communities that previously would not be affected to chemical dispersant exposure. This has generated a sizable body of research on the ecotoxicity of oil and chemical dispersants across a broad range of species. Yet, despite this, there is no overriding consensus on the toxicity of Corexit® EC9500A and other chemical dispersants. This research aims to add to this body of knowledge, specifically with regards to the impact on the blue crab, a key economic and ecological Gulf of Mexico species.

Impact: Structural and Functional Changes to Gill Epithelium

Blue crabs have highly specialized gills not only responsible for respiration, but the active regulation of hemolymph ion levels and are in direct contact with their environment [56]. Previous research has generated reports of structural loss, necrosis and edema in African catfish (*Clarius gariepinus*) gills [105], zebrafish gills, and mouse lungs and human airway epithelial cells exposed to chemical dispersants [106]. Using conventional histology and image analysis, I was able to identify and quantify cellular

damage to the gill epithelium, degradation of the gill cuticle, and evidence of fluid infiltration (edema) into the hemolymph spaces (Weiner et al., unpublished). This led to the question, does a change in structure denote a change in function. To answer this question, we looked to the transport of two substantial ions in the blue crab, Na^+ and Ca^+.

Unique, even in the marine world, blue crabs have a powerful salt uptake system housed in the posterior gill epithelium in the form of numerous Na+/K+ ATPase pumps. These pumps are the predominant facilitator of brachial ion transport. They allow blue crabs to tolerate the variable salinity gradient in the estuaries they inhabit, and as such are essential to their ongoing survival [56]. Using RT-qPCR we identified severely reduced transcript abundance of Na+/K+ ATPase mRNA in the gills after Corexit® EC9500A exposure. There is already evidence that surfactant-based oil dispersants are toxic to ATPase enzymes, specifically inhibiting Na+/K+ ATPase [107]. Taken together, this could imply that Corexit® EC9500A exposure could have devastating effects on the blue crabs' ability to tolerate changes in environmental salinity.

Ca^{2+} is a critical, ubiquitous second messenger for eukaryotic organisms. In the blue crab, sizeable amounts of $Ca^{2+}$are mobilized during molting as the carapace is demineralized and then mineralized, all the while maintaining low cytosolic Ca^{2+} levels [108]. The enzymes responsible for regulating intracellular Ca^{2+} levels (PMCA, SERCA, and NCX) have been studied in a variety of crustacean tissue including the gills and hepatopancreas [109, 110, 111]. Using RT-qPCR we demonstrated a severely reduced transcript abundance of both PMCA and SERCA mRNA in the posterior gills after exposure to Corexit. This suggests an impairment of the ATPases responsible for keeping intracellular Ca^{2+} levels low, and elevated levels of Ca^{2+}in the cytosol are

cytotoxic and can lead to cellular apoptosis [109]. Recently, there has been an emerging body of evidence suggesting a surge in intracellular Ca^{2+} could promote ecdysteroidogenesis and our lab has previously shown a pattern of intracellular Ca^{2+} concentration during a molt cycle that mimicked the quantitative and temporal ecdysteroid hemolymph titer [73]. Corexit-induced impairment of the ability to control intracellular Ca^{2+} levels could have damaging effects on the crab's ability to manage Ca^{2+} levels during a molt cycle and prevent a precocious and potentially unsuccessful molt. Molting is essential for growth, maturation, and reproduction of the species and hinderances to successful molting cycles has the potential to affect the juvenile population size and fitness.

Future Directions

Further experimentation is required to tease out the potential effects of dispersant exposure on the respiratory functions of blue crab gills. Needed are experiments to identify potential changes in ventilation rate, hemolymph O_2 levels, and lactate levels (a metabolic marker during times of hypoxia) which would provide a better understanding of how dispersant exposure perturbs the blue crabs' respiratory capacity.

