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CHEMICAL DEFENSES OF *APLYSIA CALIFORNICA* AND SENSORY PROCESSING BY PREDATORY FISHES

by

MATTHEW NUSNBAUM

Under the Direction of Charles Derby

ABSTRACT

In predator-prey interactions, prey species have complex defensive behaviors to protect themselves from predators. Chemical defenses are one tool that is employed to protect against predators, especially for slow-moving or otherwise susceptible prey. Many of these chemical defenses have been studied and the effective compounds identified, but few studies were performed on their mechanisms of detection.

In my research, I used the sea hare, *Aplysia californica*, as chemically defended prey. This slow moving mollusk is soft-bodied with no external shell, but it has adapted a number of defenses including chemical defenses. Ink is a sticky mixture of the products of the ink gland and the opaline gland which are mixed in the mantle cavity and released toward an attacker. I show that this ink secretion protects the sea hare from predation by a fish predator.

Because many deterrent compounds taste bitter, bitter taste receptors are thought to protect predators from ingesting harmful compounds in prey. Studies of deterrent taste detection have commonly utilized bitter compounds from human hedonics to study the responses in animals, such as fruit flies, fishes, rats, and monkeys. In my dissertation, I argue that the study of chemical defenses allows us to ask more questions about detection of relevant deterrents and interactions between predators and prey at the individual and population levels. My results show that diet-derived pigments in *Aplysia* ink, aplysioviolin and phycoerythrobilin, are strongly deterrent to fish predators. Electrophysiological analyses of the gustatory system show that these compounds are equipotent and cross-adapt each others' responses completely. Aplysioviolin and phycoerythrobilin produced incomplete reciprocal cross-adaptation with amino acids and adapted bile salt responses but were not significantly adapted by these latter stimuli. These results showed multiple pathways that are sensitive to aplysioviolin and phycoerythrobilin, which may have different effects on the physiology and behavior of the predatory fish. My findings demonstrate the value to the fields of chemical ecology and chemosensory biology of studying sensory processing of relevant deterrent compounds. This work lays the foundation for how a diet-derived photopigment is adapted by a species to protect itself from predators by stimulating their chemosensory systems.

INDEX WORDS: *Aplysia californica*, Aplysioviolin, *Ariopsis felis*, Catfish, Chemoreception, Deterrence, Electrophysiology, Fish, Gustation, Ink, Phycoerythrobilin, Predator-Prey, Sea hare, *Thalassoma bifasciatum*, Wrasse

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MATTHEW NUSNBAUM

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

in the College of Arts and Sciences

Georgia State University

2011

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by

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May 2011

For Erin, my beautiful bride-to-be and the inspiration for all that I do.

ACKNOWLEDGEMENTS

There are so many people I need to thank for their help, guidance, companionship, and support throughout my graduate school career. Without all of your support I never would have made it to this point and I have no idea what might have become of me! First, and foremost, Chuck who taught me not only about science and how to be a scientist but also how to think critically about not only my own ideas but the work of others. Conversations with Chuck were always thought-provoking and often left me looking at my original ideas with a new eye. I also must thank Chuck for his un-ending patience and support, without which I am sure I would have given up on frustrating experiments many times over the years. I also want to thank the rest of my committee, who always brought great insight to our meetings together. John generously invited me into his lab to learn techniques without which I would have only half of a dissertation and came to the rescue with advice and guidance many times over the years. Manfred Schmidt provided invaluable experience in the field of chemical senses and a critical eye for how I might interpret my results. Matthew Grober's insights and knowledge on everything from fish biology and behavior to evolutionary theory have made conversations with him enjoyable and enlightening over the years. I never got to spend as much time talking with any of you as I would have liked, but the time we did work together was wonderful for me and I thank each of you for being a part of my academic journey.

I would like to thank all of the members of the Derby lab, past and present, who helped make work life both enjoyable and productive. I would like to especially thank Juan Aggio for his advice, troubleshooting, conversation and friendship; it has truly been a pleasure to work with

him. Michiya Kamio, whose humorous and patient guidance into the murky world of biochemistry, helped me with a part of my project that I never thought I would take on myself.

I thank all of the other members of the Neuroscience Institute and the Biology Department who have been such good friends and colleagues along the way, including Laura Been, Vicente Colunga, Don Edwards, Charuni Gunaratne, Stephanie Gutzler, Rick Hanberry, Ko-Chun Ko, Jamie LaPraire-Beale, Josh Lilvis, Tiffany Love-Chezem, Dayna Loyd, Anne Murphy, Joe Normandin, Zeni Shabani, Mahin Shahbazi, Tizeta Tadesse, and many others.

And finally, I must thank my family for all of their love and support over the years. Without them I'm not sure I would have had the courage to come to graduate school or to stay once I got here. For as long as I can remember, I have been passionate about science and wanted to be a scientist and I am fortunate that I have a family that has always supported that goal, even when I couldn't always answer the question, "What are you going to do with that?".

TABLE OF CONTENTS

ACKNOWLEDGEMENTS v

LIST OF TABLES ix

LIST OF FIGURES x

LIST OF ABBREVIATIONS xi

1 GENERAL INTRODUCTION 1

1.1 Chemical defenses in the marine environment 2

1.2 *Aplysia californica* chemical defenses 3

1.3 Chemical Senses 5

1.4 Detection of deterrents 8

2 EFFECTS OF SEA HARE INK SECRETION AND ITS ESCAPIN-GENERATED COMPONENTS ON A VARIETY OF PREDATORY FISHES 11

2.1 Introduction 12

2.2 Materials and Methods 15

2.3 Results 22

2.4 Discussion 24

3 INK SECRETION PROTECTS SEA HARES BY ACTING ON THE OLFACTORY AND NONOLFACTORY CHEMICAL SENSES OF A PREDATORY FISH 35

3.1 Introduction 36

3.2 Materials and Methods 39

3.3 Results 46

3.4 Discussion 51

4 TASTE-MEDIATED BEHAVIORAL AND ELECTROPHYSIOLOGICAL RESPONSES TO DETERRENT PIGMENTS FROM THE INK OF THE SEA HARE *APLYSIA CALIFORNICA* BY A FISH PREDATOR *ARIOPSIS FELIS*...61

4.1 Introduction 62

4.2 Materials and Methods 66

4.3 Results 73

4.4 Discussion 75

5 GENERAL DISCUSSION……..……..…..91

5.1 Chemosensory mechanisms of chemical defenses 91

5.2 Mechanisms of action of the chemical defenses of *Aplysia californica* 93

5.3 Neurethology, neuroecology and the study of chemical defenses 97

Literature Cited 100

LIST OF TABLES

4.1 Cross-adaptation data from electrophysiological recordings from the maxillary barbel of *A. felis* 83

LIST OF FIGURES

2.1 Summary of the compounds of the escapin/L-lysine pathway in the ink and opaline secretion of sea hares 30

2.2 Responses of five fish species in ingestion assay using ink and opaline 31

2.3 Responses of five fish species in ingestion assay using the reaction products of the enzyme escapin, found in ink 33

3.1 Median (±25th and 75th interquartile intervals) number of feeding strikes by bluehead wrasses towards sea hares that released ink 57

3.2 Responses of bluehead wrasses to extraoral presentations of ink and opaline in the cloud assay 58

3.3 Responses of bluehead wrasses in the pellet assay 59

3.4 Effect of nares occlusion on feeding responses of bluehead wrasses 60

4.1 Schematic diagram of separation of deterrent compounds in *A. californica* ink, aplysioviolin (APV) and phycoerythrobilin (PEB).....84

4.2 Behavioral analysis of the concentration-response relationship of ink fractions 85

4.3 Behavioral responses in the barbel assay demonstrate that 100% full strength ink, APV, and PEB are detected by the barbels 86

4.4 Electrophysiological recordings from a branch of the facial-trigeminal nerve complex.....87

4.5 Electrophysiological concentration-response curve for APV and PEB.....88

4.6 Cross-adaptation effects of different stimuli presented to the maxillary barbel 89

LIST OF ABBREVIATIONS

AAs- Amino Acid Blend

APV- Aplysioviolin

ASW- Artificial Sea Water

CDC- Sodium Chenodeoxycholate

GDC- Sodium Glycochenodeoxycholate

OR- Olfactory Receptor Protein

ORN- Olfactory Receptor Neuron

PEB- Phycoerythrobilin

TCA- Sodium Taurocholate

TLC- Sodium Taurolithocholate

CHAPTER 1

GENERAL INTRODUCTION

In predator-prey interactions, prey species use various evasive tactics against attacking and threatening predators. Chemical defenses are used by many organisms to avoid predators, and some of the effector molecules are identified (Pawlik 1995; McClintock and Baker 2001; Matz et al. 2008). Many studies demonstrated that chemical defenses protect prey species from predation (Eisner and Meinwald 1966; Whittaker and Feeny 1971; Janzen 1977; Tachibana 1988; Paul 1992; Pawlik 1993; Berenbaum 1995; McClintock and Baker 2001; Kelley et al. 2003; Cruz-Rivera and Villareal 2006). The mechanisms behind these effects, however, have received less attention. Anti-predatory chemical defenses function as irritants, toxins, deterrents, or distracters. If an animal can prevent a predator from eating it, whether through toxins or deterrents, it enhances its likelihood to survive to increase its reproductive output. Being able to detect and respond to these defenses can protect predators from ingesting potentially harmful prey items. My dissertation is a study of the chemical defenses of the sea hare, *Aplysia californica*, their effectiveness against fish predators, the identity of the deterrent components, and the mechanisms by which these deterrents have their effects. Studying sea hare chemical defenses and fish chemoreception provides insight into the sensory processing of behaviorally relevant deterrent signals, which has not been well-studied in aquatic vertebrates.

1.1 Chemical defenses in the marine environment

Chemical defenses are widespread in marine systems and are especially concentrated in small, slow moving or otherwise unprotected species. The sources and effects of these chemical defenses are many and varied and depend on the evolutionary history of the producer as well as that of their potential consumers. Thousands of marine secondary metabolites were identified in sponges, ascidians, soft corals, bryozoans, annelids, algae, marine microbes, and many other benthic and pelagic organisms, and many of these secondary metabolites were behaviorally identified as chemical defenses (Faulkner 1991, 1993). Chemical defenses include compounds that are irritants, toxins, venoms, or deterrents, and they may have general effects or be targeted towards specific classes of predators. Sponges synthesize and maintain an enormous diversity of chemical defenses in their tissues, many of which are feeding deterrents to fishes and crustaceans (Albrizio et al. 1995; Chanas and Pawlik 1995; Pawlik et al. 1995; Wilson et al. 1999). These defenses are considered passive and constitutive because the predator has to come into direct contact with the deterrent-containing tissue in order for it to be exposed to significant concentrations of the active compounds. However, passive defenses from one species can be sequestered in the diet of a consumer, and the effect can be enhanced by the alteration or concentration of these compounds. One example is the nudibranch *Hexabranchus sanguineus*, which feeds preferentially on *Halichondria* sponges; the sponges produce oxazole macrolides that deter fish feeding (Pawlik 1993). The nudibranch alters these compounds and concentrates them in its dorsal mantle and egg masses where they serve as defenses against the nudibranch's predators. The concentrations of these deterrent compounds are relatively low in the sponge, concentrated in the nudibranch tissue, and are significantly greater in the egg masses, but even the lowest occurring concentrations produce strong deterrent responses in fish (Pawlik 1993).

Having constitutive deterrents is an effective way to avoid predation; however, if the prey can avoid any of its tissue being damaged, it may enhance its likelihood of surviving an encounter (Endler 1986, 1991; Skelhorn and Rowe 2005). To avoid any contact, the prey must release their defenses in such a way that the predator will encounter and respond to it prior to taking tissue from the prey. These active defenses can be synthesized de novo or sequestered from the diet and concentrated in such a way that they are ready for release during a predatory encounter. An efficient mechanism of action for these defenses is to take advantage of predators' existing chemical senses to produce an avoidance or rejection response. While a great deal of research has been performed in identifying compounds that function as deterrents, little is known concerning the predators' ability to detect ecologically relevant aversive cues (Hara 1994; Hay 1996; Kicklighter et al. 2005; Hayden et al. 2007; Kamio et al. 2007; Cohen et al. 2008). In this dissertation, I take advantage of the ink secretion of *A. californica* to study how it protects the sea hare from predators, how the aversive chemicals are detected and how this detection may be coded in the periphery in a predatory fish.

1.2 *Aplysia californica* chemical defenses

A. californica is a familiar species to many neurobiologists, chemical ecologists, and natural products chemists, as it is a rich source of biologically active chemistry and its nervous system has been well-studied (Kandel 1979; Kinnel et al. 1979; Walters and Erickson 1986; Gillette et al. 1991; Yamazaki 1993; Pennings 1994; Frost and Kandel 1995; Wright and Carew 1995; de Nys et al. 1996; Painter et al. 1998; Gallimore and Scheuer 2000; Ginsburg and Paul 2001; Cummings et al. 2005). *A. californica* is not known to have any natural predators, but some generalist predators will attack it on occasion (Johnson and Willows 1999). Like *A.*

californica, many prey species have to deal with the complex problems of defending themselves against a diversity of predators with a limited number of defenses (Endler 1986; Pearson 1989). Sea hares are well-suited for the study of chemical defenses, as they are generally lacking other types of protection, such as speed, cryptic coloration, or hard outer shells and must rely on defensive chemistry for protection.

Aplysia californica is a bottom dwelling shell-less gastropod mollusk that can be found in subtidal and intertidal waters in the Pacific Ocean from Northern California to Baja, California. *A. californica* can release ink when disturbed as early as the 1-mm long, post-metamorphic juvenile stage (Kriegstein 1977). The sea hare produces an ink consisting of secretions from its ink and opaline glands. Sea hares of the genus *Aplysia* obtain a variety of secondary plant toxins and pigments exclusively from a red algae diet (Winkler and Dawson 1963; Darling and Cosgrove 1966; Irie et al. 1969; Chapman and Fox 1969; Winkler 1969; Watson 1973; Blankenship et al. 1975; Kinnel et al. 1979; MacColl et al. 1990; Kamio et al. 2010a, 2010b). The two glandular products, ink and opaline, are typically released simultaneously (Tritt and Byrne 1980; Prince et al. 1998; Nolen and Johnson 2001). Ink is diffusible and purple, while opaline is cloudy and highly viscous. Ink contains red-algal derived pigments, secondary metabolites, proteins, free amino acids, and other chemicals (MacColl 1990; Pennings and Paul 1993; Johnson and Willows 1999; Petzelt et al. 2002, Kicklighter et al. 2005). Opaline contains algal secondary metabolites, proteins, free amino acids, and other compounds (Johnson and Willows 1999; Rogers et al. 2000, Kicklighter et al. 2005).

Mechanisms of chemical defense by ink of *A. californica* were previously described for two potential predators, the California spiny lobster, *Panulirus interruptus*, and a Pacific sea anemone, *Anthopleura sola* (Nolen et al. 1995; Kicklighter et al. 2005; Kicklighter and Derby

2006). *A. californica* ink deters predation by *P. interruptus* through a variety of mechanisms including unpalatability, sensory disruption, and phagomimicry. In spiny lobsters, stimulation by the ink and opaline secretions occurs in both the gustatory and olfactory systems, as demonstrated by electrophysiological recordings (Kicklighter et al. 2005). Injection of ink from *Aplysia dactylomela* into pieces of fish fillet caused rejection by 95% of laughing gulls *Larus atricilla* (DiMatteo 1981). Of the 37 species of *Aplysia*, 30 have an ink gland that can release ink when the animal is attacked by a predator (reviewed in Nolen et al. 1995; Johnson and Willows 1999). Recent work identified compounds in *A. californica* ink that are deterrent to blue crabs, *Callinectes sapidus* (Kamio et al. 2010a, 2010b). Further, these same compounds, Aplysioviolin (APV) and Phycoerythrobilin (PEB), are deterrent to fish at natural concentrations and in serial dilutions to 0.01% full strength. Chapter 2 of this dissertation examines the deterrent effects of the components of the ink secretion, ink and opaline, against a group of predatory fishes representing a variety of predatory styles and habitats. Chapter 3 tests the fish chemosensory systems affected by ink and the effectiveness of the ink secretion in protecting the sea hare from predatory fishes.

1.3 Chemical Senses

Vertebrates can possess a number of chemosensory structures, including taste buds, olfactory organs, vomeronasal organs, septal organs, the Grüneberg ganglion and solitary chemoreceptor cells. Gustation is defined as the chemical sense that is mediated by taste receptor cells within taste buds. Olfactory responses are mediated through specific protein receptors expressed in the dendrites of primary olfactory receptor neurons (ORNs). The other chemosensory organs are not as well-studied for their physiological or behavioral effects and

will not be a focus in this dissertation. The behaviors mediated by olfaction and gustation can often discriminate the two systems. Gustation mediates simple and reflexive behaviors, most commonly consummatory feeding behaviors, whereas olfaction is often involved in more complex behaviors, such as searching for distant chemical sources, courtship behavior, and chemosensory learning behaviors (Atema 1977).

Olfactory systems are involved in the detection and discrimination of a vast number of biologically relevant compounds used to identify and locate prey, conspecifics, mates, or spawning habitats (Sorensen and Caprio 1998). Initially, the olfactory system detects odorants with an assortment of olfactory receptor molecules (ORs) located within the dendritic membranes of ORNs. The majority of vertebrate ORs are members of the superfamily of seven-transmembrane domain G-protein coupled receptors, but small groups of ORNs that do not express ORs were identified that express trace amine-associated receptors (TAARs), transient receptor potential (TRP) channels or V1R receptors in addition to GC-D neurons which express the receptor guanylyl cyclase GC-D and utilize a cGMP-mediated cascade to transduce chemosensory stimuli (Buck and Axel 1991; Munger et al. 2009). Canonical ORNs generally express one of ~1,000 OR genes in mammals (Buck and Axel 1991) or one of ~100 in fish (Ngai et al. 1993; Barth et al. 1996) which encode for molecular receptors. Evidence indicates a correlation exists in the species of teleosts investigated between the anatomical shape of ORN, the class of molecular receptor expressed, the type of biologically relevant odorant detected, the signal transduction cascade activated, and the portion of the olfactory bulb (OB) that processes the odorant information (Friedrich and Korsching 1998; Hansen et al. 2003; Hara and Zhang 1996; Nikonov and Caprio 2001; Sato et al. 2005). Though Chapter 4 focuses on processing of defenses by the gustatory system, the olfactory system in fishes is important for food search and

learned behaviors and may play an important role in the detection and processing of chemical defenses (Valentinčič et al. 2000; Valentinčič 2005; Derby and Sorensen 2008).