Impact: Inducement of Reactive Oxygen Species in the Gill Epithelium

The generation of reactive oxygen species is at the most basic level, a byproduct of respiration and enzymatic activity. ROS are generated by a series of enzymes, including

NADPH oxidase (NOX) complexes [112] and C-reactive protein [113]. NOX4, found in the endoplasmic reticulum, has been shown to produce ROS via H^2O^2 in airway epithelial cells leading to apoptosis [114]. In smooth muscle vasculature, CRP directly induces NOX4 activation leading to ROS generation [113]. Li et al., (2015) showed that exposure to Corexit® EC9500A resulted in lung and gill epithelium inflammation and permeability which led to the production of ROS and cleaved-Caspase 3 mediated apoptosis cause by the upregulation of NOX4 and CRP. Expounding on this, we used IHC to demonstrate evidence of NOX4, CRP and CC3 upregulation in blue crab gills exposed to Corexit® EC9500A. Combined with our previous demonstration of inflammation in the gill epithelium (Weiner, et al., unpublished), it is highly probable that the gill epithelium experiences cellular apoptosis in response to CE exposure. To this end, reports of acute and chronic respiratory distress are among the most reported symptoms of oil spill responders [115, 116, 117, 118, 119]. That being said, the use of IHC has limitations and to strengthen my argument I attempted, though unsuccessfully, RT-qPCR for NOX4 and CRP. Multiple primers were designed based on the human genetic sequences for NOX4 and CRP, and primer sequences found in the primary literature. The genetic sequences of NOX4 and CRP have not yet been determined for *Callinectes sapidus*, which may have contributed to my unsuccessful attempts. However, the IHC results warrant further investigation, potentially in other benthic species, such as shrimp and oysters, with similar environmental and economic value.

Impact: Crustacean Hyperglycemic Hormone Mediated Stress Response

CHH is a pleiotropic hormone responsible for glucose metabolism and the regulation of environmental stress [120]. A CHH-mediated stress response causes a hyperglycemic response [121], can suppress the molting cycle [101], inhibit reproduction [122], and modify ion transport (specifically affecting Na^+/K^+ATPase) [104]. Despite this, there has not to our knowledge been any prior investigations on a possible CHH-mediated response to chemical dispersants. The determine if there is indeed a stress response, we began by examining glucose levels from zero to four hours after CE exposure. The hemolymph glucose titer rose, peaked at one hour and then fell back to basal levels by the four-hour time mark. To tie this to a CHH-mediated response we examined CHH mRNA transcript abundance in the eyestalks at the one-hour time mark. CHH mRNA transcript abundance was significantly increased compared to control levels, so we can deduce that the rise in glucose is most likely due to an increase in CHH. As mentioned previously, we observed a significant decrease in Na^+/K^+ATPase mRNA transcript abundance in response to CE exposure. CHH has a direct effect on ion transport by causing an increase in Na^+ influx and an experimentally determined 50% increase in Na^+/K^+ATPase gill activity. This defensive affect in response to environmental stress could be hindered by the CE-mediated loss of Na^+/K^+ATPase mRNA.

It is well documented that MIH is the predominant hormone responsible for control of the molting cycle. That being said, physiologically relevant levels of CHH have been shown to suppress ecdysteroidogenesis in blue crabs [123]. We examined ecdysteroid levels in the hemolymph at the one-hour time mark but did not observe a statistically significant change. It is possible that the duration or magnitude of the CHH

surge was not adequate to illicit a significant response, or the response occurred beyond the scope of the four-hour time course.

FUTURE DIRECTIONS

We chose to identify changes in the osmoregulatory ion transport function of the gills. Further experimentation is required to tease out the potential effects of dispersant exposure on the respiratory functions of blue crab gills. Needed are experiments to identify potential changes in ventilation rate, hemolymph O_2 levels, and lactate levels (a metabolic marker during times of hypoxia). These experiments would provide a better understanding of how Corexit-exposure perturbs the blue crabs' respiratory capacity and potentially causes a state of hypoxia, eliciting a CHH-mediated stress response [87].

We explored the aforementioned, proposed mechanism of Corexit cytotoxicity utilizing IHC for NOX4, CRP and CC3. Cytochrome C is released from the mitochondria during cellular apoptosis [106, 112], and there are commercially available kits that identify the movement of Cytochrome C from the mitochondria into the cytosol. Needed are experiments to identify the presence of markers of the intrinsic apoptosis cascade, for example Cytochrome C in blue crab gills, and given the probable conserved nature of the mechanism, potentially in other benthic species, such as shrimp and oysters, with comparable environmental and economic value.