Taste information is transmitted from peripheral gustatory receptors to the central nervous system via cranial nerves VII, IX and X. Gustatory receptor molecules are expressed by taste cells organized into structures called taste buds in the epithelium of the oropharyngeal cavity and, in the case of many fishes, on the surface of the body. Gustatory receptor cells express T1R and T2R molecular receptors which are G-protein coupled receptors (Ishimaru et al. 2005). In fishes, T1Rs function as dimers and detect amino and nucleic acids while T2Rs have not been shown to form dimers and detect aversive compounds (Oike et al. 2007). Brockhoff et al. (2010) showed that individual T2Rs are broadly-tuned to respond to a wide variety of bitter and toxic compounds, allowing taste cells possessing a small number of T2R receptor types to respond to a broad spectrum of aversive compounds. Vertebrate gustatory receptor cells are different from olfactory receptor cells that are primary neurons in that taste cells are modified epithelial cells and therefore synapse on specific cranial nerves to relay taste information to the central nervous system. Some taste cells express more than one receptor type, which also distinguishes them from ORNs (Ishimaru 2005). In fishes, taste buds lying within the oropharyngeal cavity are innervated by cranial nerves IX and X, while those positioned on the exterior are innervated by cranial nerve VII (Atema 1971). Cranial nerve VII innervated extraoral taste buds are implicated in the localization of food, whereas those within the oral cavity are innervated by IX and X cranial nerves and are involved in ingestion and rejection behaviors (Atema 1971; Finger and Morita 1985; Morita and Finger 1985). Both systems are highly sensitive to amino acids and contain specific selective fibers that in channel catfish are most responsive to L-arginine (L-arg), L-alanine (L-ala), and L-proline (L-pro), and these fibers

are in different proportions in the IX and VII systems (Ogawa and Caprio 2010). The fish gustatory system not only has high specificity for amino acids, but exhibits diverse sensitivities to organic and inorganic chemicals including bile acids, polyamines, nucleotides, quinine and carbon dioxide (Yoshii et al. 1979, 1980; Hara et al. 1984; Yamamori et al. 1988; Yamashita et al. 1989, 2006, Michel et al. 2003; Rolen et al. 2003; Caprio and Derby 2008). Many amino acids, polyamines and nucleotides are thought to be attractive, food related stimuli for fishes (Carr et al. 1996; Kasumyan and Døving 2003). The behavioral relevance of gustatory detection of bile salts and the mechanisms underlying detection of deterrents have not been well studied to date. Taste information, detected at the periphery, is transmitted to the dorsal parts of the facial and vagal lobes, the primary gustatory nuclei of the CNS, for further processing and eventual translation into appropriate behavioral responses (Atema 1971). In Chapter 4, I show that the catfish gustatory system is sensitive to components of the sea hare ink secretion and that these components also reduce responsiveness to a blend of stimulatory amino acids.

1.4 Detection of deterrents

Because many toxic metabolites taste bitter, bitter gustatory receptors are thought to protect predators against the ingestion of poisonous compounds in prey (Garcia and Hankins 1975; Glendinning 1994; Glendinning et al. 1999). Studies examining aversive taste discrimination found differences between invertebrate systems and vertebrate systems. In vertebrate systems, most commonly studied in mice and rats, a bitter sensitive gustatory receptor cell expresses many different T2Rs, which allows it to respond to a broad range of bitter compounds (Chandrashekar et al. 2000; Mueller et al. 2005). Behavioral studies have come to different conclusions on whether animals can discriminate between different bitter stimuli (Dahl

et al. 1997; Aspen et al. 1999; Scott et al. 1999; Spector and Kopka 2002). The current prevailing notion is that each T2R-expressing cell functions as a separate broadly-tuned bitter detector that can respond to a wide diversity of compounds but is not necessarily able to discriminate among them. Each cell expresses a subset of the bitter T2Rs, so that the population can differentially express all of the bitter receptor proteins. With this receptor expression pattern vertebrates may not be able to discriminate deterrents at the gustatory receptor cell level, but neuronal innervation and activation patterns may serve as a mechanism by which deterrent identity can be assessed. Two types of gustatory nerve fibers in channel catfish are affected by quinine; Group I fibers fire action potentials after quinine presentation and Group II fibers lose responsiveness to amino acids when they are mixed with the deterrent compound (Ogawa et al. 1997). The behavioral consequences of the observed activity patterns in gustatory fiber types are not currently known, but it is possible that they reinforce each others' effects so that the animal is deterred by aversive compounds and has reduced sensitivity to attractive compounds. In invertebrate systems, evidence is accumulating that the gustatory system functions differently. Invertebrate gustatory receptors are expressed in primary gustatory neurons as opposed to the modified epithelial cells found in vertebrates (Derby and Sorensen 2008). Some of these gustatory neurons are broadly tuned, as in vertebrates, but others are narrowly tuned to specific relevant compounds and may function as part of a labeled line for those compounds (Clyne et al. 2000; Moon et al. 2006; Weiss et al. 2011).

Studies in invertebrates revealed a great deal about their detection and processing of chemical defenses and the pathways involved in these processes. *Drosophila* behaviorally discriminate between bitter tastants because the population of gustatory receptor molecules is differentially expressed in different types of taste neurons (Meunier et al. 2003; Weiss et al.

2011). Meunier et al. (2003) showed that specific sensilla on *Drosophila* prothoracic legs respond with dose-dependent latency to bitter compounds and identified the responding cells as S, W, and L2 cells. These cells responded with similar latencies, but had opposite activity patterns. The behavioral effects of these different cell types are not known, but they could reinforce each other to ensure an effective deterrent response. Weiss et al. (2011) found that the specificity and sensitivity of different classes of taste neurons differed, which could allow for a combinatorial code for identification of specific bitter tastants. These activity patterns may aid in differentiating the identity of deterrent compounds and determining the appropriate contextual behavioral response. Thus, deterrents, which are a class of molecules that are behaviorally important, are robustly detected through very different systems in vertebrates and invertebrates. A great deal more needs to be done to understand the detection of behaviorally relevant deterrent compounds by vertebrates. Chapter 4 takes what I have learned about the chemical defenses of *A. californica* and uses this information to examine mechanisms of detection of ecologically relevant deterrents by an electrophysiological model for vertebrate gustation, the sea catfish *Ariopsis felis*. By using identified natural compounds to study the detection and response to deterrents I can begin to ask a number of questions that allow me to not only characterize the function of the chemosensory systems, but also to address ecological interactions at many other levels of organization from chemical synthesis to population dynamics. I propose that the study of chemosensory pathways involved in the detection of chemical defenses should be the focus of sensory biologists who wish to understand the evolution and function of chemosensory systems as well as chemical ecologists interested in the evolution and population dynamics of secondary metabolites.

CHAPTER 2

EFFECTS OF SEA HARE INK SECRETION AND ITS ESCAPIN-GENERATED COMPONENTS ON A VARIETY OF PREDATORY FISHES

Acknowledgments: Funding was provided by NSF IBN-0614685, a GSU Brains & Behavior fellowship, and support from the NSF IGERT program at Georgia Institute of Technology.

Previously published as: Nusnbaum, M, Derby CD (2010) Effects of sea hare ink secretion and its escapin-generated components on a variety of predatory fishes. Biol Bull 218: 282-292

2.1 Introduction

Predator-prey interactions can exert strong selection pressure that affects the evolution of anti-predation defenses (McClintock and Baker 2001; Paul *et al*. 2007; Zimmer and Ferrer 2007; Hay 2009). These defenses include behavioral adaptations, body coloration, mechanical defenses, and chemical defenses. To be effective, an anti-predator defense must disrupt the predation event at the point of detection, approach, capture, or acceptance of the prey (Endler 1986). Chemical defenses can be either passive, such as compounds constitutively found in tissues, or actively released, as in the nematocysts of a sea anemone. The adaptations are restricted by the natural history of the species, and they control the relationship that prey species have with their potential predators. Prey species that may encounter a variety of predators must be adapted for protection against a variety of predation methods and must have defenses that affect organisms with very different sensory systems and adaptations of their own.

Mollusks in general, and opisthobranch mollusks in particular, have an impressive array of defenses against a broad range of predators from diverse taxa, including sea anemones, sea stars, crustaceans, fishes, and humans (Kinnel et al. 1979; Denny 1989; Avila et al. 1991; Cimino and Ghiselin 2001; Cimino and Gavagnin 2006). Opisthobranchs, which include sea hares, are soft-bodied and slow-moving benthic snails that live in many marine habitats (Carefoot 1987; Wägele and Klussmann-Kolb 2005). No predator is known to make a regular meal of them, but a number of generalist predators, notably fish, crustaceans, and sea anemones, have been reported in field studies to occasionally consume them (Winkler and Tilton 1962; Pennings 1990; Paul and Pennings 1991; Johnson and Willows 1999; Ginsburg and Paul 2001; Pennings et al. 2001). Sea hares would be highly vulnerable to predators if not for the possession of a variety of defenses that include escape behaviors, large size, crypsis, and chemicals

(Carefoot 1987; Johnson and Willows 1999). Chemical defenses of sea hares include both passive and active forms. Passive chemical defenses include deterrent and toxic molecules in the skin and other tissues that are highly effective against many predators (Winkler 1969; Watson 1973; Stallard and Faulkner 1974a, b; Ambrose et al. 1979; Kinnel et al. 1979; Paul and Pennings 1991; Pennings and Paul 1993; de Nys et al. 1996; Pennings et al. 1999; Ginsburg and Paul 2001; Wägele and Klussmann-Kolb 2005; Kamiya et al. 2006; Wägele et al. 2006; Derby 2007), but can also include having flesh of low nutritional value (Pennings 1990; Penney 2002). Inking is an active chemical defense that is used as a late line of deterrence during attacks. Sea hare ink secretion is a sticky, purple mixture of the products of two glands (Nolen et al. 1995; Johnson and Willows 1999): ink, a product of the ink gland, is a deep purple color; opaline, a product of the opaline gland, is white and highly viscous. Ink and opaline are co-secreted, mixed in the mantle cavity, and released toward the source of the attack.

Ink secretion has been shown to protect sea hares against a number of predators, especially invertebrates such as crustaceans and sea anemones, though the identity of bioactive molecules and mechanisms of its effects are largely unexplored (see reviews by Carefoot 1987; Johnson and Willows 1999; Derby 2007). Mechanisms of action of sea hare ink secretion are best studied for *Aplysia californica* and two of its invertebrate predators, the spiny lobster *Panulirus interruptus*, and the sea anemone *Anthopleura sola*. Ink secretion reduces predation by *P. interruptus* through a variety of mechanisms including unpalatability, sensory disruption, and phagomimicry (Kicklighter et al. 2005; Shabani et al. 2007; Aggio and Derby 2008). Against sea anemones, ink secretion is an unpalatable deterrent that causes tentacular withdrawal (Nolen et al. 1995; Kicklighter and Derby 2006). Recent work on the blue crab *Callinectes sapidus*, has

determined that one of ink's purple pigments, aplysioviolin, is a chemical deterrent (Kamio et al. 2010).

Much less is known about the effects of sea hare ink secretion on another dominant class of predators in marine habitats—predatory fishes. Ink secretion from *Dolabella auricularia* is unpalatable to reef fishes (Pennings et al. 1999), and ink secretion from *Aplysia dactylomela* induced increased swimming activity in a puffer and goby (Carefoot et al. 1999). Ink, but not opaline, from *A. californica* is unpalatable to the sea catfish *Ariopsis felis* (Sheybani et al. 2009). In fact, opaline and its amino acid fraction are appetitive to sea catfish, suggesting that opaline might contribute to the effect of the ink secretion through sensory disruption or phagomimicry (Sheybani et al. 2009).

The current study had two goals. The first was to evaluate the efficacy of sea hare ink secretion as a chemical deterrent against fish, with a future aim of examining mechanisms of its effect on this group of predators. I examined five species of fishes, which represent a variety of predation styles and habitats, since these variations might influence the effectiveness of a particular defensive strategy. The second goal was to test the deterrent effects of a set of components in ink—those produced by the escapin pathway— on these fish predators. Escapin is an L-amino acid oxidase that oxidizes its substrates, L-lysine and L-arginine in opaline, when ink and opaline are secreted simultaneously, and produces a complex set of compounds that are mild deterrents against *Panulirus interruptus* and *Callinectes sapidus* (Yang et al. 2005; Johnson et al. 2006; Kamio et al. 2007, 2009; Aggio and Derby 2008) (Fig. 1).

2.2 Materials and Methods

Animals

To test for the effects of the *Aplysia californica* ink secretion on predatory fishes, I performed an ingestion assay on five species of fishes with different feeding styles, ranging from those that engulf prey whole to those that peck small pieces from larger prey items. I included in my study species that are strongly suspected of being predators of sea hares as well as some that are practical laboratory models that can be used in future mechanistic studies.

The bluehead wrasse *Thalassoma bifasciatum* represents a good laboratory model as well as a potential predator of the sympatric sea hare *Aplysia dactylomela,* and I have performed further experiments with this species to examine mechanisms of deterrent effects (Nusnbaum and Derby 2010). Bluehead wrasses are found in the waters around Florida and the Caribbean islands (Feddern 1965). The advantages of using this species for aquarium bioassays have been detailed previously (Pawlik et al. 1987). It is a common fish species for testing anti-predatory chemical defenses because it is easy to maintain and train to feed on artificial diets (Lindquist and Hay 1996; Hay et al. 1998; Kubanek et al. 2000; Odate and Pawlik 2006). For my study, juvenile animals, 5–10 cm long, in the yellow phase were wild-caught in south Florida and maintained at Georgia State University in individual 40-liter glass aquaria (50 cm x 25 cm x 30 cm) containing filtered and aerated (Whisper Filters Tetra, Blacksburg, VA) seawater (Instant Ocean, Aquarium Systems, Mentor, OH) at a salinity of 28 ppt and a temperature of about 21 °C. Fish were fed shrimp and brine shrimp *ad libitum* twice daily. Fish were kept on a 14:10 light/dark cycle and maintained in the same aquaria in which they were tested.

The other fishes that I tested are señorita wrasses *(Oxyjulis californica),* bonnethead sharks *(Sphyrna tiburo),* mummichogs, or killifish *(Fundulus heteroclitus),* and pinfish *(Lagodon*

rhomboides). Señorita wrasses are sympatric with *A. californica* at intermediate depths in the Pacific (Bray and Ebeling 1975). Although there are no records of predation events between these species, it is possible for adult señorita wrasses, which can reach 25 cm in total length, to eat juvenile sea hares. Bonnethead sharks are found along the east and west coasts of North and South America and could potentially encounter one of a number of *Aplysia* species including *A. californica* (Enric et al. 1996). This shark is a bottom-feeding predator that eats a wide variety of molluscs and crustaceans. Mummichogs are small generalist predators that typically feed on insect larvae, small crustaceans, and molluscs, and live in intertidal waterways or salt marshes throughout the Atlantic coastal areas (Bigelow and Schroeder 1953). It is unlikely that this species would encounter a sea hare or attack one in nature, but it represents a generalist predator that can easily be trained to feed on artificial diets. Pinfish are unlikely to attack a sea hare, but they represent a predatory fish species with a variable diet and have been used in studies of the efficacy of chemical defenses (Huang et al. 2008).

Señorita wrasses, each about 15 cm long, were wild-caught by Marinus Inc. (Garden Grove, CA), shipped to my laboratory, and kept individually in aquaria in the same conditions as bluehead wrasses. Pinfish averaging 12 cm in length were obtained by dropping lines and hooks off a dock into waters near the Whitney Laboratory (St. Augustine, FL). Mummichogs were also obtained from the Whitney Laboratory; 10 cm long fish were caught in traps in shallow marshy areas. Pinfish and mummichogs were kept individually in 20-liter (40 cm x 20 cm x 20 cm) plastic containers supplied with flowing seawater and fed pieces of shrimp throughout the experiment. Bonnethead sharks were caught by personnel at Mote Marine Laboratory (Sarasota, FL) and held in that facility. The bonnethead sharks, about 20–90 cm long, were housed in a single group of 20 animals in a 227,000-liter aquarium (15 m in diameter and 3 m in depth) and

fed *ad libitum* on a combination of shrimp and fish during an acclimation period. The acclimation period lasted until the fish fed reliably on introduced food for between 3 and 5 days for all species tested. For the assays, they were fed sparingly on shrimp to maintain hunger levels. After completion of these studies, which took 1–2 weeks, pinfish and mummichogs were returned to the waters where they were caught, and bonnethead sharks were used for further behavioral analyses by other researchers at the Mote Marine Laboratory. Señorita wrasses and bluehead wrasses were maintained for 1–3 months and tested in multiple behavioral assays before being euthanized because they could not be returned to the waters where they were caught.

Collection of sea hare secretions

Ink and opaline were collected from adult sea hares wild-caught by Marinus Inc. (Garden Grove, CA) immediately after their arrival in my laboratory. The diet of these wild-caught individuals is not known, but the presence of purple ink indicated that their diet included red algae. Secretions were collected from the dissected ink and opaline glands. Ink glands were gently squeezed to release ink. Opaline glands were centrifuged at 30,000 x g for 1 h at 4° C to separate opaline secretion from gland tissue. Secretions collected from individual animals were pooled to reduce any effect of individual variability in contents of glands. Secretions were frozen at -80 °C until needed.

Preparation of other stimuli

Escapin, an L-amino acid oxidase in ink of *A. californica*, was purified from ink by using an AKTA 100 Automate fast protein liquid chromatography (FPLC; Amersham Pharmacia Biotech, Piscataway, NJ). A preparative grade Hi-load Superdex 200 16/60 column (Amersham

Pharmacia Biotech) was used for initial size separation, with fractions collected in an automated fraction collector. The mobile phase consisted of 50 mmol l^{-1} potassium phosphate buffer at pH 7.6. Fractions containing escapin had a yellow color and eluted separately from the purple pigments in the ink (Yang et al. 2005). To make escapin end products for L-lysine or L-arginine (Fig. 1), escapin was incubated with 145 mmol l^{-1} L-lysine or 350 µmol l^{-1} L-arginine at 30 °C in 50 mmol l^{-1} potassium phosphate buffer for 48–72 h. These are the natural concentrations of L-lysine and L-arginine found in opaline of wild-caught animals (Kicklighter et al. 2005; Derby et al. 2007), and therefore products were tested at these maximal concentrations. Production of escapin intermediate products for lysine or arginine (Fig. 1) followed the same protocol as for escapin end product except that 4 mg/ml of catalase (C1345, Sigma- Aldrich, St. Louis, MO) was added to the solution to scavenge H_2O_2 and prevent the completion of the reaction. Escapin and catalase were removed from the solution by filtration, and the solution was lyophilized for storage at -20°C. H_2O_2 and ammonia were tested at 145 mmol l^{-1}, since L-lysine is present at this concentration in *A. californica* ink (Derby et al. 2007) and therefore 145 mmol l^{-1} is the highest concentration that H_2O_2 and ammonia could reach in a reaction. The combination of lysine intermediate products + H_2O_2 is much more bactericidal than either alone (Yang et al. 2005; Ko et al. 2008). I tested this mixture, as well as mixtures of other escapin products + H_2O_2 or ammonia, to determine if they are more effective deterrents than their components.

Ingestion assay

Preparation of pellets.

Pellets were created to test the effect of added stimuli on feeding behavior, especially the acceptance of the food, as described in Hay et al. (1998) and as used previously in Sheybani et al. (2009). To make the pellets, shrimp purchased at a local seafood market were freeze-dried, then ground into a powder using a mortar and pestle. Powdered shrimp and alginate (Sigma-Aldrich) were combined in a 5:3 ratio by weight, and 8 g of this mixture was added to 100 ml of deionized water. Opaline, seawater, and uncolored escapin products were colored with 0.1% red food color (McCormick & Co., USA: listed contents are water, propylene glycol, FD&C reds 40 and 3, and propylparaben). The addition of food color to these stimuli was intended to control for the color and intensity of ink. This shrimp-alginate solution was drawn into a 50 µl pipette and exuded into a 0.25 mol l-1 $CaCl_2$ solution, creating a solid cylinder of 1 mm diameter that was cut into pellets 3 mm long. Unflavored alginate pellets were produced by following the same procedure except that shrimp was not added. Preliminary behavioral tests showed that shrimp-alginate pellets were attractive to fish, whereas unflavored alginate pellets were not. Shrimp-alginate pellets could be treated with test solutions by combining 1 ml of test solutions per 3 ml of alginate gel, to create test pellets. This creates pellets containing 25% full-strength test stimulus, which is likely in the range of secretion concentrations that fish are likely to encounter when attacking live, juvenile sea hares.

Behavioral testing.

For bluehead wrasses, señorita wrasses, pinfish, and mummichogs, individually held animals were acclimated to hand feeding with a food stimulus, and only those fish that ate were used in subsequent testing. During the experiment, each individual of these four species was presented once with each of the 16 test substances and the control. The time between consecutive stimulus presentations was at least 20 min. Fish were tested no more than eight times each day to maintain high hunger levels. Food was fed to fish between each test, and data for a test were not used if the fish rejected or ignored the food. Alginate pellets flavored with freeze-dried shrimp powder were used in all ingestion assays except for those requiring immediate feeding after mixing of the stimuli, since the formation of the pellets requires time to gel. In these cases, 2-mm cubes of freeze-dried shrimp were treated with test substances. Using freeze-dried shrimp was especially important in tests mixing escapin intermediate products with H_2O_2, because these two products combine in a non-enzymatic reaction. The kinetics of that reaction (Kamio et al. 2009) requires that these stimuli be fed to the fish immediately upon mixing. Some unstable and transient products from the reactions are hypothesized to be involved in the deterrent effects, and they could be at undetectable levels within 1 min of mixing. Therefore, all of the experiments in which escapin intermediate products were mixed with H_2O_2 or NH_3 used freeze-dried shrimp, as did experiments with ink and opaline mixed together (ink + opaline). In these cases, the substances were applied dropwise onto the pieces of freeze-dried shrimp and immediately presented to fish. Ink and opaline were applied together in this manner, with two pipettes simultaneously releasing secretions onto the same piece of shrimp to allow mixing.

Bonnethead sharks were fed freeze-dried shrimp soaked in test substances, rather than pellets, because pellets could not be formed that would be large enough to be bite-size. Each shrimp (*ca.* 32 mm long) was peeled and saturated with test substances applied dropwise onto the flesh before being immediately presented to the sharks with a pair of forceps. Each of the nine test substances and the control were presented eight times to the group of 20 sharks, and each test substance was followed by a piece of food to ensure normal feeding by the sharks. At least 15 min was allowed to pass between presentations of consecutive test substances. Control and test substances were presented in a randomized order, and no more than 10 presentations were given to the sharks in a single day. The experimenter could not discriminate the identity of the sharks in the group, and thus we cannot exclude multiple treatments of a substance with the same shark.

Fish were hand-fed food held in a pair of forceps. The food items, which contained different substances as described below, were presented in a random order to avoid order effects, and they were presented blind to protect against observer bias; however, due to the deep color of ink it was not possible to completely hide its nature from the researcher. I used acceptance or rejection of food as a measure of its palatability. Acceptance is defined as taking the food into the mouth, followed by swallowing it during test. Rejection is defined as the food not being swallowed and remaining in the aquarium at the end of the test period. When encountering a piece of food, the fish typically brought it into its mouth and flushed water through the mouth and out the gills. If the food was palatable, the fish kept the item in its mouth and swallowed it. If the food was strongly aversive, the fish either did not take it into the mouth or took it in and immediately ejected it. If the food was not strongly aversive, the fish often repeatedly brought it into its mouth and ejected it. The outcome was rated “rejection” if the food had not been swallowed by the end of the test period. The fish would generally take the food into its mouth

immediately upon presentation and then would either swallow it or spit it out. The fish was observed for about 30 s after ingestion to ensure that it did not later reject a previously accepted food item. A satiety control was presented after each test sample; if the fish did not accept a control food sample, the prior response was not used in the data analysis. Responses were recorded as either "rejection" or "acceptance," and were analyzed using Cochran's *Q* test with *post hoc* testing employing one-tailed McNemar's tests.

2.3 Results

Responses to food treated with sea hare ink or opaline

Four species of fishes – señorita wrasses, bluehead wrasses, mummichogs, and pinfish – were tested with alginate pellets of different composition. All fish were tested individually (see Fig. 2 for number of animals for each species), and all individuals used in the study accepted shrimp-flavored pellets (a positive control) and rejected unflavored pellets (a negative control). All individual fish of each species also rejected shrimp-flavored pellets containing either ink or ink + opaline, and they accepted shrimp-flavored pellets containing opaline (Fig. 2 A-D). Thus, ink or ink + opaline cause significant rejection of otherwise palatable food in these four species of fish (see statistics in Fig. 2 A-D).

Bonnethead sharks were tested as a single group of 20 animals rather than individually because of housing limitations. Sharks were fed freeze-dried shrimp rather than alginate pellets because pellets could not be made of sufficiently large size for the sharks. The group of sharks was presented eight times with each test substance. The group accepted all eight presentations of shrimp or shrimp containing opaline (Fig. 2E). The group accepted 5 of 8 presentations (62.5%) of shrimp containing either ink or ink + opaline. Overall, there was a significant effect of

treatment (Cochran's Q test, Q=9, df=3, *P*=0.029). Pair-wise testing failed to reveal a significant difference between any test substance and the control, although there was a strong but non-significant trend (*P*=0.06) for shrimp treated with ink or ink + opaline to be rejected more than the control. My behavioral observations revealed that those sharks that ate ink-treated shrimp handled them differently from plain shrimp: they repeatedly spit them out and took them back in their mouth before finally accepting and swallowing them.

Thus, four of the five tested species of fishes showed clear and statistically significant rejection of ink-treated food, and the other species showed a strong tendency towards rejection as well as qualitative differences in handling of ink-treated food.

Responses to food treated with escapin products

Two of the five fish species – bluehead wrasses and señorita wrasses – significantly rejected shrimp-flavored pellets or shrimp containing some of the products of escapin's activity on lysine and arginine (Fig. 3 A, B). Pellets with lysine intermediate products + H_2O_2 were rejected by 26% of bluehead wrasses and 26% of the señorita wrasses. Pellets with arginine intermediate products alone were rejected by 22% of bluehead wrasses. H_2O_2 alone did not significantly deter feeding by any of the species tested (Fig. 3 A-E). Mummichogs, pinfish, and bonnethead sharks were not significantly deterred by any escapin products (Fig. 3 C-E). Since the concentrations of escapin's intermediate and end products tested were near the theoretically highest concentrations that they might occur in the secretions, these results indicate that for the two species of wrasses, escapin products are at most minor contributors to the deterrence of sea hare secretions, and for pinfish, mummichogs, and bonnethead sharks, escapin products do not contribute to the deterrence.

2.4 Discussion

Animals have a diversity of defenses against predators (Endler 1986; McClintock and Baker 2001; Paul et al. 2007; Zimmer and Ferrer 2007; Hay 2009). These defenses function to disrupt the sequence of a predatory attack at the point of detection, approach, capture, or acceptance of the prey. Prey animals can utilize multiple mechanisms of protection from different predators and in different contexts (Endler 1986). Molluscs have an impressive array of defenses to protect themselves from a broad host of predators from diverse taxa including sea anemones, sea stars, crustaceans, fishes, and humans. Some molluscs are protected by shells, but many are not. Some, such as the squid, take advantage of speed and acute vision for protection. Chemical defenses are used extensively by both shelled and shell-less mollusks. The mucus secreted by molluscs can function as a mechanical and a chemical defense as well as a carrier for defenses (Branch 1981; Rice 1985; Avila et al. 1991; Ehara et al. 2002; Kicklighter et al. 2005). The skin of marine gastropods has deterrent chemicals, many of which are diet derived (Stallard and Faulkner 1974; Pennings and Paul 1993; Pennings 1994; de Nys et al. 1996; Ginsburg and Paul 2001). Mucus and deterrent-rich skin and egg masses are examples of passive defenses, but molluscs also possess a variety of active chemical defenses that are only released upon predatory attack. These chemical defenses include the ink of gastropods such as the sea hare *Aplysia californica* but also include ink of cephalopods which may act as a visual mimic, distracter, or smoke screen in addition to its potential chemosensory effects (Caldwell 2005; Derby et al. 2007; Wood et al. 2008).

Sea hare ink secretion as a chemical defense against a diversity of predators

Chemical defenses play a large role in the life of sea hares. Inking is a defense used only when sea hares are severely disturbed (Leonard and Lukowiak 1985). My observations show that sea hares will tolerate physical manipulation without inking, for example, pecking by bluehead wrasses, poking and biting by crustaceans, ingesting by sea anemones, and handling by humans. Thus, inking is a high threshold behavior, typically only produced in severe attacks, such as when taken into the mouth of a large fish or following vigorous pecking by smaller fishes. This would be expected if acquisition and sequestration of the active compounds in ink is energetically costly.

A. californica ink is broadly effective as a chemical defense against an array of predators. I have not found a species that does not show some aversive response to ink secretion, and many and diverse species, including cnidarians, crustaceans, and fishes are known to be affected by external presentation of ink (DiMatteo 1982; Nolen et al. 1995; Rogers et al. 2000). Ink is even a powerful antimicrobial agent (Ko et al. 2008) or a toxin for some animals (Flury 1915). An animal that would otherwise be vulnerable to attack from a variety of predators must have defenses that protect them from this same variety. A chemical defense that affects sensory systems of members of many different phyla functions as a good broad spectrum protection.

Our study examined the use of ink by sea hares as a chemical defense against vertebrate predators, based on an ingestion assay with five species of predatory fishes: bluehead wrasses *Thalassoma bifasciatum*, señorita wrasses *Oxyjulis californica*, pinfish *Lagodon rhomboides*, mummichogs *Fundulus heteroclitus*, and bonnethead sharks *Sphyrna tiburo*. My results demonstrate that sea hare ink secretion is unpalatable to all five species. All species showed aversive responses to otherwise palatable food when it was impregnated with the sea hare ink

secretion (Fig. 2). This was clearest with bluehead wrasses, señorita wrasses, pinfish, and mummichogs, which significantly rejected food laced with ink. The aversion of bonnethead sharks to ink secretions was weaker but still evident, as indicated by a statistically significant effect of secretions on acceptance of food and a change in handling of food treated with ink. The lower rejection rates in the feeding assay in sharks may be explained by the fact that these experiments were performed with whole freeze dried shrimp rather than shrimp-flavored pellets as in the other fishes. Palatability and attractiveness of potential food sources are controlled by many factors including hunger level, the presence and concentration of attractant molecules such as amino acids, the presence and concentration of deterrent molecules, and the perceived nutritional value of the food (McClintock and Baker 2001; Cruz-Rivera and Hay 2003). My ability to discern finer levels of deterrence may be affected by hunger level, concentration of attractive molecules and the predation style of the fish species.

Our test species included fish with predation styles ranging from those that would likely engulf a sea hare (bonnethead shark) to others that would likely attack sea hares by pecking small pieces from it (wrasses, pinfish, mummichogs). Some are more likely than others to be predators of sea hares (bonnethead sharks, wrasses, pinfish) (Bigelow and Schroeder 1953; Bray and Ebeling 1975; Enric et al. 1996; Huang et al. 2008). Some are proven behavioral models in studies of chemical defenses and good candidates for future physiological mechanistic studies (bluehead wrasses) (Pawlik et al. 1987; Lindquist and Hay 1996; Hay et al. 1998; Kubanek et al. 2000; Odate and Pawlik 2006).

Similar effects of sea hare ink secretion on food acceptance were observed with sea anemones (Nolen et al. 1995; Kicklighter and Derby 2006), spiny lobsters (Kicklighter et al. 2005; Aggio and Derby 2008), crabs (DiMatteo 1982), reef fishes (Pennings et al. 1999), sea

catfish (Sheybani et al. 2009), and sea gulls (DiMatteo 1981). Together, these results demonstrate that ink secretion is unpalatable to a broad array of marine predators.

Chemical defenses, such as sea hare ink, can have effects on different phases of attack by predators. The process of predatory attack involves two phases: approach and capture of food, when the prey is taken into the mouth, and the acceptance phase, when the prey is swallowed and consumed (Endler 1986; Ritson-Williams and Paul 2007). When ink is presented as a cloud, as might happen before a predator actually bites or attempts to ingest sea hares, it can cut off an attack (Nolen et al. 1995; Kicklighter et al. 2005; Nusnbaum and Derby in press). When presented in food, as might happen when a predator takes a bite of a sea hare and simultaneously gets a mouthful of ink, it causes egestion (DiMatteo 1982; Rogers et al. 2002; Kicklighter et al. 2005; Nusnbaum and Derby in press). In bluehead wrasses, these varied effects are due to responses by the olfactory system and the gustatory system respectively (Nusnbaum and Derby in press). Understanding how a potential chemical defense is detected by the predators' sensory systems gives insight into both the co-evolution of these signals and the sensory biology of deterrence. There are many examples of plant chemical defenses against insects and the identity of the insects' sensors that detect them (e.g. Stowe et al. 1995; Bernays et al. 1989; Glendinning et al. 1990). For example, some tannins produce deterrent effects on herbivores, mediated by taste receptors on mouthparts, and at high concentrations tannins can produce systemic toxicity (Mueller-Harvey 2006). Herbivores' detection of deterrent compounds and association of this effect with the tannin source can help it to avoid toxic effects and protect the tannin producer from predation. Alternatively, toxic or aversive plants can produce volatiles (which may or may not be directly associated with the toxic effects) that herbivores may associate with the somatosensory or gustatory experiences and learn to avoid such defended prey (Woolfson and

Rothschild 1990; Rothschild et al. 1994). The deterrent responses that fishes and other predators display toward sea hare ink may function as both a protection from immediate predation as well as a chemical stimulus for learned aversion (Long and Hay 2006).

Identity of the components in sea hare ink secretion that are deterrents against fish

Sea hare ink secretion is a mixture of ink from the ink gland and opaline from the opaline gland. When combined, ink and opaline form a more persistent, sticky secretion than ink alone. To determine whether the defensive chemicals in ink secretion are present in ink, opaline, or some of the identified components of the ink secretion, I used the same five species of predatory fishes. I show that it is ink, not opaline, that is highly unpalatable (Fig. 2). When these two secretions combine, at least one enzyme and its substrate are combined: escapin in ink is mixed with high concentrations of L-lysine and L-arginine in opaline (Yang et al. 2005; Johnson et al. 2006). There are likely other compounds formed by the mixing of the two secretions, which may contribute to the efficacy of ink. So far, escapin compounds have been tested on several species of predators, and they have proven to be relatively unimportant contributors to overall deterrence. Escapin's reaction products, which constitutes a complex mixture (Fig. 1; Kamio et al. 2009a), had limited effects on palatability of food for my test fishes (Fig. 3). For señorita wrasses and bluehead wrasses, shrimp containing a mixture of lysine intermediate products and H_2O_2, which are products of escapin's activity on lysine, was rejected significantly more than plain shrimp, though rejected less than shrimp containing ink secretion. This mixture of lysine intermediate products and H_2O_2 is also responsible for the secretion's powerful bactericidal effects (Yang et al. 2005; Ko et al. 2008). Blue crabs and spiny lobsters are also deterred by high levels of H_2O_2 which is released during the enzyme catalyzed reaction (Aggio and Derby 2008;

Kamio and Derby unpublished). While escapin reaction products are likely not the major deterrents against species that have been tested, they may contribute to the overall effectiveness of the secretion and may be maximally effective against other predators.

Thus, having a defensive secretion composed of many active compounds is useful for a species that is potentially so vulnerable to so many predators. Some compounds may be fairly specific to certain predators, so the prey species may benefit from possession of many compounds of diverse functional types. Other compounds may be broadly effective such as H_2O_2 or phagomimetic levels of amino acids (Kicklighter et al. 2005). The molecular identities of the compounds accounting for most of the unpalatability of ink to any predatory species are mostly unknown, though the purple pigment aplysioviolin has recently been identified as being effective against both invertebrate and fish predators (Kamio et al. 2010). This complement of chemical defenses, combined with other (non-chemical) defenses, results in a well-defended animal.

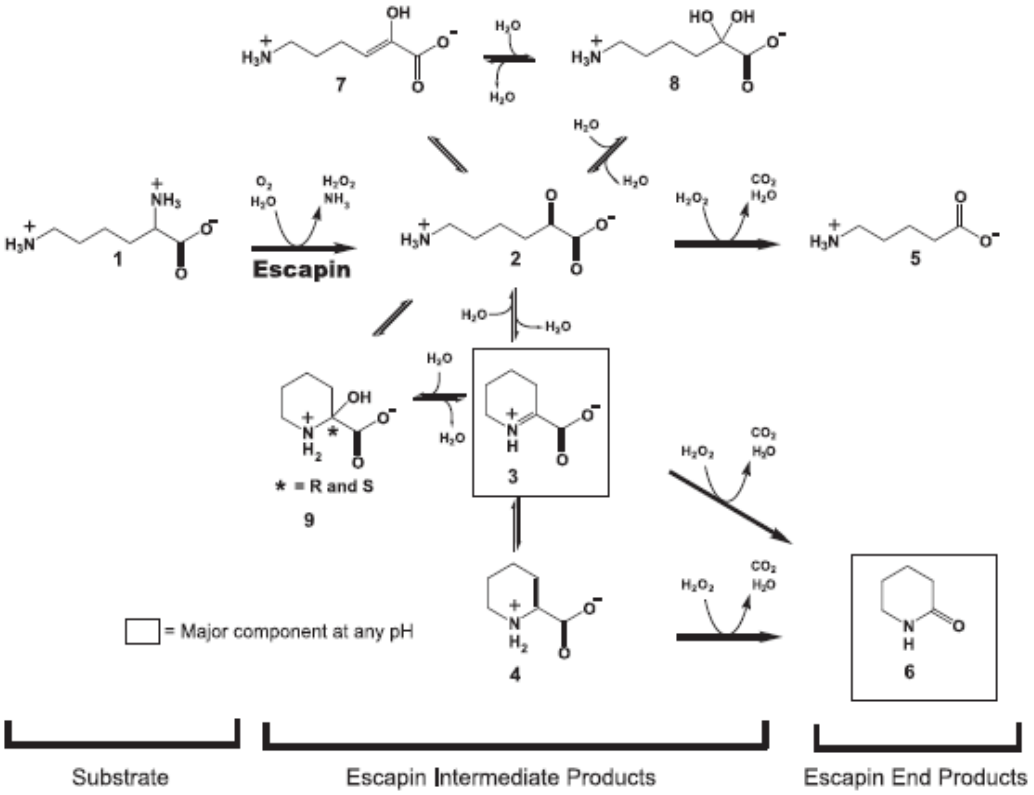

Figure 2.1 Summary of the compounds of the escapin/L-lysine pathway in the ink and opaline secretion of sea hares. Escapin is an L-amino acid oxidase in ink that is mixed with its substrates, L-lysine and L-arginine in opaline, when ink and opaline are secreted simultaneously, producing a complex set of compounds (Yang et al. 2005; Johnson et al. 2006; Kamio et al. 2009). First, escapin oxidatively deaminates L-lysine (1) to form "escapin intermediate products" of lysine; these products are a mixture of α-keto-ε-aminocaproic acid (2), Δ^1-piperidine-2-carboxylic acid (3), Δ^2-piperidine-2-carboxylic acid (4), 6-amino-2-hydroxy-hex-2-enoic acid (7), 6-amino-2,2-dihydroxy-hexanoic acid (8), 2-hydroxy-piperidine-2-carboxylic acid (9), ammonium, and hydrogen peroxide. Then, these components non-enzymatically react with hydrogen peroxide to form "escapin end products" of L-lysine, composed of a mixture of δ-aminovaleric acid (5) and δ-valerolactam (6). The concentration of escapin's products of lysine can be in the millimolar range. Escapin intermediate and end products of L-arginine are also formed but to a much lesser degree since L-arginine is 300 times less concentrated than L-lysine in the secretion. Escapin's products of lysine are known to have bacteriostatic and bactericidal effects (Ko et al. 2008), but their effects on predators have been reported for only three species. Hydrogen peroxide evoked aversive behaviors from spiny lobsters *Panulirus interruptus*, including mouthpart rubbing, tail flipping, and deterring of feeding (Aggio and Derby 2008). Hydrogen peroxide is also a mild deterrent against blue crabs *Callinectes sapidus* (Kamio et al. 2007). Escapin's products were reported as having no deterrent effects on sea anemones *Anthopleura sola* (Kicklighter and Derby, 2006). Adapted from figure 1 of Kamio et al. (2009), with permission from *Chemistry* (see References for complete reference).