We did not identify a change in the hemolymph ecdysteroid titer in response to Corexit-exposure over a 4-hour period. However, further experimentation is required to

tease out the potential effects of Corexit-exposure on ecdysteroidogenesis and the molt cycle. As mentioned previously, MIH is the predominant hormone in control of the molting cycle. Needed are experiments to determine the effects of Corexit-exposure to MIH levels at different stages of the molt cycle and changes to the length of time between juvenile molts, which would provide a better understanding of how dispersant exposure disturbs blue crab reproduction, juvenile population density and fitness.

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APPENDIX A:

CONTRIBUTION TO SCIENCE

Peer-Reviewed Manuscripts from PhD Dissertation

Effect of a Chemical Dispersant (Corexit® EC9500A) on the Structure and Ion Transport Function of Blue Crab (Callinectes sapidus) Gills

Weiner, A.C.; Roegner, M.; Watson, R.D. (under review 2020)

Chemical dispersants are mixtures of surfactants and solvents that have become important tools in the remediation of spilled oil. We report here the impact of a chemical dispersant, Corexit® EC9500A, on the structure and ion transport function of blue crab (*Callinectes sapidus*) gills. Exposure of blue crabs to Corexit® EC9500A for 24 hours (0- 300 ppm in artificial seawater under static conditions) revealed a 24-h lethal concentration 50 (LC_{50}) value of 210 ppm. A histological analysis of gills from crabs exposed for 24 h to a sub-lethal concentration of Corexit® EC9500A (125 ppm) revealed evidence of loss or disruption of cuticle, and an increase in stained amorphous material in the hemolymph spaces of gill lamellae. Quantitative image analysis of stained gill sections revealed the area/length ratio of gill lamellae in crabs exposed to Corexit® EC9500A (24 h, 125 ppm), was greater than that in gill lamellae from control crabs. Quantitative PCR was used to measure the relative abundance of transcripts encoding three ion transport proteins (Na^+/K^+ ATPase, plasma membrane Ca^{2+} ATPase (PMCA), and sarcoplasmic reticulum/endoplasmic reticulum Ca^{2+} ATPase (SERCA)) in gills from Corexit-exposed and control crabs. In general, the abundance of transcripts encoding each ion transport protein was lower in gills from dispersant-exposed crabs than in gills

from control crabs. The combined results are consistent with the hypothesis that 24-h exposure of blue crabs to a sublethal concentration of Corexit® EC9500A impacts both the structure and ion transport function of gills.

This manuscript is currently under review. Full text of this manuscript is included herein as the chapter titled “Effect of a Chemical Dispersant (Corexit® EC9500A) on the Structure and Ion Transport Function of Blue Crab (*Callinectes sapidus*) Gills.”

Exposure of Blue Crabs (Callinectes sapidus) to a Chemical Dispersant (Corexit® EC9500A) Induces in Gills Cellular Events Linked to Cytotoxicity and Elicits an Endocrine-Mediated Physiological Stress Response

Weiner, A.C.; Palmer, J.W.; Watson, R.D. (in preparation)

Although use of chemical dispersant has become an important tool in oil spill response strategies, there remain concerns about possible effects of dispersant on marine life and public health. We have previously reported that exposure of adult blue crabs (*Callinectes sapidus*) to sub-lethal concentrations of the dispersant Corexit® EC9500A disrupts both the structure and ion transport function of gills. Follow-up studies reported here were designed to investigate the cellular mechanisms that underlie the cytotoxic effects of dispersant on gills; specifically, to determine whether Corexit® EC9500A promotes creation of reactive oxygen species (ROS) leading to caspase-3-dependent apoptosis. Results of immunohistochemical studies indicated that exposing blue crabs to Corexit® EC9500A (125 ppm for 24 h) increased NADPH oxidase (NOX4) immunoreactivity, C-reactive protein (CRP) immunoreactivity, and cleaved (active) caspase-3 (CC3) immunoreactivity in lamellae of anterior and posterior blue crab gills. The results are consistent with the hypothesis that exposure of blue crabs to a sublethal concentration of Corexit® EC9500A triggers in gills oxidative stress, NOX4- and CRP-mediated production of ROS, and subsequent caspase-3-dependent apoptosis. Another aim of the current studies was to determine whether exposure of blue crabs to Corexit® EC9500A elicits a physiological stress response characterized by hyperglycemia. In Crustacea, a physiological stress response is mediated by an eyestalk neurohormone, crustacean hyperglycemic hormone (CHH). We found that exposing blue crabs to