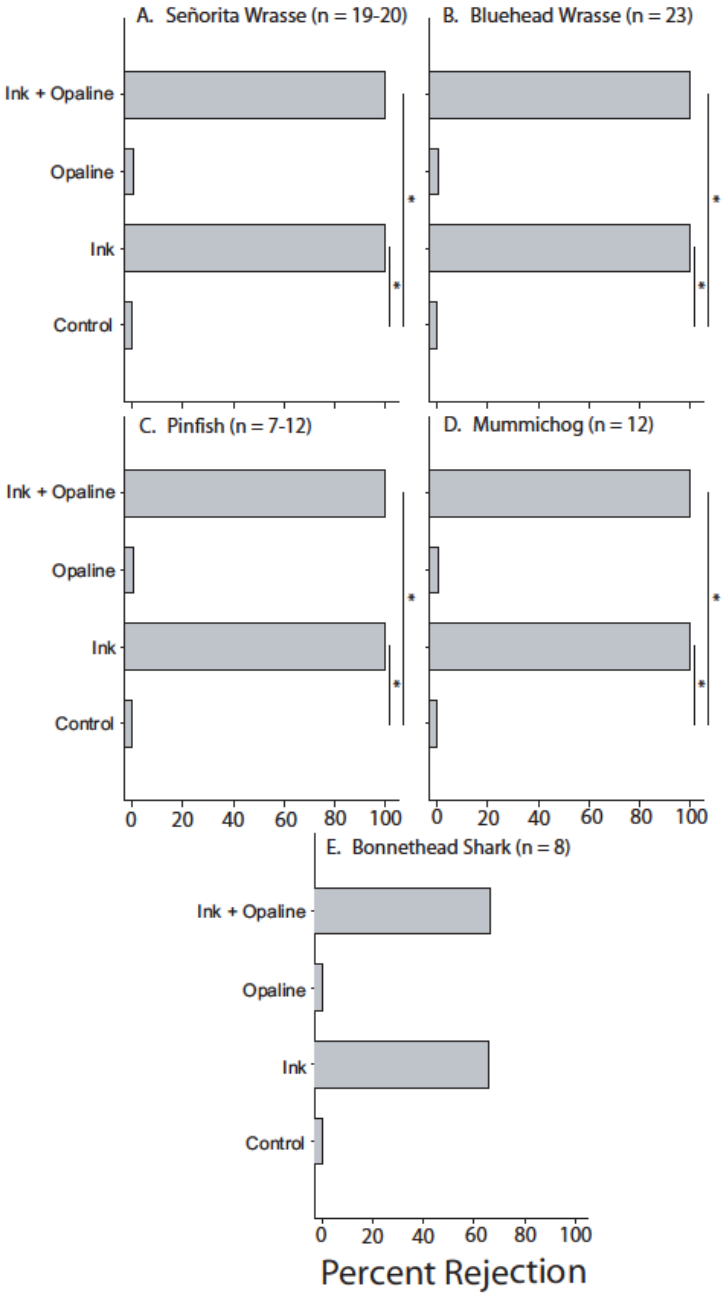

Figure 2.2 Responses of five fish species in ingestion assay using ink and opaline. Ink and opaline were collected and presented as described in the Materials and Methods. Responses in A–D represent the percentage of fish that rejected shrimp-flavored alginate pellets or freeze-dried shrimp to which the indicated substance had been added, where *n* = number of individual fish on which each substance was tested. Responses in E represent the percentage of trials in which a single group of 20 sharks rejected freeze-dried shrimp to which the indicated substance had been added, where *n* = number of trials in which each substance was tested on the group of sharks. Rejection of shrimp-flavored pellets or shrimp containing the indicated substance was statistically compared to rejection of shrimp-flavored pellets or shrimp without an additive (which all fish ate before and after the experimental pellet or shrimp), using Cochran's *Q* test and

post hoc one-tailed McNemar's tests. Ink or ink + opaline produced significant rejection (P = 0.0001), as indicated by asterisks, in A–D: (A) señorita wrasses, Q = 51.08, df = 3, P = 0.0001; (B) bluehead wrasses, Q = 61.23, df = 3, P = 0.0001); (C) pinfish, Q = 30, df = 3, P = 0.0001); (D) mummichogs, Q = 33.90, df = 3, P = 0.0001. For bonnethead sharks (E), there was an overall difference in the responses to the substances (Q = 9, df = 3, P = 0.029); however, none of the test substances was significantly different from the seawater control, although there was a strong but non-significant trend (P = 0.06) for shrimp treated with ink or ink + opaline to be rejected more than the control. For all five species of fishes A–E, opaline did not cause rejection (P = 0.05).

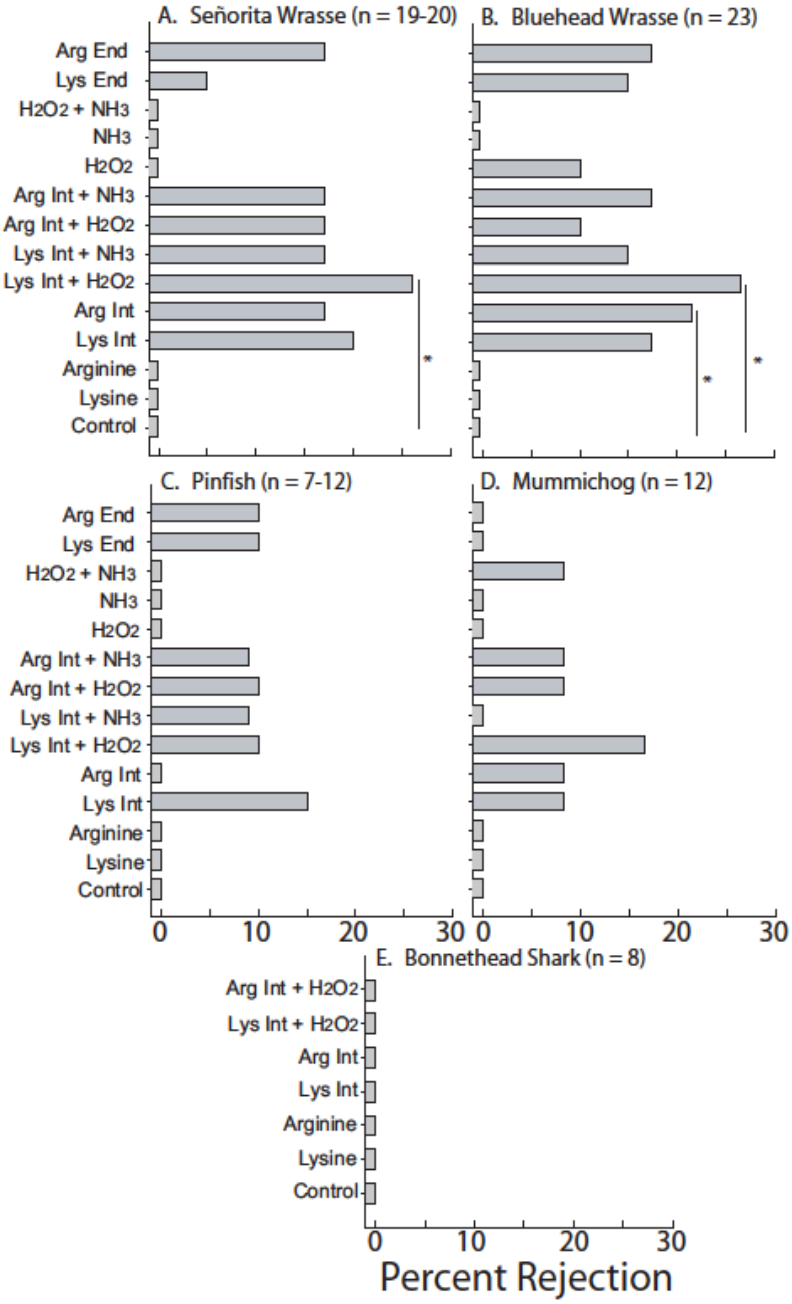

Figure 2.3 Responses of five fish species in ingestion assay using the reaction products of the enzyme escapin, found in ink. The reaction products and pathway are shown in Fig. 2.1, and how I produced them is described in the Materials and Methods. They include Lys Int = lysine intermediate products, Arg Int = arginine intermediate products, Lys End = lysine end products, Arg End = arginine end products, H_2O_2 = hydrogen peroxide, and NH_3 = ammonia. Responses in A–D represent the percentage of fish that rejected shrimp-flavored alginate pellets or freeze dried

shrimp to which the indicated substance had been added, where *n* = number of individual fish on which each substance was tested. Responses in E represent the percentage of trials in which a single group of 20 sharks rejected freeze-dried shrimp to which the indicated substance had been added, where *n* = number of trials in which each substance was tested on the group of sharks. Cochran's *Q* test and then *post hoc* testing with one-tailed McNemar's tests were used to compare rejection of shrimp-flavored pellets or shrimp containing the indicated substance with rejection of shrimp-flavored pellets or shrimp without an additive (which all fish ate before and after the experimental pellet), with an asterisk indicating significance at $P = 0.05$. Cochran's *Q* test values: A: $Q = 23.64$, df = 12, $P = 0.023$; B: $Q = 23.37$, df = 12, $P = 0.025$; C: $Q = 12$, df = 12, $P = 0.446$; D: $Q = 12.36$, df = 12, $P = 0.417$; E: $Q = 6$, df = 6, $P = 0.423$. For señorita wrasses (A), lysine intermediate products + H_2O_2 ($P = 0.032$) produced significant rejection. For bluehead wrasses (B), lysine intermediate products + H_2O_2 ($P = 0.016$) and arginine intermediate ($P = 0.032$) produced significant rejection.

CHAPTER 3

INK SECRETION PROTECTS SEA HARES BY ACTING ON THE OLFACTORY AND NONOLFACTORY CHEMICAL SENSES OF A PREDATORY FISH

Acknowledgments: Funding was provided by National Science Foundation (NSF) grant IBN-0614685, by a Georgia State University Brains & Behavior fellowship, and by support from the NSF IGERT program at Georgia Institute of Technology.

Previously published as: Nusnbaum M, Derby CD (2010) Ink secretion protects sea hares by acting on the olfactory and nonolfactory chemical senses of a predatory fish. Animal Behav 79: 1067-1076.

3.1 Introduction

Animals use a wide variety of defences against predators, including speed, stealth, crypsis, size, physical defenses, and chemicals (Pawlik 1993; McClintock & Baker 2001; Hay 2009). Opisthobranch mollusks, which include sea hares, are soft bodied and slow moving, and thus would be highly vulnerable to predators if not for the possession of a variety of defences. These include cryptic coloration and behaviour, large size, ability to produce copious mucus, and, most notably, chemical defences (Carefoot 1987; Johnson & Willows 1999; Wägele and Klussmann-Kolb 2005). Chemical defenses of sea hares include passive ones, which are constitutively present, and active chemical defenses, which are released only when the animal is attacked by a predator (Nolen et al. 1995; Johnson & Willows 1999). One active chemical defense is inking, which is the release of a purple, sticky secretion. The ink secretion of sea hares is the product of two glands that co-release their contents: the ink gland, which releases a purple fluid; and the opaline gland, which releases a white, highly viscous substance. These secretions are mixed in the sea hare's mantle cavity and squirted out of the body through the muscular pumping of the mantle.

Sea hares use ink to defend themselves from a diversity of predators using a variety of mechanisms. Mechanisms of chemical defense by ink of *Aplysia californica* have been described for two potential predators, a Pacific sea anemone, *Anthopleura sola*, and the California spiny lobster, *Panulirus interruptus*. Ink reduces predation by *P. interruptus* through a variety of mechanisms including unpalatability, sensory disruption, and phagomimicry (Kicklighter et al. 2005; Shabani et al. 2007; Aggio & Derby 2008). Against sea anemones, ink is an unpalatable deterrent that causes tentacular withdrawal (Nolen et al. 1995; Kicklighter & Derby 2006). Injection of ink from *Aplysia dactylomela* into pieces of fish fillet resulted in

rejection by laughing gulls *Larus atricilla* (DiMatteo 1981). Studies on a number of sea hare species indicate that diets consisting of chemically depauperate plants alter the ink secretion and reduce its efficacy as an feeding deterrent, indicating that some chemical defenses are diet derived (Pennings & Paul 1993; Nolen et al. 1995; Prince et al. 1998; Ginsburg & Paul 2001; Pennings et al. 2001). Thus, ink has the potential to chemically defend sea hares from predatory invertebrates, fish, birds, and perhaps even marine or terrestrial vertebrates.

To expand our understanding of sensory mechanisms of chemical defense by sea hare ink, the current study was undertaken to examine a fish predator. Fish occupy the niche of top predators in most marine systems and represent a potentially strong selective pressure for the slow-moving, soft-bodied sea hares. There is little evidence of fish predation on sea hares in the wild, likely due to a combination of defenses including chemical defenses such as ink release during an attack (Carefoot 1987; Johnson & Willows 1999). Fish are good model systems to study mechanisms of chemical senses, as their chemosensory systems are well characterized and they can be effectively studied both behaviorally and electrophysiologically (Nikonov & Caprio 2001; Rolen et al. 2003; Sato & Sorensen 2003; Caprio & Derby 2008; Cohen et al. 2008; Sheybani et al. 2009). The process of predatory attack, in general and by fish, involves two phases: approach and capture of food, when the prey is taken into the mouth, and the acceptance phase, when the prey is swallowed and consumed (Endler 1986; Ritson-Williams & Paul 2007). The approach and capture of prey by fish can be controlled by many senses. Of the chemical senses, the olfactory system is often involved in this phase, but other extra-oral chemical senses, such as external gustatory systems, can also control this behavior in some fish (reviewed in Caprio & Derby 2008). The acceptance and consumption of food is controlled by intra-oral gustation (Valentinčič & Caprio 1994; Kasumyan & Døving 2003; Caprio & Derby 2008).

Chemical defenses might function at either or both of these phases and be effective in protecting potential prey species (Ritson-Williams & Paul 2007). Deciphering the phases in predation in which chemical defenses function will allow further identification of the chemosensory modalities involved and therefore further elucidation of the functional mechanisms of the defenses.

I chose to use the bluehead wrasse *Thalassoma bifasciatum* in my study because it is a good laboratory model as well as a potential predator of the sympatric sea hare *A. dactylomela*. Bluehead wrasses are found in the waters around Florida and the Caribbean islands, often associated with reefs but also found in inshore non-reef areas and sea grass beds (Feddern 1965; Clifton & Motta 1998). *A. dactylomela* occupies a similar ecological niche as *A. californica*. Like *A. californica*, *A. dactylomela* releases purple ink and white opaline, and its ink and opaline contain many of the same or similar diet-derived and metabolized defensive compounds, including ammonia, amino acids, the enzyme escapin and the pigment phycoerythrobilin (which can act as phagomimics and sensory disruptors), and L-amino acid oxidases (dactylomelin P in *A. dactylomela* and escapin in *A. californica*), aplysioviolin and phycoerythrobilin, which are or generate aversive compounds (Melo et al. 2000; Kicklighter et al. 2005; Derby et al. 2007; Kamio et al. submitted). The advantages of using the bluehead wrasses for aquarium bioassays have been detailed previously (Pawlik et al. 1987). It is a common fish species for testing anti-predatory chemical defenses, since it is easy to maintain and train to feed on artificial diets (Lindquist & Hay 1996; Kubanek et al. 2000; Odate & Pawlik 2006). In other studies, I found that ink of *A. californica* is an effective deterrent against five other fish species, including wrasses sympatric with *A. californica*, señorita wrasses *Oxyjulis californica*, as well as pinfish *Lagodon rhomboides*, mummichogs *Fundulus heteroclitus*, and bonnethead sharks *Sphyrna*

tiburo. All of these fish responded to presentation of *A. californica* secretions in the same way as *T. bifasciatum* and sea catfish *Ariopsis felis* (Sheybani et al. 2009; Nusnbaum & Derby submitted).

To test the protective capabilities of the ink secretion, I presented either normal or de-inked *Aplysia californica* to bluehead wrasses and observed if inking affected predatory attacks. To test if ink acts extra-orally as a chemical defense to prevent fish from taking sea hares into their mouths, I presented food to bluehead wrasses in a cloud of ink and examined if that condition reduced food capture. To test for phagomimicry, I added to an alginate pellet a mixture of amino acids at concentrations identical to those in natural ink and opaline to determine if this increased acceptance. To test for unpalatability, I added ink and/or opaline to shrimp-flavored alginate pellets and examined if this affected whether or not bluehead wrasses accepted the pellets. I inferred palatability, or lack thereof, from the results of the pellet assays. To examine the role of olfaction in the effect of ink on fish, I performed nares occlusions and tested anosmic fish in cloud assays as well as pellet assays.

3.2 Materials and Methods

Animals

Juvenile yellow phase bluehead wrasses (*Thalassoma bifasciatum*), 5-10 cm long, were wild caught in south Florida and maintained at Georgia State University in individual 40-liter glass aquaria (50 cm x 25 cm x 30 cm) containing 28 ppt sea water (Instant Ocean, Aquarium Systems, Mentor, OH) that was filtered and aerated (Whisper Filters: Tetra, Blacksburg, VA) and ca. 21° C. Fish were fed frozen shrimp and brine shrimp ad libitum twice daily. Fish were

kept on a 14:10 L:D cycle and maintained in the same aquaria in which they were tested. Small (~ 1 g) specimens of *Aplysia californica* were obtained from the NIH National Resource for *Aplysia* (Miami, FL) and kept in separate 40-liter glass aquaria before being used in the feeding assay. Sea hares were raised on an exclusive diet of laboratory-grown *Gracilaria ferox* prior to being shipped to my laboratory and were not fed during the 1-week period following their arrival at my laboratory prior to experimentation. Wrasses were kept in captivity for no longer than 3 months during behavior assays and were euthanized at the end of the study.

Collection of sea hare secretions

Ink and opaline were collected from adult sea hares caught in waters off the coast of California by Marinus Inc. (Garden Grove, CA) immediately after their arrival in my laboratory. The diet of these wild-caught individuals is not known, but the presence of purple ink indicated that their diet included red algae. Secretions were collected from dissected ink and opaline glands. Ink glands were gently squeezed to release ink. Opaline glands were centrifuged at 30,000 × g for 1 hr at 4°C to separate opaline secretion from gland tissue. Secretions collected from individual animals were pooled to reduce any effect of individual variability in contents of glands. Secretions were frozen at −80°C until needed.

Feeding assay using live sea hares

Small specimens of *A. californica*, ~ 1 g and 2.5 cm in length, were fed to bluehead wrasses to examine effects of inking on attacks by predatory fish. The fish were food deprived for one week to ensure that they would readily attack the unfamiliar prey item. Twenty-nine individual fish were each tested with a single sea hare that was either normal (i.e., with ink) or

de-inked. Each fish was tested once to avoid biasing the data due to predator experience. Fifteen sea hares were de-inked by repeatedly applying high concentrations of sea salt to the containing water, which induced head retraction and ink release. These sea hares were rinsed in sea water and allowed to rest for 5 min between salt applications and allowed at least 1 hr to rest before being used in feeding assays. If a de-inked sea hare did not return to normal mobility and behavior, it was not used in the feeding assay. Fourteen sea hares were fed to the fish without de-inking, four of which did not release ink during the encounter, likely due to low intensity of attack by the fish, and thus were not included in the analysis.