Corexit® EC9500A (125 ppm for 4 h) produced a transient (60 min) increase in hemolymphatic glucose, and a transient increase in abundance of the CHH transcript in eyestalk neural ganglia. The results are consistent with the hypothesis that exposure of blue crabs to Corexit® EC9500A promotes a CHH-driven physiological stress response that includes an increase in hemolymphatic glucose.

This manuscript is currently in preparation. Full text of this manuscript is included herein as the chapter titled “Exposure of Blue Crabs (Callinectes sapidus) to a Chemical Dispersant (Corexit® EC9500A) Induces in Gills Cellular Events Linked to Cytotoxicity and Elicits an Endocrine-Mediated Physiological Stress Response.”

Calcium Signaling and Regulation of Ecdysteroidogenesis in Crustacean Y-organs

Weiner, A.C.; Chen, H-Y.; Roegner, M.; Watson, R.D. (in peer review)

Crustacean Y-organs secrete ecdysteroid molting hormones. Ecdysteroids are released in increased amount during premolt, circulate in hemolymph, and stimulate the events in target cells that lead to molting. During much of the molting cycle, ecdysteroid production is suppressed by molt-inhibiting hormone (MIH), a peptide neurohormone produced in the eyestalks. The suppressive effect of MIH is mediated by a cyclic nucleotide second messenger. A decrease in circulating MIH is associated with an increase in the hemolymphatic ecdysteroid titer during pre-molt. Nevertheless, it has long been hypothesized that a positive regulatory signal or stimulus is also involved in promoting ecdysteroidogenesis during premolt. Data reviewed here are consistent with the hypothesis that an intracellular Ca^{2+} signal provides that stimulus. Pharmacological agents that increase intracellular Ca^{2+} in Y-organs promote ecdysteroidogenesis, while agents that lower intracellular Ca^{2+} or disrupt Ca^{2+} signaling suppress ecdysteroidogenesis. Further, an increase in the hemolymphatic ecdysteroid titer after eyestalk ablation or during natural premolt is associated with an increase in intracellular free Ca^{2+} in Y-organ cells. Several lines of evidence suggest elevated intracellular calcium is linked to enhanced ecdysteroidogenesis through activation of Ca^{2+}/calmodulin dependent cyclic nucleotide phosphodiesterase, thereby lowering intracellular cyclic nucleotide second messenger levels and promoting ecdysteroidogenesis. Results of transcriptomic studies show genes involved in Ca^{2+} signaling are well represented in Y-organs. Several recent studies have focused on Ca^{2+} transport proteins in Y-organs.

Complementary DNAs encoding a plasma membrane Ca^{2+} ATPase (PMCA) and a sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) have been cloned from crab Y-organs. The relative abundance of PMCA and SERCA transcripts in Y-organs is elevated during premolt, a time when Ca^{2+} levels in Y-organs are likewise elevated. The results are consistent with the notion that these transport proteins act to maintain the Ca^{2+} gradient across the cell membrane and re-set the cell for future Ca^{2+} signals.

This manuscript is currently in peer review. Full text of this manuscript is included herein as the chapter titled “Calcium Signaling and Regulation of Ecdysteroidogenesis in Crustacean Y-organs.”

APPENDIX B:

IACUC APPROVAL LETTER

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

MEMORANDUM

DATE: 24-May-2019

TO: Watson, R Douglas

FROM: Robert A. Kesterson, Ph.D., Chair

Institutional Animal Care and Use Committee (IACUC)

SUBJECT: **NOTICE OF APPROVAL**

The following application was approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on 24-May-2019.

Protocol PI:	Watson, R Douglas
Title:	Effects of Chemical Dispersant on Crab Gill Function
Sponsor:	UAB DEPARTMENT
Animal Project Number (APN):	IACUC-20154

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

This protocol is due for full review by 23-May-2022.