During the feeding assay, either a normal or a de-inked sea hare was placed in the aquarium with a bluehead wrasse and the behavior was observed for 2 min. The sea hare was taken out of a holding aquarium by a researcher wearing latex gloves and slowly placed into the bottom of an aquarium containing one fish. The trial began after the hand was removed and the sea hare remained on the aquarium floor. During the encounter, if the fish did not approach the sea hare within 30 sec, then the trial was concluded. Measurements included the number of times the sea hare was struck, whether or not an inking episode occurred, and the damage to the sea hare. Since the bluehead wrasse pecks at food that is larger than its mouth (Clifton & Motta 1998), I observed that without a strong and prolonged attack period it was unlikely that the sea hare was killed by the fish. There is evidence to demonstrate that sea hares have multiple lines of defense, including potential chemical defenses that make the flesh distasteful (Carefoot 1987; Johnson & Willows 1999; Kamiya et al. 2006; Wägele & Klussmann-Kolb 2005; Derby 2007). Therefore, I assumed that the number of times the pecking predator struck the sea hare represents an approximation of the intensity of the attack and therefore the likelihood of significant damage to the prey species. To determine the effects of the lack of ink, I calculated the number of fish

strikes in an encounter in which the sea hare could not release ink. A one-tailed Mann-Whitney U test was performed to determine if the median number of strikes was greater toward de-inked sea hares (N = 15) compared to control sea hares (N = 10) (α = 0.05).

Cloud assay

The cloud assay was performed to determine the effect of a cloud of ink on a fish's response to a piece of food. This was accomplished by injecting 1 ml of one of four test stimuli into the water between the fish and an attractive food. The test stimuli were ink+opaline, ink, or opaline, each at full-strength, or sea water. Opaline and sea water were colored with 0.1% red food color (McCormick & Co., USA: listed contents are water, propylene glycol, FD&C reds 40 and 3 and propylparaben). The addition of food color to these two stimuli was intended to control for the color and intensity of ink. UV-visual spectral analysis of ink and food color showed that ink and food color had similar though non-identical spectra, both with peak absorbance at 510-570 nm and 330-340 nm (Supplemental Figure 1), thus serving my purpose of having controls with roughly the same color and intensity as ink.

A stimulus was drawn into a pipette and the pipette was lowered into the aquarium. The experimenter simultaneously placed a 3 mm x 3 mm piece of shrimp into the aquarium while releasing a cloud of 1 ml of stimulus between it and the fish. The cloud was ca. 4 cm in diameter when first introduced, reached ca. 15 cm after 30 sec which was wide enough to cover the width of the aquarium, and spread over half of the aquarium with considerable dilution by the conclusion of the experiment. During this time, the small piece of shrimp typically sank in the water column but remained behind the spreading cloud relative to the fish. Each trial lasted until a fish touched the food or took the food into its mouth (i.e. capture), with a maximum trial

duration of 2 min if the fish never touched the food. At the end of the trial, if the fish had not reached and eaten the shrimp, the shrimp remained on the aquarium bottom. Fifteen blue head wrasses were tested once on each of the four stimuli, presented individually in random order. I attempted to present these stimuli in a blind fashion, i.e., using a code for the stimuli, the nature of which the experimenter was unaware. But because of differences in color and/or viscosity of the stimuli, the blind procedure was not successful in all cases. Between trials, the filtration of the aquarium was sufficient to remove the substance from the water within 15 min, as indicated by color changes. Time to reach the shrimp averaged 7.2 sec and for those fish that reached it ranged from 2 to 20 sec. Recorded data included if the fish touched, captured, or accepted (i.e. consumed) the food, and the amount of time for the fish to touch the food. Cochran's Q test, followed by one-tailed McNemar post-hoc tests, was used to determine which substances impaired the ability of bluehead wrasses to touch and capture food, with the assumption that a substance will decrease the food-finding ability of fish ($N = 15$, $\alpha = 0.05$). Friedman's test, followed by one-tailed Wilcoxon matched-pairs post-hoc tests, was used to determine which substances caused animals that found the food to take a significantly greater time to reach it ($N = 10$, $\alpha = 0.05$). In addition, I recorded descriptions of the fish's movements and its position and behavior relative to the cloud of ink and to a control cloud of food color.

Pellet assay

Preparation of pellets.

Pellets were created to test the effect of added stimuli on feeding behavior, as described in Hay et al. (1998) and as used previously in Sheybani et al. (2009). To make the pellets,

shrimp purchased at a local seafood market were freeze dried, then ground into a powder using a mortar and pestle. Powdered shrimp and alginate (Sigma-Aldrich) were combined in a 5:3 ratio by weight, and 8 gm of this mixture was added to 100 ml deionized water. Red food color (McCormick& Co. USA, as described above and in Supplemental Figure 1) was added to the mixture to allow the normally uncolored pellets to be visualized by the researcher as well as to control for the deep color in ink. This shrimp-alginate solution was drawn into a 50-µl pipette and exuded into a 0.25 M $CaCl_2$ solution, creating a solid matrix that could be cut into 3-mm long and 1-mm wide pellets. Unflavored alginate pellets were produced by following the same procedure except that shrimp was not added. Preliminary behavioral tests showed that shrimp-alginate pellets were attractive to fish, whereas unflavored alginate pellets were not. Shrimp-alginate pellets and unflavored pellets were treated with ink, opaline, ink+opaline, a mixture of the amino acids in ink (AAI, Supplemental table 1), a mixture of the amino acids in opaline (AAO, Supplemental table 1), or sea water, by combining 1 ml of full-strength secretion, AAI, AAO, or sea water per 3 ml of alginate gel, to create test pellets. This creates pellets containing 25% full-strength ink, opaline, AAI, or AAO, which is in the range of secretion concentrations that fish are likely to encounter when attacking live, juvenile sea hares.

Behavioral testing

The fish were acclimated to hand feeding with a food stimulus, and only those fish that ate were used in subsequent testing. Alginate pellets flavored with freeze-dried shrimp powder were used as food. Hand feeding was performed using a pair of forceps. Food was presented to each fish and behavior was observed. I used acceptance of food, indicated by the fish swallowing and consuming it, and the converse, rejection of food (i.e. fish took the food into the

mouth, did not consume it, but rather spit out the food) as a measure of its palatability. When a fish encountered a piece of food, the fish typically captured it and flushed water through the mouth and out the gills. If the food was palatable, the fish accepted it. If the food was strongly unpalatable, the fish either did not capture it, or if it did, immediately rejected it. If the food was not strongly unpalatable, the fish often repeatedly captured and spit it out. The outcome was rated 'rejection' if at the end of the test period the food was not consumed. Responses were recorded as either 'rejection' or 'acceptance', and analyzed using Cochran's Q test with post-hoc testing using one-tailed McNemar's tests ($\alpha = 0.05$).

Nares occlusion

An occlusion of the olfactory system of bluehead wrasses was performed by plugging the nares with petroleum jelly (Vaseline, Chesebrough-Ponds, USA) a procedure used by others (Wisby & Hasler, 1954; Hasler & Scholz, 1983; Yano & Nakamura, 1992; Mitamura et al., 2005). To perform the plugging procedure, the fish was restrained in a moistened Kim-Wipe and loosely held in the researcher's hand. The front of the head was then patted dry and a cotton swab coated in petroleum jelly was gently rubbed across the nares. This applied the jelly to the nares and left a thin coating across the immediate surrounding region. Fish were returned to the aquarium to recover, and the procedure lasted no longer than 2 min. Sham animals were subjected to the exact same procedure, except the cotton swab was moistened with sea water. This method had no effect on swimming or feeding behavior in black rockfish (Mitamura et al., 2005) and did not alter these behaviors in my experiments. Following the procedure, fish were given a day to recover and then caught and visually inspected to verify that petroleum jelly remained in place before behavioral assays were performed. To examine if nares blockage had

any effects on behavior, I examined how nares occluded animals vs. sham animals responded to food. Fish were fed following the procedure and experiments were not performed until they fed normally. Sham and occluded animals took the same amount of time (one day) to return to normal feeding behavior and fish were able to orient toward food pellets dropped into the aquarium and immediately swam to them, captured and swallowed them. Cloud and pellet assays were performed with occluded and sham animals. The procedure for these assays was identical to that outlined above, except that fish were tested with ink but not opaline, as opaline was shown to be inactive in prior experiments. I also tested sea water and the mixture of amino acid components of ink (AAI, Supplementary table 1) as control stimuli.

3.3 Results

Responses of bluehead wrasses to live sea hares

I examined the behavior of 25 bluehead wrasses, with 10 fish exposed to a normal (i.e. ink-containing) sea hare that released ink during the encounter, and 15 fish exposed to a de-inked sea hare and thus did not release ink during the encounter. During a typical encounter, the fish approached the sea hare and swam around it for a number of seconds before making its first strike. If the strike was hard and the sea hare contained ink, the sea hare released its ink secretion by squeezing the mantle cavity rhythmically. Typically, there were 1 to 5 squeezes of the mantle lasting from 5 to 30 sec. Each squeeze released approximately 0.25 ml ink, based on visual comparison with release of sea hare ink of known volumes using a pipette. The ink released by a sea hare trailed out of the mantle cavity and slowly diluted in the water column within 2-3 cm from the sea hare. No strikes were forceful enough to seriously damage the sea hare. In only one trial (with a de-inked sea hare) was a small piece removed from the mantle and

in this case the fish mouthed and rejected the flesh multiple times but did not continue its attack on the sea hare. In none of the 25 interactions was a sea hare killed. An example of an encounter between a bluehead wrasse and sea hare, including inking, is shown in Supplemental video 1.

The protective effect of ink was examined by comparing the number of times a wrasse struck a sea hare during an encounter with an ink-releasing sea hare vs. during an encounter with a de-inked sea hare (Fig. 1). In encounters of fish with sea hares that released ink (N = 10), there was a median of 1 strike, with a range of 1 to 3 strikes. In five of these encounters, the sea hare released ink after the first strike and was then not struck again. The five other inking episodes occurred following the second or third strike. In only four cases was the sea hare struck after it released ink, and it was never more than once. On the other hand, in encounters of fish with sea hares lacking ink (N = 15), the median number of strikes was 3, with a range of 2 to 7. This value is significantly greater than the number of strikes during encounters with ink-releasing sea hares (Fig. 1). This demonstrates that inking decreases the likelihood that a sea hare will be attacked.

Cloud assay

Responses of bluehead wrasses to extra-oral ink.

In this assay, a cloud of full-strength ink, colored full-strength opaline, or colored sea water was presented between a fish and a piece of food, the fish was scored according to whether or not it reached the food during the 2-min trial and the time required to reach the food, and qualitative descriptions of the fish's behavior in relation to the cloud were recorded. Opaline and sea water had food color added to them, as described in the Methods, to simulate the color of ink. Significantly fewer fish reached the food when an ink cloud was present compared to a sea water

cloud: 10 of 15 fish reached the shrimp with an ink cloud while 14 of 15 succeeded with a colored sea water cloud (Fig. 2A). The animals that succeeded in reaching the food in the presence of an ink cloud required a significantly longer time than in the presence of a sea water cloud, with median times of 15 sec and 6 sec respectively (Fig. 2B). A cloud of colored opaline did not affect the ability of bluehead wrasses to reach the shrimp and did not produce any noticeable change in behavior compared to a cloud of colored sea water (Fig. 2).

Behavioral observations revealed that fish presented with a colored cloud always spent 1-2 sec attending to and swimming in front of the cloud before performing one of four behaviors: 1) swim into and through the cloud without pausing before reaching the food; 2) swim into and through the cloud, but pausing for 1-2 sec before reaching the food; 3) avoid the cloud while moving toward the food and moving around the cloud until it reached the food; or 4) swim away from the cloud and never reaching the food. These behaviors were generally exclusive and a fish would display only one of them during a trial. When presented with a cloud of colored sea water, fish commonly (53%) swam into and through the cloud without pausing before reaching the food (Fig. 2C). Less often (20%), they swam around avoiding the cloud to reach the food. Unlike in the colored sea water cloud, fish presented with the ink cloud frequently (67%) swam away from the cloud and never reached the food. If a fish reached the shrimp, it usually (20%) did so by avoiding the cloud while moving toward the food, bending its body to keep its head toward the food and moving around the cloud until it reached the shrimp. The distribution of responses to a cloud of colored sea water significantly differed from that to a cloud of ink (Fig. 2C). A cloud of colored opaline produced a set of behaviors similar to a cloud of colored sea water (Fig. 2C).

Pellet assay

Responses to plain pellets treated with ink, opaline, or their amino acid components.

Bluehead wrasses did not accept plain alginate pellets. I added ink, opaline, or a mixture of ink and opaline to these plain alginate pellets to determine if ink and/or opaline increases the pellets' palatability by causing them to be accepted and consumed, and thus is a 'phagomimic'. Neither ink + opaline, ink, nor opaline at full strength led to an increase in acceptance of unflavored pellets as no pellets were eaten with any of these treatments ($N = 23$). Thus, the secretions of sea hares cannot make neutral stimuli palatable. Pellets containing the amino acid components in ink or opaline (AAI and AAO respectively in Supplemental table 1) were palatable to bluehead wrasses, which accepted these pellets as frequently as shrimp pellets (20 out of 23 individuals for all three types of pellets). Pellets containing AAI or AAO were accepted more often than pellets containing ink (McNemar test: $N = 23$, $P < 0.0001$).

Responses to food treated with ink or opaline.

I examined the feeding responses of bluehead wrasses to shrimp-alginate pellets treated with ink, opaline, or a combination of these secretions to determine if these added chemicals decrease palatability. Results are shown in Figure 3 and are expressed as a percentage of animals rejecting the food items ($N = 23$). Fish were only used in assays if, prior to experimentation, they ate a shrimp-alginate pellet. Ink+opaline caused rejection of these otherwise palatable food items. The rejection was clear and strong: pellets with ink+opaline were rejected in 100% of the trials. Rarely did any fish take a pellet with ink+opaline into its mouth a second time. Ink alone also caused all individuals to reject otherwise palatable pellets.

Opaline alone did not cause rejection of palatable food items. Thus, ink is responsible for the secretion's unpalatability.

Nares Occlusion

Cloud Assay.

Each fish, whether sham or occluded, was tested with clouds of three concentrations (1%, 10%, and 100% of full strength) of ink, clouds of the same three concentrations of AAI (Supplemental table 1), and a cloud of sea water (Fig. 4A). The AAI and sea water clouds were colored with food color, as described above. When presented with 100% ink, 8 of 10 occluded animals reached the shrimp; with 10% ink, 9 of 10 occluded fish reached the shrimp; and with 1% ink, 8 of 10 occluded fish reached the shrimp. In all other stimulus and treatment combinations, all 10 fish reached and ate the shrimp. Thus, nares occlusion did not affect the percentage of fish that reached and captured the shrimp. However, nares occlusion did affect the time it took for fish to reach shrimp. Compared to sham fish, fish with occluded nares took significantly less time to reach and capture food when in the presence of an ink cloud (repeated measures ANOVA: df = 13, $P < 0.0001$). This was the case for all three ink concentrations (Bonferroni test: 1% ink $P = 0.001$, 10% ink $P = 0.01$, 100% ink $P = 0.001$). Occlusion did not affect the fish's ability to find food in a cloud of colored AAI or sea water. Occluded fish did not show any noticeable change in behavior aside from their response to the otherwise deterrent cloud of ink: there was no qualitative difference in feeding, swimming behavior, or head shaking, and there were no overt displays of distress in treated fish.

Pellet Assay.

Sham and occluded animals were fed shrimp-alginate pellets with no additives (sea water), with ink at four concentrations (0.01%, 0.1%, 1%, 10% and 100% full-strength ink), and with the mixture of amino acids in ink (AAI, Supplemental table 1) at the same four concentrations (Fig. 4B). Sham animals only rejected pellets containing ink, at any concentration. Nares occlusion did not affect the fish's rejection of pellets containing ink at any concentration: sham and nares occluded animals rejected ink-containing food pellets at all concentrations tested, but they did not reject control food pellets. At the lowest concentration of ink tested (0.01%), 60% of the sham and nares occluded fish rejected ink-laced food pellets.

3.4 Discussion

The goal of my study was fourfold. First, I wanted to determine if the ink secretion of sea hares *Aplysia californica* protects sea hares during attacks by a predatory fish, the bluehead wrasse *Thalassoma bifasciatum*. Second, I wanted to test which of the ink secretion's two glandular components – ink or opaline – is responsible for the activity and whether phagomimicry plays a role in the defense. Third, I wanted to determine whether the defensive chemicals function extra-orally or intra-orally, including the role of the olfactory system.

The release of ink protects sea hares during predatory attacks

To test for the protective effects of the ink secretion in interactions between sea hares and predatory fish, I manipulated small sea hares so that they could not secrete ink and placed them with bluehead wrasses, and I determined whether inking decreases the number of predatory

strikes. I found that a sea hare was struck significantly less frequently if it released ink (Fig. 1). This demonstrates a reduction in predatory attacks as a result of inking. My results are supportive of similar studies of interactions between sea hares and predatory sea anemones (Nolen et al. 1995) or spiny lobsters (Kicklighter et al. 2005). There are other known sources of chemical defenses in sea hares and other opisthobranch molluscs besides ink. These include sequestered secondary metabolites in the skin and digestive glands (Paul & Pennings 1991; Paul & Van Alstyne 1988; Pennings & Paul 1993; Kamiya et al. 2006). Undoubtedly, such non-ink chemical defenses contribute to the protection of sea hares against fish in my assay, and they probably explain why the number of predatory strikes by fish was relatively low and why none of the sea hares were killed in my experiments. Nonetheless, I demonstrated that inking adds a layer of chemical protection against predatory fish, perhaps when other defenses are not completely effective (Pearson 1989).

Ink acts as a chemical defense during different phases of predatory attacks

I examined whether ink or opaline functions by preventing fish from taking sea hares into their mouth (i.e. extra-orally) or by increasing rejection of sea hares once taken into the mouth (i.e. intra-orally) by performing two assays. Using a cloud assay, in which 1 ml of ink or opaline was presented between the fish and a piece of food and the behavior toward the food was examined, I found that bluehead wrasses were able to detect ink from a distance and actively avoid it (Fig. 2). My observations of live sea hares releasing ink indicated that ca. 0.25 ml of ink would be released during each of the 1-5 pumps of the mantle, as compared by eye to known volumes of ink, indicating that 1 ml of ink is a realistic volume that a predator would encounter from a 1 g sea hare. Ink was so effective that in some instances the fish would not reach the food

as a result of exposure. Opaline did not have an effect. Ink was effective as a deterrent in the cloud assay at concentrations from full strength to a 100 times dilution (Fig. 4). Thus, ink should be effective in various contexts and distances, from close to full strength as when a predator takes the sea hare into its mouth at which time ink is released, to a dilution as when a predator approaches an inking sea hare from a distance. Furthermore, my results indicate that the deterrent effects of ink are able to function through distance chemoreception, such as olfaction or extra-oral gustation. A similar conclusion about extra-oral effects was drawn by Ritson-Williams & Paul (2007) from field studies of the effects of chemicals from marine invertebrates on reef fish.

Using a pellet assay, in which food-flavored alginate pellets were treated with ink or opaline and rejection of the food was quantified, I demonstrated that ink, but not opaline, is highly unpalatable (Fig. 3). Bluehead wrasses rejected otherwise palatable food when it was impregnated with ink or a combination of ink and opaline, but not opaline alone. Ink caused significant rejection of food at concentrations as low as 10,000 times dilutions of full-strength ink (Fig. 4).

The results of the pellet assay do not support the hypothesis that ink as a whole defends through phagomimicry, since wrasses did not eat plain alginate pellets containing ink or opaline. However, wrasses did eat plain alginate pellets containing the amino acid component of either ink or opaline. This is not surprising, given that free amino acids evoke feeding responses in many fishes, and several amino acids, including proline, alanine, and arginine, can evoke reflexive biting at concentrations as low as 0.1 mM (Valentinčič & Caprio 1994; Valentinčič et al., 1999). Thus, my results suggest that ink and opaline contain appetitive components for wrasses, but that the deterrent compounds in ink and opaline overcome these appetitive

components, resulting in a deterrence. While the bluehead wrasse is not a sympatric predator of *A. californica*, I found that the señorita wrasse *Oxyjulis californica* responds in the same way to pellets treated with ink and opaline and is sympatric with juvenile California sea hares, which remain in the deeper waters where they are born before moving into shallower regions as adults. *O. californica* is found at depths from tidepools to 42 m, and in kelp beds, reefs and rocky bottoms where *A. californica* congregates in red algae (Goodson 1988). Bluehead wrasses feed on a diverse selection of prey, including small molluscs and crustaceans similar in size to juvenile sea hares (Carefoot 1987). Similar effects of the ink secretion and its components were observed with sea anemones (Nolen et al. 1995 ; Kicklighter & Derby 2006), spiny lobsters (Kicklighter et al. 2005; Aggio & Derby 2008), sea gulls (DiMatteo 1981), and a number of fish species including wrasses, bream, and goatfish (DiMatteo 1981, 1982; Pennings et al. 1999, 2001). This demonstrates that the deterrent effects of the ink secretion can function through intra-oral chemoreception. Thus, my experiments suggest that both extra-oral and intra-oral chemoreceptors mediate the effects of the ink defensive compounds.

Ink acts through both olfactory and intra-oral chemical senses of fish

I followed this set of experiments with a series in which I temporarily inactivated the olfactory system of the fish through nares occlusion, and repeated the cloud and pellet assays. These experiments demonstrate that both extra-oral and intra-oral chemoreception function in the detection of chemical defenses and behavioral aversion, but these two sets of receptors function in different phases of the predation event. Nares occlusion reduced ink's effect on the capture of food, but it had no effect on ink's ability to cause rejection of food once taken into the fish's mouth. The occluded fish took less time to reach food than the sham fish when an ink cloud, but

not a colored opaline or sea water cloud, was present, indicating that removal of olfactory stimulation results in a behavioral insensitivity to external presentations of ink. Thus, the olfactory system is responsible for behavioral deterrence from a cloud of ink, and non-olfactory chemical senses, likely intra-oral, possibly gustation, are responsible for rejection of ink-treated food if taken into the mouth. Fish rejected ink impregnated pellets only after they were taken into the mouth and fish do not possess retro-nasal connections to the oral cavity. Together with the results of the nares occlusion assays, these considerations lead me to hypothesize that an intra-oral chemical sense, probably gustation, is responsible for this rejection. My results also show that a single chemical defense can function through multiple sensory channels to affect predator behavior and protect the prey species at different stages in the predatory encounter.

Principles derived from studies of sea hare chemical defenses

The sea hare is a soft-bodied, slow-moving animal that takes advantage of a number of defenses to protect itself from a variety of predators. Why should a sea hare, or any animal, have so many chemical defenses? The answer to this question is likely evolutionary: the animal uses different levels of defenses to protect itself from different predators and different stages of a predatory encounter. These defenses have different degrees of cost and effectiveness as well. In the case of the sea hare, passive chemical defenses such as those found in the skin and mucous have a different cost: benefit than active chemical defenses such as ink released only after a sustained predatory encounter (Nolen & Johnson 2001). These multiple lines of defense can affect different predators, and some compounds may work on olfactory pathways and others through gustatory pathways. The different chemicals may affect the behavior of the predator

through different sensory pathways and in different ways as in the nares occlusion experiments (Fig. 4).

Chemical defenses may also act multimodally by affecting chemosensory systems while also functioning as visual cues. Coloration of the body or secretions is used by many animals (Young & Bingham 1987; Harvey et al. 1988; Vences et al. 2003), including marine gastropods (Becerro et al. 2006; Ritson-Williams & Paul 2007), as aposematic cues. I did not find evidence that ink functions as an aposematic indicator of the snail's distastefulness. My experiments showed that a cloud of purple ink negatively affected approach of food by wrasses, but a similarly colored cloud of sea water or amino acids did not, a result contrary to the expectation if ink were an aposematic signal (Figs. 2, 4). Furthermore, sea hares *A. californica* and *A. dactylomela* are cryptically colored, which would seem to be at odds with an aposematic ink. The function of the coloration of ink may in fact be other than as a visual signal to predators: the purple color of ink is largely due to the compound aplysioviolin, which is a chemical deterrent against predatory crabs and possibly other predators (Kamio et al. submitted). All together, my results favor the idea that ink functions as a secondary chemical defense to deter predators that may otherwise not be deterred by all of the sea hare’s other lines of defense.

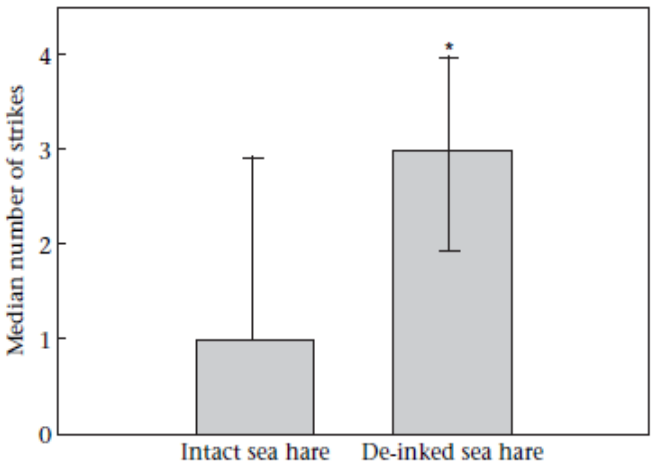

Figure 3.1 Median (±25th and 75th interquartile intervals) number of feeding strikes by bluehead wrasses towards sea hares that released ink (intact, N = 10 events) and towards sea hares that were prevented from releasing ink (de-inked, N = 15 events). *P < 0.05.

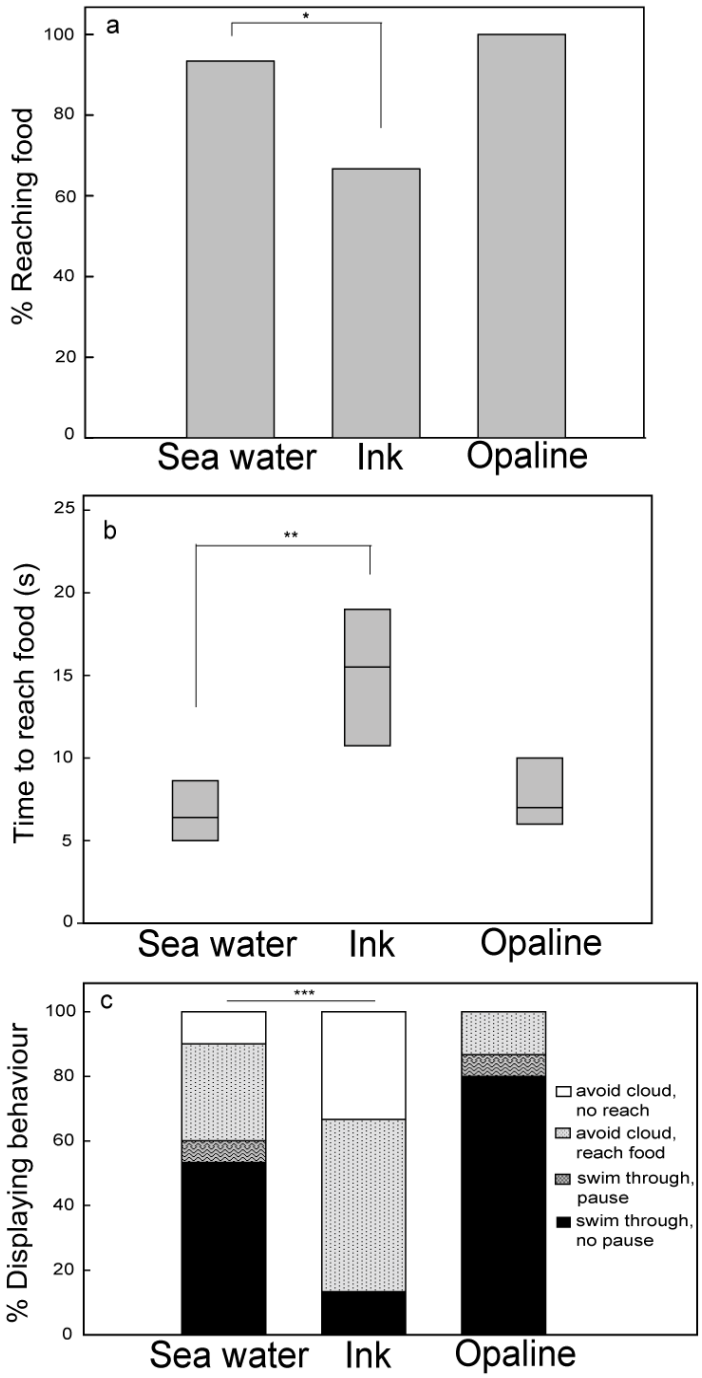

Figure 3.2 Responses of bluehead wrasses to extraoral presentations of ink and opaline in the cloud assay. (a) Percentage of fish (out of 15 tested) that reached the food (shrimp pellet) presented behind a 1 ml cloud of the indicated stimulus. Ink and opaline were presented at full strength. (b) Median time (horizontal line) and the 25th and 75th percentile ranges (lower and upper limits of the box) to reach the food. (c) Responses of bluehead wrasses to a cloud of the indicated stimulus. *P < 0.05; **P < 0.001; ***P < 0.0001.

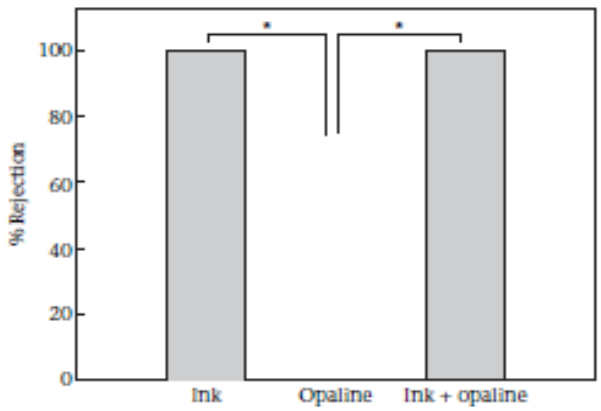

Figure 3.3 Responses of bluehead wrasses in the pellet assay. Percentage of fish that rejected shrimp-flavored alginate pellets treated with ink, opaline or ink + opaline. *P < 0.001.

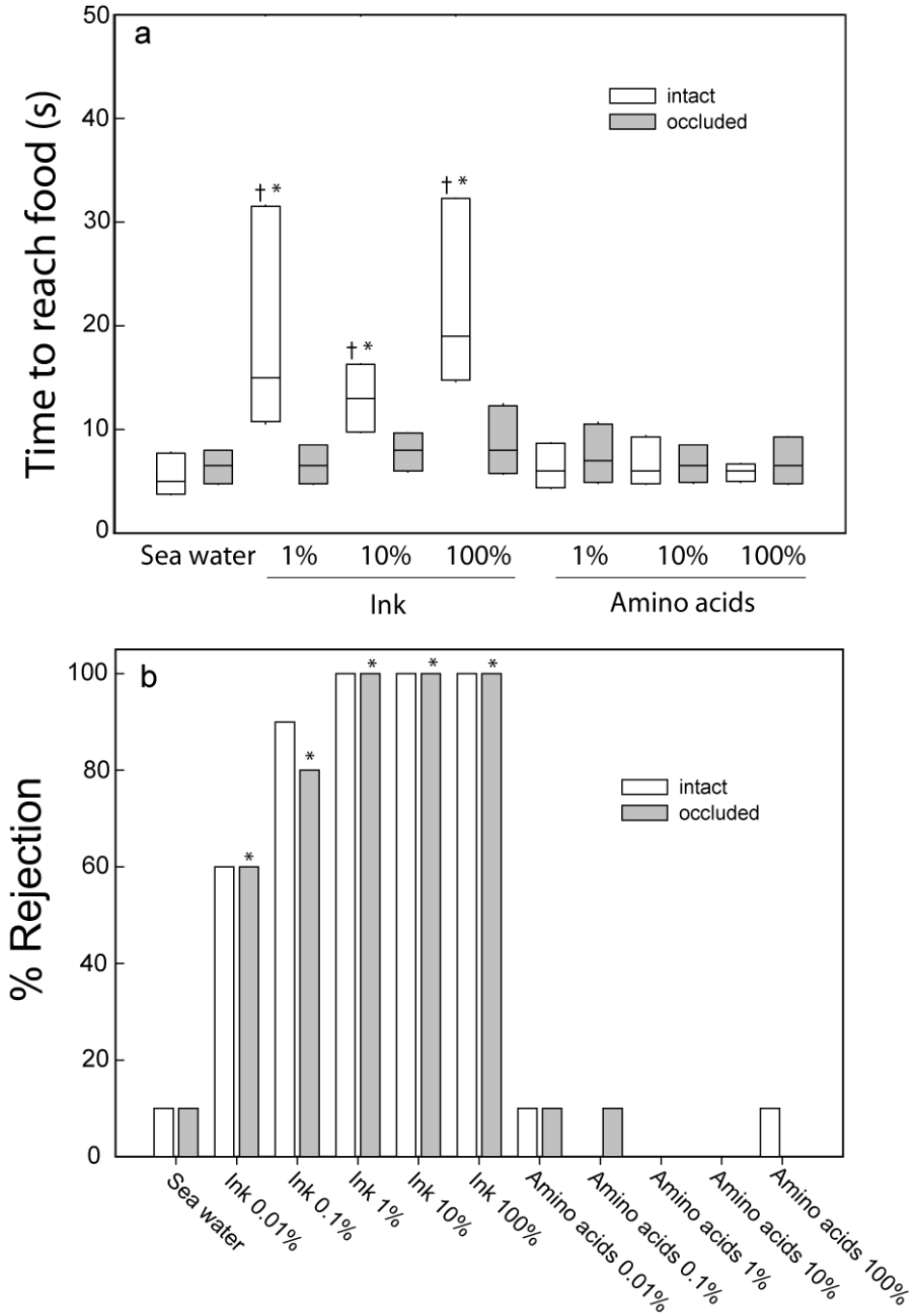

Figure 3.4 Effect of nares occlusion on feeding responses of bluehead wrasses: (a) median time (horizontal rule) and 25th and 75th interquartile ranges (lower and upper limits) to reach the food in the cloud assay and (b) percentage of fish that rejected pellets in the pellet assay. ▭: nares occluded (N = 10); □ : sham (N = 10). In (a), symbols denote either a significant difference from colored sea water (†P < 0.01), or a significant difference from sham (*P = 0.001). In (b), an asterisk denotes a significant difference from sea water (*P < 0.01).

CHAPTER 4

TASTE-MEDIATED BEHAVIORAL AND ELECTROPHYSIOLOGICAL RESPONSES TO DETERRENT PIGMENTS FROM THE INK OF THE SEA HARE *APLYSIA CALIFORNICA* BY A PREDATORY FISH *ARIOPSIS FELIS*

Acknowledgements: Funding was provided by grants from NSF (IOS-0614685, IOS-1036742) and a GSU Brains & Behavior Fellowship.

4.1 Introduction

Anti-predator defenses can take many forms, including behavioral adaptations (e.g. stealth and speed), visual defenses (e.g. camouflage and aposematic coloration), mechanical defenses (e.g. spines, claws and exoskeletons), and chemical defenses (e.g. venoms, irritants, and deterrents) (Endler 1986; Hay et al. 1998; Caro 2005). Chemical defenses are used by many organisms to deter predators, and some of the effector molecules have been identified (Pawlik 1993; McClintock and Baker 2001). Chemical defenses can protect prey species from predation by acting on the predator's chemosensory systems (Eisner and Meinwald 1966; Tachibana and Gruber 1988; Paul and van Alstyne 1992; Pawlik 1993; Berenbaum 1995; McClintock and Baker 2001; Kelley et al. 2003). To be effective, chemical defenses must act on the attacking predator, and activating the predator's chemosensory systems is a direct way to produce a desired response. Selection/rejection of food by some fish is influenced by their detection of deterrent molecules, such as alkaloids and acids (Derby and Sorensen 2008). Chemical defenses are well-studied by chemical ecologists (Hay 1996; Kicklighter et al. 2005; Hayden et al. 2007), but much less is known about their detection by chemosensory systems (Hara 1994; Kamio et al. 2007; Cohen et al. 2008).

Sea hares of the genus *Aplysia* obtain a variety of secondary plant compounds as well as ink pigments exclusively from a red seaweed diet (Winkler and Dawson 1963; Darling and Cosgrove 1966; Irie et al. 1968; Chapman and Fox 1969; Winkler 1969; Watson 1973; Kinnel et al. 1979; Blankenship et al. 1983; MacColl et al. 1990). *Aplysia californica* is a bottom dwelling gastropod mollusk with a reduced and internalized shell which lives in subtidal and intertidal waters in the Pacific Ocean from Northern California to Baja, California. *Aplysia californica* can release ink when disturbed as early as post-metamorphic juveniles, around 1-mm long

(Kriegstein 1977). Mechanisms of chemical defense by ink of *A. californica* have been described for two potential predators, the California spiny lobster, *Panulirus interruptus*, and a Pacific sea anemone, *Anthopleura sola* (Nolen et al. 1995; Kicklighter et al. 2005; Kicklighter and Derby 2006). Ink from *A. californica* deters predation by *P. interruptus* through a variety of mechanisms, including unpalatability, sensory disruption, and phagomimicry (i.e. chemically stimulating the feeding pathway to distract a predator's attention). In spiny lobsters, ink and opaline secretions stimulate gustatory and olfactory systems as demonstrated by electrophysiological recordings (Kicklighter et al. 2005). However, the identity and response properties of the deterrent compounds in ink and opaline secretions were not examined for *P. interruptus*. In previous work, I found that ink, but not opaline, is deterrent to fishes (Nusnbaum and Derby 2010a, 2010b). Here, I test components of ink that were identified through bioassay guided fractionation that were deterrents to blue crabs, *Callinectes sapidus*, (Kamio et al. 2010a, 2010b).

Gustation is a well-studied chemosensory modality for eliciting aversive responses to chemical stimuli (Garcia et al. 1968; Smith and Theodore 1984; Wiggins et al.1989; Kang et al. 2010). Responding with aversion upon tasting a deterrent stimulus is an adaptive response that can protect the predator from ingesting a toxic or noxious prey item. In mammals, the perception of deterrent compounds is mediated by a family of gustatory receptors, T2Rs, and these compounds include a broad spectrum of unrelated chemical structures that share only the behavioral response they elicit, namely aversion (Meyerhof 2005; Behrens and Meyerhof 2006; Roper 2007). Individual T2Rs are broadly tuned to respond to a wide variety of deterrent and toxic compounds, allowing taste cells possessing a small number of T2R receptor types to respond to a broad spectrum of aversive compounds (Brockhoff et al. 2010). Behavioral

aversion to stimuli that taste bitter to humans is well-documented among vertebrates, including fishes, amphibians, and mammals (Hidaka et al. 1978; Jones and Hara 1985; Brining et al. 1991; Takeuchi et al. 1994; Lamb and Finger 1995; Caicedo et al. 2002). Animals have the capacity to detect and respond to aversive compounds that may be toxic or taste bad. These behavioral responses to deterrents can broadly be described as aversion or rejection.

Chemosensory systems in fishes convey information about food sources, conspecifics, and other environmental factors (Sorensen and Caprio 1998). An important difference between chemosensation in fishes and terrestrial vertebrates is that the stimuli for fishes are dissolved in their aqueous environment. The compounds that were behaviorally tested in fishes are predominantly attractive and most were studied because they elicited ingestive behaviors. The classes of compounds found to be effective gustatory stimuli amongst fishes include small, water-soluble molecules, such as amino acids, nucleotides, polyamines, and bile salts (Michel et al. 2003; Rolen et al. 2003; Yamashita et al. 2006; Caprio and Derby 2008). Most of the knowledge gained on fish gustation over the past 30 years of research has focused on the transduction and discrimination of amino acid stimuli (Michel and Caprio 1991; Caprio et al. 1993; Valentinčič and Caprio 1994; Valentinčič et al. 1999; Caprio and Derby 2008). In many fishes, taste buds are located not only in the oropharyngeal cavity, as in mammals, but also over the external portions of the head and lips, and in catfishes, taste buds are located on barbels as well as distributed over the entire external body surface. These taste buds are innervated by branches of the facial nerve (cranial nerve VII) which form gustatory neural pathways that are broadly- or narrowly-tuned to specific classes of molecules, such as L-amino acids, which are involved in food search behaviors (Caprio et al. 1993). Integrated multi-unit and single-unit recordings show that different populations of nerves can be broadly or narrowly tuned to groups

of amino acids (Michel and Caprio 1991; Kohbara and Caprio 1996; Ogawa and Caprio 1999; Yamashita et al. 2006). The neural processing of bile salt gustatory information occurs through relatively independent neural pathways as well as fibers that can also be responsive to amino acids (Rolen et al. 2003; Yamashita et al. 2006). Some studies have been performed to understand the receptors and pathways involved in detection of deterrents, however much of this work used well-known, but behaviorally irrelevant, stimuli, such as quinine and denatonium (Ogawa et al. 1997, Caicedo et al. 2002, Oike et al. 2007). The chemosensory systems of animals are constrained by their evolutionary history and are best suited for detecting compounds that are behaviorally relevant. Quinine, a compound with unknown ecological relevance for fishes, activates a population of gustatory fibers and suppresses amino acid responses in another population of fibers (Ogawa et al. 1997). Both, or either, of these neural mechanisms may be involved in the signals leading to the aversive response elicited by quinine.

To learn how animals detect and respond to deterrent compounds, it is logical to test ecologically and behaviorally relevant stimuli. Aplysioviolin (APV) and phycoerythrobilin (PEB) are two structurally related deterrent compounds that were purified from the ink secretion of *A. californica* (Kamio et al. 2010a, 2010b) (Fig. 1 and Suppl. Fig. 1). *A. californica* derives PEB from phycobilin, a photosynthetic pigment found in its red algal diet. The sea hares convert most of the PEB into APV and store both in the ink gland, with APV being ten times more concentrated than PEB in the ink secretion (Kamio et al. 2010a, 2010b). The present report describes electrophysiological and behavioral research using sea catfish, *Ariopsis felis*, a chemosensory model, to investigate the detection and signal processing of identified deterrent compounds from the chemical defenses of *A. californica*. *Ariopsis felis* are found in the Gulf of

Mexico and are sympatric with *Aplysia dactylomela*, which also possess APV and PEB in their ink (Kamio et al. 2010a).

4.2 Materials and Methods

Animals

Sea catfish, *Ariopsis felis* (male and female, 12-30 cm), were collected by Gulf Specimen Marine Laboratory (Panacea, FL) and the Whitney Marine Laboratory (St. Augustine, FL). They were maintained at Georgia State University in individual 40-liter glass aquaria containing filtered and aerated sea water (ASW, Instant Ocean, Aquarium Systems, Mentor, OH) at a salinity of 28 ppt. They were kept on a 12:12 L: D cycle and fed frozen shrimp ad libitum. Adult sea hares, *Aplysia californica* (15-30 cm), were collected in California by Marinus Scientific (Garden Grove, CA, USA).

Collection and purification of sea hare secretions

Upon arrival in the laboratory, sea hares were placed in ice water and then injected with isotonic $MgCl_2$ to anesthetize them prior to dissection. Ink glands were dissected and immediately frozen at -80°C and stored until used. Ink was collected by gently squeezing defrosted ink glands in a Petri dish with the blunt end of a scalpel handle; the resultant is considered full strength, or 100%, ink. To extract APV and PEB from the ink secretion, methods were adapted from Kamio et al. (2010). Briefly, ink glands (86.43 g wet weight) were freeze-dried in a lyophilizer (Labconco, Kansas City, MO), macerated in 100% MeOH, and centrifuged to remove insoluble tissue, proteins, and polar compounds. The resulting pellet had a purple color, and the purple extract was washed from the pellet with 100% MeOH until the pellet

became white. The purple supernatant was separated using HP-20SS (Diaion, Mitsubishi Chemical USA Inc, Japan) in an open column with a H_2O/MeOH stepwise gradient. The 100% MeOH fraction was further separated by RP-HPLC on a C18 (Phenomenex Luna, Torrance, CA) column (10x250 mm) and a gradient of 40–100% MeOH in H_2O; 8.0 ml/min. In this scheme, PEB elutes at 3-4 min and APV at 4-6 min (Fig. 4.1). A mixture called Ink – (APV+PEB) was created by recombining the fractions of the ink secretion that were taken from these steps except for APV and PEB. This includes the pellet removed in the MeOH wash, the other fractions from HP-20SS separation, and the HPLC fractions that did not include the peaks of APV and PEB (Fig. 4.1). Following HPLC separation, samples of the fractions containing APV and PEB were analyzed for free amino acid content (AminoAcids.com, St. Paul, MN USA), to ensure that any behavioral or electrophysiological response to these fractions was not due to free amino acids.

Preparation of pellets

Shrimp-flavored pellets were created to examine the effect of test stimuli on catfish feeding behavior, especially the ingestion of food, as described in Hay et al. (1998) and as used previously in Nusnbaum and Derby (2010a, 2010b). To make the pellets, shrimp purchased at a local seafood market were freeze dried and ground into a powder using a mortar and pestle. Powdered shrimp and alginate (Sigma-Aldrich) were combined in a 5: 3 ratio by weight, and 8 gm of this mixture was added to 100 ml deionized water. Red food color, 0.1% by volume (McCormick & Co., USA), was added to the mixture to allow the normally uncolored pellets to be visualized by the researcher as well as to simulate the deep color in ink. This shrimp-alginate solution was drawn into a 50-µl pipette and exuded into a 0.25 M $CaCl_2$ solution, creating a solid matrix that could be cut into 3-mm long pellets. Shrimp-flavored pellets and unflavored alginate

pellets (which lacked shrimp powder) were also treated with different concentrations of test stimuli by combining those stimuli with the alginate gel, to create ‘test pellets’.

Pellet Assay

A pellet assay was performed as previously described in Nusnbaum and Derby (2010a, 2010b) and commonly used to test deterrence of natural products against predatory fishes (Kubanek et al. 2000; Pawlik 1987, 1993; Pawlik et al. 1987, 1995). Briefly, shrimp-flavored pellets were used as positive controls. I used acceptance of food, indicated by the fish swallowing and consuming it, and the converse, rejection of food, as positive and negative measures, respectively, of its palatability. Hand-feeding was performed using a pair of forceps. The outcome was rated ‘rejection’ if at the end of the test period the pellet was not consumed. Responses were recorded as either ‘rejection’ or ‘acceptance’, and analyzed using Cochran’s Q test with post-hoc testing using one-tailed McNemar’s tests ($\alpha = 0.05$). Thirteen sea catfish were tested with all test pellets and were given control pellets before and after each test pellet. Fish were tested no more than eight times each day to maintain high hunger levels. Control pellets were fed to fish between each test, and data for a test were not used if the fish rejected or ignored the control. Time between test pellets was at least 20 min. The pellet assay tested the palatability of ink, APV, PEB, and ink – (APV+PEB) at log-step concentrations from 100% to 0.0001% ink in ASW. Due to the deep color of ink, APV, and PEB, it was not possible to present these stimuli with the experimenter completely unaware of their identity.

Barbel Assay

The results of the pellet assay for sea catfish were similar to previous studies with several other fish species that demonstrated that ink is a deterrent to food ingestion (Nusnbaum and Derby 2010a, 2010b). Consequently, a barbel assay was performed to determine (a) if the barbels mediate the sea catfish's detection of the deterrent compounds in ink, and (b) if an electrophysiological recording from the barbel nerve could be used to study the detection of these compounds. Cubes of freeze dried shrimp (8 mm^3) were used in this assay to test the effectiveness of the test stimuli (ink, APV, PEB, and ASW) on contact with the barbel of the catfish. The shrimp cubes were treated with 250 μl of the full-strength concentration of each test stimulus, which was enough to saturate the shrimp. The cube was held in the water column with a pair of forceps for the fish to approach. When the fish approached, the cube, the cube was slowly moved to brush across the barbel. The intensity and speed of the brush were maintained as qualitatively consistent as possible and the binary nature of the responses indicates that this motion did not significantly affect the fishes' responses to the test stimuli. The fish either turned towards the food and ingested it or turned and swam away. The number of fish performing these behaviors was compared using Cochran's Q test with post-hoc testing using one-tailed McNemar's tests ($\alpha = 0.05$). Due to the deep color of ink, APV, and PEB, it was not possible to present these stimuli with the experimenter completely unaware of their identity.

Electrophysiological Methods

Fish were immobilized with an intramuscular injection of gallamine triethiodide (Flaxedil, ~1.0 mg/kg body weight, Sigma-Aldrich, St. Louis, MO), covered with wet tissue paper, and secured to a wax block. Gill irrigation was provided by a flow of ~50 ml/min aerated

ASW into which the general anesthetic (2-phenoxyethanol (0.2 ml/l: Sigma-Aldrich) was added. Supplemental Flaxedil was added as necessary to maintain animal immobility. The maxillary barbel was inserted into a tube and fixed in place so that it was continuously bathed with a flow of 10 ml/min ASW (without 2-phenoxyethanol) from a pressurized reservoir during the experimental period. This flow provided a carrier flow for test stimuli during recordings. Procedures for the surgical exposure of the facial/trigeminal nerve complex to the maxillary barbel, surgical isolation, and electrophysiological recording of integrated taste activity were previously described (Wegert and Caprio 1991). Briefly, tetracaine (3%) was used as a local anesthetic on the skin 5 min prior to surgery to expose the branches of the facial-trigeminal nerve complex that innervates the rostral portion of the head. A branch of the facial-trigeminal nerve complex, which innervates the maxillary barbel, was isolated and carefully cleaned of connective tissue. Neural activity was recorded with a glass pipette suction electrode with an Ag-AgCl wire, AC amplified (P511, Grass Instruments, Quincy, MA), connected to an audio monitor, and the signals were recorded on a computer's hard drive for analysis using Spike2 software (Cambridge Electronic Design, Cambridge, England). Multifiber signals were integrated with a 0.1 s time constant and measured as the maximum amplitude of the signal above the baseline pre-stimulus level. The amplitude of the response above baseline was normalized by subtracting the amplitude of the signal following ASW presentation and dividing by the pre-stimulus amplitude to account for baseline activity and possible artifacts.

Stimulus Delivery

Stimuli were delivered using an injection loop with a gravity-feed ASW flow. A maxillary barbel was inserted into a silicone tube and continuously bathed in ASW (8-10

ml/min) without anesthetic, or continuously bathed in adapting solution during cross-adaptation experiments (described below). A loop was filled with 0.5 ml of stimulus solution that was then introduced to the maxillary barbel by diverting the flow through the loop via a 3-way solenoid valve (MTV Series, Takasago Electric, Nagoya, Japan). Background ASW flow and flow through the loop were maintained at the same rate by a pinch valve. The electric valve was controlled by software, and stimulation periods were aligned with response recordings for later analysis. With the exception of the period when stimuli were being delivered, the barbel was continuously bathed in ASW to prevent desiccation and avoid introducing mechanical artifacts during stimulus delivery. The tube was flushed with ASW for at least 1 min between stimulus applications. An amino acid blend (AAs: 10^{-4} M L-ala, D-ala, L-arg, gly, and L-pro), which are the most stimulatory amino acids to the gustatory system of sea catfish (Michel and Caprio 1991), was presented at least every four presentations to check the stability of the recording. If responsiveness changed by more than 20%, no data from the preceding recordings were used for analysis. Stimuli were presented to the barbel for as long as the recorded response amplitude to AAs remained within 20% of initial response values. Multiple nerve branches were tested within a preparation, but no more than three branches were tested in one preparation, and data presented here represent 28 recordings taken from 11 individuals.

Cross-Adaptation Experiments

Electrophysiological cross-adaptation experiments were performed to analyze the relative independence of the neural pathways for APV, PEB, and other test stimuli. These experiments consisted of three recording phases. 1) In Pre-adaptation, ASW continuously bathed the maxillary barbel, and stimuli were introduced as indicated in the previous method section. The

bile salts, sodium taurolithocholate (TLC), sodium taurocholate (TCA), sodium chenodeoxycholate (CDC), and sodium glycochenodeoxycholate (GDC), were chosen because of their effectiveness as taste stimuli in channel catfish (Rolen and Caprio 2008). Bile salts and AAs were tested at 10^{-4} M each. Further experiments with AAs were performed using L-ala and D-ala because these amino acids were identified as being most stimulatory to independent populations of facial taste fibers in the sea catfish (Michel and Caprio 1991). 2) During adaptation, an adapting solution replaced ASW and continuously bathed the maxillary barbel. The adapting solution was allowed to flow for at least 2 min before any test stimuli were presented. Adapting and test stimuli were diluted to test concentration in ASW. If responses to the test stimuli were suppressed by the adapting stimulus to baseline activity the test and adapting stimuli were considered to share the same neural pathway(s) and possibly the same molecular receptors. If responses to the test stimuli during adaptation remained significantly above baseline but below unadapted levels the test and adapting stimuli bound to at least some partially independent receptor sites and were processed by some relatively independent peripheral neural pathways. If the adapting stimulus failed to reduce the response to a test stimulus, the test stimulus was determined to be completely independent and therefore bound to molecular receptors independent from those to the adapting stimulus and also did not share peripheral neural pathways. 3) During Post-adaptation, ASW replaced the adapting solution and continuously bathed the maxillary barbel for at least 5 min before stimuli were tested again. Stimuli were identical to those in pre-adaptation and data were not used unless the pre- and post-adaptation response amplitudes were within 20% of each other. This standard for consistency ensured that recordings were only taken while the signal was stable (Ogawa and Caprio 1999). The degree of adaptation was calculated with the following formula:

$$PercentAdaptation = \frac{\frac{Rb+Ra}{2} - Da}{\frac{Rb+Ra}{2}} x100$$

where R_b is the response to a stimulus before adaptation, D_a is the response during adaptation, and R_a is the response after recovery from adaptation (Sheybani et al. 2009). Each nerve branch was tested with one or two adapting stimuli and four or five test stimuli. To test whether an adapting stimulus affected the amplitude of the response to each test stimulus, ANOVA followed by post-hoc Dunnett's tests compared cross-adaptation to self-adaptation ($\alpha = 0.05$). Responses to self-adaptations were statistically compared to an expected 100% adaptation using a single sample t-test. For those cross-adaptations that were significantly different from self-adaptation, one sample t-tests determined whether the percent adaptation was significantly different from the unadapted control (i.e. 0% adaptation) (GraphPad Prism version 4.04, GraphPad Software, San Diego CA USA).

4.3 Results

Behavioral Responses

Pellet Assay

Thirteen fish were tested individually, and all individuals used in the study accepted untreated shrimp pellets. Ink, APV, and PEB were each tested at log-step dilutions from 100% to 0.001% full strength (Fig. 4.2). To assess additive effects and necessity of APV and PEB for the ink's deterrence, APV+PEB and ink – (APV+PEB) were also tested. Ink and APV+PEB were significantly deterrent from 100% to 0.01% full strength (McNemar's test, $n = 13$, $p < 0.01$), APV and PEB were significantly deterrent from 100% to 0.1% (McNemar's test, $n = 13$, p

< 0.05), and ink – (APV+PEB) was significantly deterrent from 100% to 1% full strength (McNemar's test, n = 13, p= 0.03).

Barbel Assay

When a sea catfish's barbel was touched with freeze dried shrimp that had been soaked with ASW, the fish turned towards the shrimp and took it into its mouth (12/12). When the shrimp was treated with Ink, APV, or PEB at full-strength, fish turned away from the contact and did not ingest the shrimp (McNemar's test, n = 12, $p \leq 0.001$) (Fig. 4.3).

Electrophysiological Responses

Concentration-Response Functions for APV and PEB

Integrated multiunit responses to APV and PEB were recorded from branches of the facial-trigeminal complex innervating the maxillary barbel, of which the facial nerve components contain taste fibers. The responses from each nerve branch were normalized to the responsiveness to APV at 10^{-3} M to account for variations in signal amplitude and responsiveness between branches. Phasic responses were recorded for APV and PEB as well as AAs and bile salts, and these recordings were used for analysis (Fig. 4.4). Neural activity increased as stimuli came into contact with the barbel and returned to baseline levels following stimulation. APV and PEB were strongly stimulatory and had equivalent concentration-response curves (RMANOVA, n = 7, F = 0.629, df = 3, $p > 0.05$) (Fig. 4.5). Concentration had an effect on response magnitude to APV and PEB (RMANOVA Within-Subject Contrasts, n = 7, F =25.27, df = 3, $p < 0.05$). Responses to 10^{-4} M AAs were 95.5±15.6% of 10^{-3} M APV. Purified APV and PEB fractions were tested for free amino acid content, because these compounds are known to be

stimulatory and attractive and could affect the behavioral and electrophysiological results. These analyses were sensitive to amino acid concentrations as low as 1-4 µM. The only free amino acid (taurine) above measurement threshold was found in the APV fraction at 2.4 µM. After dilution for electrophysiological analyses, the final concentration of taurine was 24 nM, which is four log units below the test concentrations of AAs (10^{-4} M). Further, taurine was the least effective stimulus in the sea catfish from a group of 28 amino acids tested in single unit recordings (Michel et al. 1993)

Cross-Adaptation Experiments

Cross-adaptation experiments were performed with approximately equi-effective concentrations of each of the adapting and test stimuli. Tested were 10^{-4} M bile salts, 10^{-4} M AAs, 10^{-4} M APV, 10^{-4} M PEB, 10^{-4} M L-ala, and 10^{-4} M D-ala. Table 1 shows the response amplitudes of integrated recordings during continuous application of the adapting stimulus, represented as a percent of the unadapted response for each test stimulus (Fig 4.6. a-f). Results of statistical analyses of these data are presented in the figure legend. Post-adaptation responses had to return to within 20% of pre-adaptation amplitude for the recordings to be used for analysis. All adapting stimuli significantly cross-adapted at least one test stimulus. APV and PEB showed complete reciprocal cross-adaptation in which adapted responses were reduced to control levels. AAs completely adapted responses to APV and PEB, but APV and PEB adaptation only partially adapted responses to amino acids, indicating cases of incomplete reciprocal cross-adaptation. Bile salts as an adapting stimulus had little effect on responses to APV and PEB and only slightly cross-adapted responses to amino acids. PEB significantly, but incompletely, adapted responses to bile salts. APV adapted bile salt responses to a similar

degree as PEB; however, the small sample size (n = 2) for this cross-adaptation resulted in a lack of statistical significance. L-ala cross-adapted responses to APV, PEB, and the mixture of AAs by ~50% and D-ala by ~75%.

4.4 Discussion

The ability to detect and respond appropriately to noxious or potentially dangerous stimuli is an important adaptation for a predator to survive in an environment filled with defended prey species. The sea hare *Aplysia californica* produces a sticky, purple secretion that is deterrent to a variety of predators including spiny lobsters, blue crabs, sea anemones, sea gulls, and several species of fishes including sea catfish (DiMatteo 1981; Nolen et al. 1995; Kicklighter et al. 2005; Kamio et al. 2010a, 2010b; Nusnbaum and Derby 2010a, 2010b). Aplysioviolin (APV) and phycoerythrobilin (PEB) are purple components of the ink secretion that are derived from the algal photopigment phycobilin and are deterrent to blue crabs, *Callinectes sapidus*, and bluehead wrasses, *Thalassoma bifasciatum* (Kamio et al. 2010a, 2010b; Nusnbaum unpublished observation). In this report, I show that: (a) APV and PEB are deterrent to sea catfish; (b) APV and PEB are detected by taste buds on the barbels of the sea catfish and that this barbel gustatory system mediates avoidance behavior by these deterrents; and (c) APV and PEB are processed by both independent and shared gustatory neural pathways, including some that are also sensitive to behaviorally important amino acids.

The catfish is well-studied as a chemosensory model because of its sensitive gustatory system and numerous and distributed taste buds. Gustatory systems of catfishes and other fishes are highly sensitive to amino acids as well as nucleotides, polyamines, and bile salts (Michel et al. 2003; Rolen et al. 2003; Yamashita et al. 2006; Caprio and Derby 2008). Most of these

stimulatory compounds are involved in food acquisition and acceptance, though there are likely other classes of gustatory stimuli, including deterrents and bile salts, whose behavioral and electrophysiological effects have not yet been analyzed. The gustatory system of fishes is organized similarly to other vertebrate taste systems, with a notable exception being the increased extra-oral taste bud distribution (Caprio et al. 1993). Within taste buds, taste cells synapse onto primary gustatory fibers, with each fiber typically receiving input from cells in multiple taste buds. Numerous studies using receptor binding and electrophysiological cross-adaptation indicate that several independent receptors and transduction pathways exist for amino acids and other compounds in fish taste systems (Caprio 1978; Kumazawa et al. 1990; Wegert and Caprio 1991; Rolen et al. 2003; Rolen and Caprio 2008). In catfish, receptor binding and single fiber studies demonstrated independence in the pathways for detecting L-alanine and L-arginine in channel catfish (Teeter et al. 1991; Caprio et al. 1997) and L-alanine, D-alanine and glycine in sea catfish (Michel and Caprio 1991; Michel et al. 1993; Kohbara and Caprio 1996), highly stimulatory feeding attractants. The expression and connectivity properties of taste cells result in gustatory fibers that respond to multiple classes of chemical stimuli but still with specificity. This study represents some of the first evidence for pathways that respond specifically to natural deterrents.

APV and PEB, major feeding deterrents in sea hare ink, are detected through multiple gustatory pathways in sea catfish

APV and PEB are relevant deterrents to sea catfish. The pellet and barbel assays demonstrate that sea catfish rejected food or food-flavored pellets treated with low concentrations of whole ink, APV, and PEB, respectively (Fig. 4.2, 4.3). In addition, APV and

PEB represent the most dominant and potent deterrent compounds in ink. Ink lacking only APV and PEB (i.e. ink – [APV + PEB]) was deterrent, but when diluted it lost activity significantly more than whole ink (Fig. 4.2). The behavioral response threshold of sea catfish to ink in the pellet assay is similar to that of bluehead wrasse which significantly reject ink at 0.01% dilutions (Nusnbaum and Derby 2010b). I then used the sea catfish to examine mechanisms involved in the detection of deterrents using APV and PEB.

Deterrent specific sensory pathway

The electrophysiological results indicated that APV and PEB are detected by the same gustatory nerve pathways and are equally effective as deterrents. APV and PEB produce spiking activity in the facial-trigeminal nerve complex innervating the maxillary barbels, and APV and PEB have similar concentration-response relationships (Fig. 4.5). APV and PEB also show complete reciprocal cross-adaptation (Fig. 4.6a, b). This result is expected due to the close structural similarity of these two compounds (Fig. 4.1). APV and PEB activated at least two sets of gustatory pathways as indicated by cross-adaptation experiments. Cross-adaptation with bile salts, L-ala, and D-ala did not completely eliminate responses to APV or PEB (Fig 4.6 d-f). These results indicate that there are fibers specific for APV and PEB, and therefore taste receptors and taste receptor cells, which are independent from the other taste stimuli tested in these experiments. Amino acid independent fibers stimulated by deterrents are found in channel catfish, where quinine specifically activates a group of taste fibers (Ogawa et al. 1997), as well as in a number of other species (Frank 1991; Danilova et al. 2002; Geran and Travers 2006). Fibers specific for APV/PEB may function as a labeled-line for processing deterrent signals, resulting in rejection or avoidance behaviors. In catfish, the facial taste nerve innervates taste

buds in the anterior portion of the mouth and on the external surface of the animal and projects to the facial lobe, whereas glossopharyngeal and vagal taste fibers innervate taste buds located in the oropharyngeal cavity and project to the vagal lobe which is involved in determination of ingestion, reflex swallowing, or rejection (Atema 1971; Morita and Finger 1985; Whitehead and Finger 2008). The function of APV/PEB specific facial nerve fibers innervating the maxillary barbel may be to produce the avoidance response observed in the barbel assay (Fig 4.3). Though not directly tested in this dissertation, similarly tuned taste fibers in the vagal or glossopharyngeal nerves innervating the oral cavity may play a role in the rejection response in the pellet assay (Fig 4.2). A subset of quinine specific rat glossopharyngeal fibers projecting to the nucleus of the solitary tract are involved in reflex rejection responses, but not in behavioral avoidance which is eliminated by decerebration (Travers et al. 1987; King et al. 1999). This study represents some of the first evidence in fishes for neural pathways that respond specifically to natural deterrents. The subset of fibers whose response properties indicated shared pathways may also contribute to behavioral rejection/avoidance, or they may have different functions.

Shared neural pathways between deterrents and other classes of taste stimuli

Cross-adaptation with APV or PEB reduced, but did not eliminate, responses to AAs and bile salts (Fig. 4.6 a, b), indicating that there are at least some pathways sensitive to amino acids and bile salts that are affected by presentation of APV or PEB. This adaptation was not complete, indicating that there are amino acid and bile salt sensitive fibers that are insensitive to APV and PEB (Fig. 4.6 c, d). Incomplete reciprocal cross-adaptation could be due to differential effects of APV and PEB on the pathways that are sensitive to different amino acids. APV and PEB were significantly cross-adapted by L-ala, but not by D-ala, and may interact differentially

with other neural pathways for untested AAs (Fig. 4.6 d, e). Adaptation with bile salts did not reduce responses to APV or PEB, suggesting that APV and PEB bind mostly to receptors independent of those to bile salts; however, some bile salt responsive fibers were affected by APV and PEB (Fig. 4.6 a, b). Though the integrated multi-unit recordings showed significant effects on response magnitude between APV/PEB and AAs, I cannot determine whether APV and PEB function as activators or suppressors without presentation of mixtures and single fiber analyses. My recordings do show that amino acids, which are feeding stimuli for fishes, share some sensory fibers with APV and PEB and these shared pathways with food stimuli may enhance APV/PEB's behavioral effectiveness as deterrents (Michel and Caprio 1991; Caprio et al. 1993). The existence of a shared pathway between bile salts and AAs, APV, and PEB indicates a difference in signal processing that could have behavioral implications (Rolen et al. 2003).

Behavioral results demonstrate that APV and PEB affect responses to stimuli that would otherwise induce ingestion. These results indicate that APV and PEB affect the fishes' normal feeding behavior towards palatable stimuli, likely amino acids, which produce snapping and swallowing behavior in fishes (Valentinčič and Caprio 1994; Valentinčič et al. 1999). There are two testable hypotheses for how APV, PEB, and amino acids interact at the sensory level. First, they could share signal transduction mechanisms, such as shared gustatory receptor molecules or second messenger cascades in the same gustatory receptor cells. Second, the activity of one signal transduction pathway could inhibit the activity of another. Cross-adaptation between L-alanine and either APV or PEB was incomplete in both directions, suggesting that there are at least two pathways that respond to APV and PEB – L-alanine sensitive and L-alanine insensitive. The same is likely for bile salts, which partially cross-adapted with AAs, APV and PEB. If APV

and PEB function as receptor antagonists, the activities of amino acid-shared and -independent pathways could be additive in their behavioral effects, with one transmitting deterrent information while another reduces the predator's ability to detect attractive amino acids. The behavioral responses to the gustatory detection of bile salts have not yet been determined (Rolen and Caprio 2008), but their shared neural fibers with APV and PEB is an interesting result that should be examined further.

Because many toxic metabolites taste bitter, bitter taste receptors are thought to protect the predator against the ingestion of poisonous food compounds (Garcia and Hankins 1975; Glendinning 1994; Glendinning et al. 1999). It is, therefore, evolutionarily advantageous for predators to be able to detect and respond to these compounds, and redundancies in this system may be conserved. In vertebrates, individual gustatory receptor cells express many different T2Rs and function as broadly tuned bitter detectors that are sensitive to many different classes of bitter compound (Chandrashekar et al. 2000; Mueller et al. 2005). Multiple nerve fibers receive input from these bitter-detecting taste cells and these fibers can be most responsive to bitter substances and also receive significant input from receptors that mediate other classes of tastes. Single taste fibers responding to structurally, and behaviorally, different classes of stimuli were demonstrated by activity in a variety of species including vertebrates and invertebrates (Zeng and Hidaka 1990; Kitada et al. 1998; Li et al. 2001; Frank et al. 2005; Lemon and Smith 2005). Two groups of gustatory nerve fibers in channel catfish respond with excitation to both amino acids and low concentrations (~10^{-4} M) of quinine. Quinine (10^{-2} M) suppresses the amino acid responses of Group II fibers by 89-100%, whereas Group I fibers are significantly less affected (Ogawa et al. 1997). APV and PEB may similarly activate and inactivate pathways that enhance their behavioral effectiveness as deterrents. The behavioral consequences of the observed

activity patterns in gustatory receptor cells and gustatory fiber types are not currently known, but it is possible that they reinforce each others' effects. These patterns may aid in differentiating the identity of deterrent compounds and determining the appropriate contextual behavioral response.

Table 4.1 Cross-adaptation data from electrophysiological recordings from the maxillary barbel of *A. felis*. Adaptation data are presented as mean percentage ± standard deviation of the unadapted response for each adapting stimulus-test stimulus pair (see text for formula). Sample size for each data set is in parentheses.

	Test Stimulus	AAs	Bile Salts	APV	PEB	L-ala	D-ala
Adapting Stimulus							
AAs		1±7% (10)	59±7% (4)	15±19% (10)	12±14 % (9)	--	--
Bile Salts		69±19% (4)	0±2% (4)	97±6% (4)	96±10% (4)	--	--
APV		29±21% (10)	21 ±14 % (2)	7±11% (10)	12±18% (10)	--	--
PEB		32±28% (10)	24±26% (9)	0±6 % (9)	1±3% (8)	--	--
L-ala		48±9% (4)	--	46±6% (4)	49±5% (4)	1±4% (4)	25±12% (4)
D-ala		62±15% (4)	--	85±13% (4)	85±8% (4)	54±17% (4)	1±5% (4)

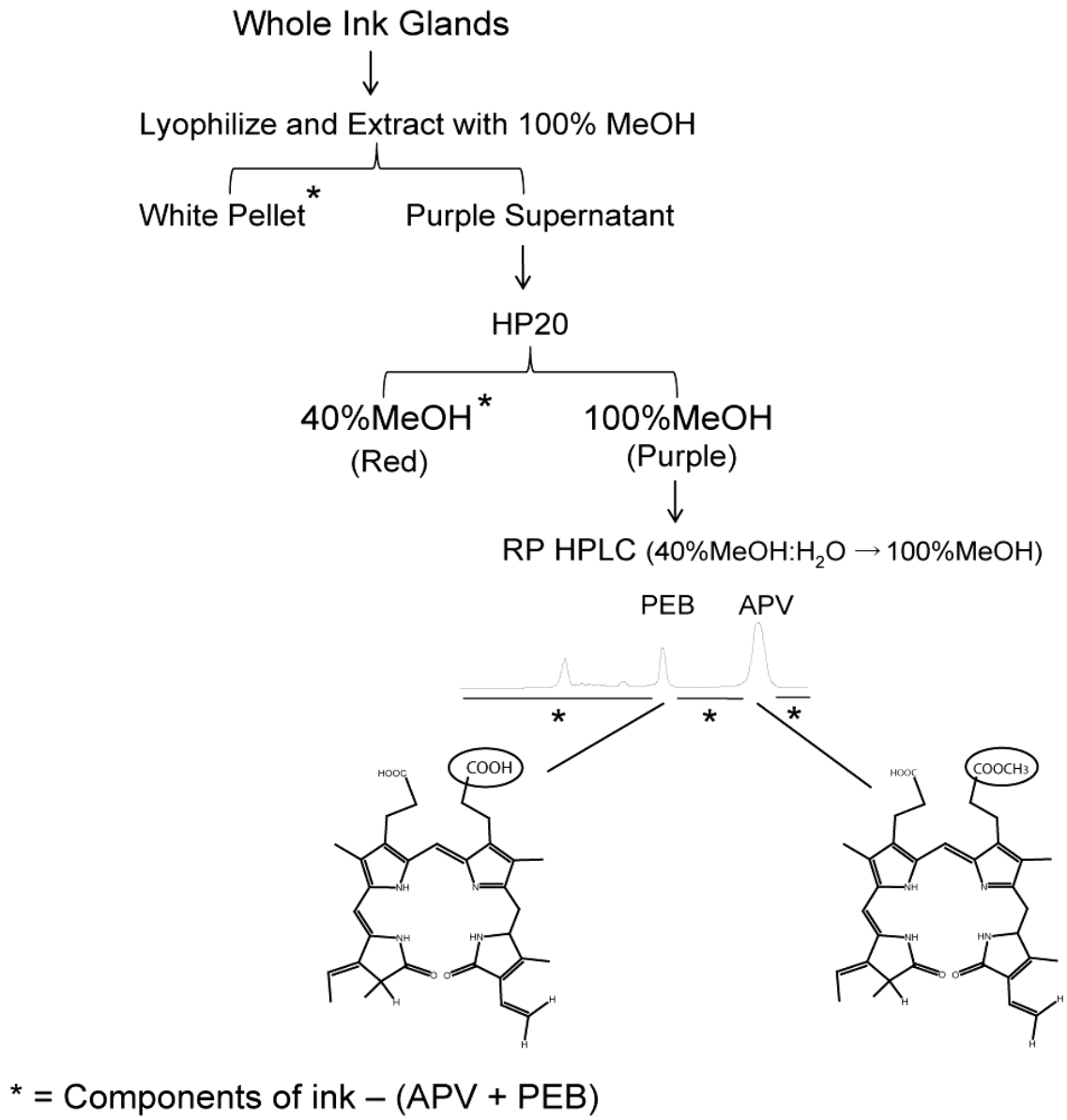

Figure 4.1 Schematic diagram of separation of deterrent compounds in *A. californica* ink, aplysioviolin (APV) and phycoerythrobilin (PEB). Ink was extracted from glands by lyophilization followed by grinding in 100% MeOH. The resulting purple supernatant was dried down, dissolved in 40% MeOH, and passed through an HP20SS column. The column was then flushed with 100% MeOH to release the purple components. The 100% MeOH fraction was further separated by RP-HPLC on a C18 column (10x250 mm) and a 40–100% MeOH in H_2O gradient; 8.0 ml/min. Peaks for PEB and APV eluted around 15 and 21 min respectively. Asterisks denote the portions of the extract that were recombined to form ink – (APV + PEB). See Supplementary Figure 1 (Online Resource 1) for UV-Visual spectral analysis of the fractions.

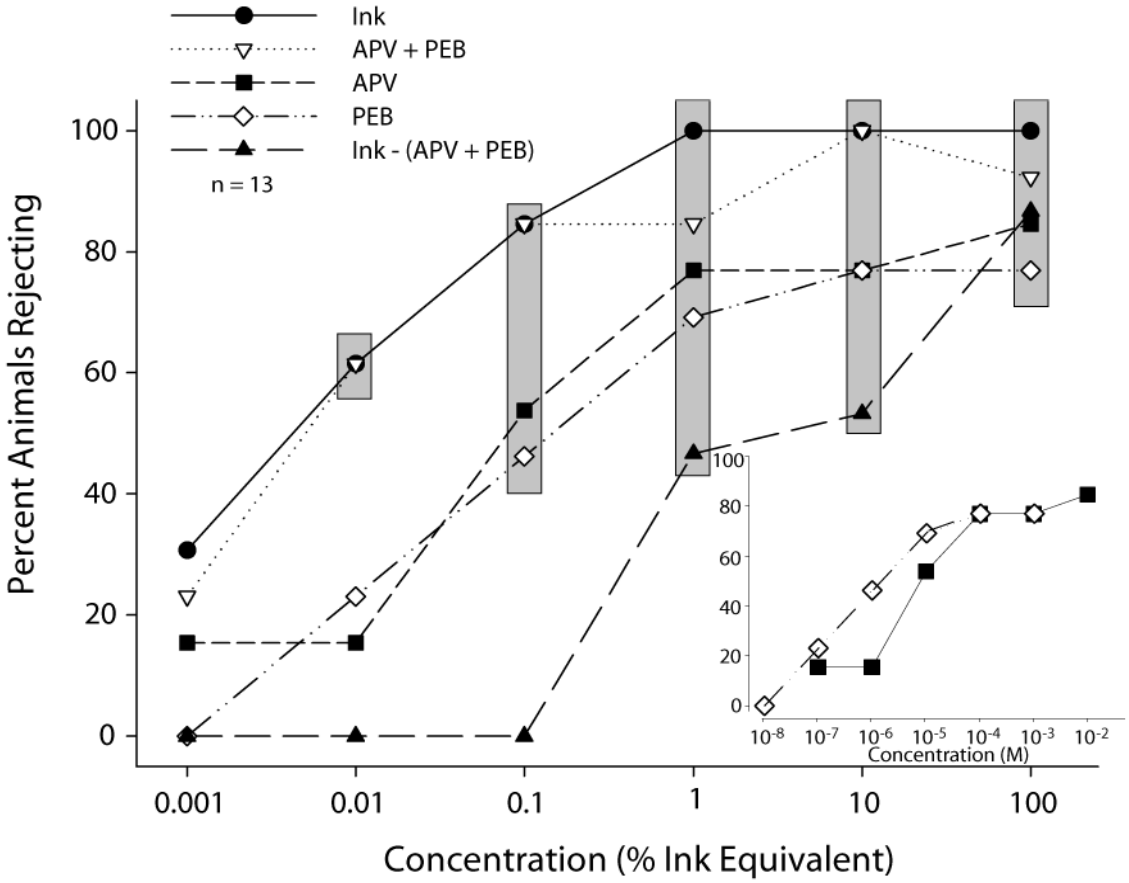

Figure 4.2 Behavioral analysis of the concentration-response relationship of ink fractions. Each stimulus was incorporated into shrimp-alginic acid pellets and offered to thirteen fish. Ink and APV+PEB were significantly deterrent at 0.01% full strength and higher. APV and PEB were significantly deterrent at 0.1% full strength and higher. Ink – (APV+PEB) was deterrent at full strength but not at 1%. .□ - response is significantly above 0%. Inset: Concentration-response relationship between APV and PEB at log molar concentration steps. APV and PEB were not significantly differently deterrent at equimolar concentrations.

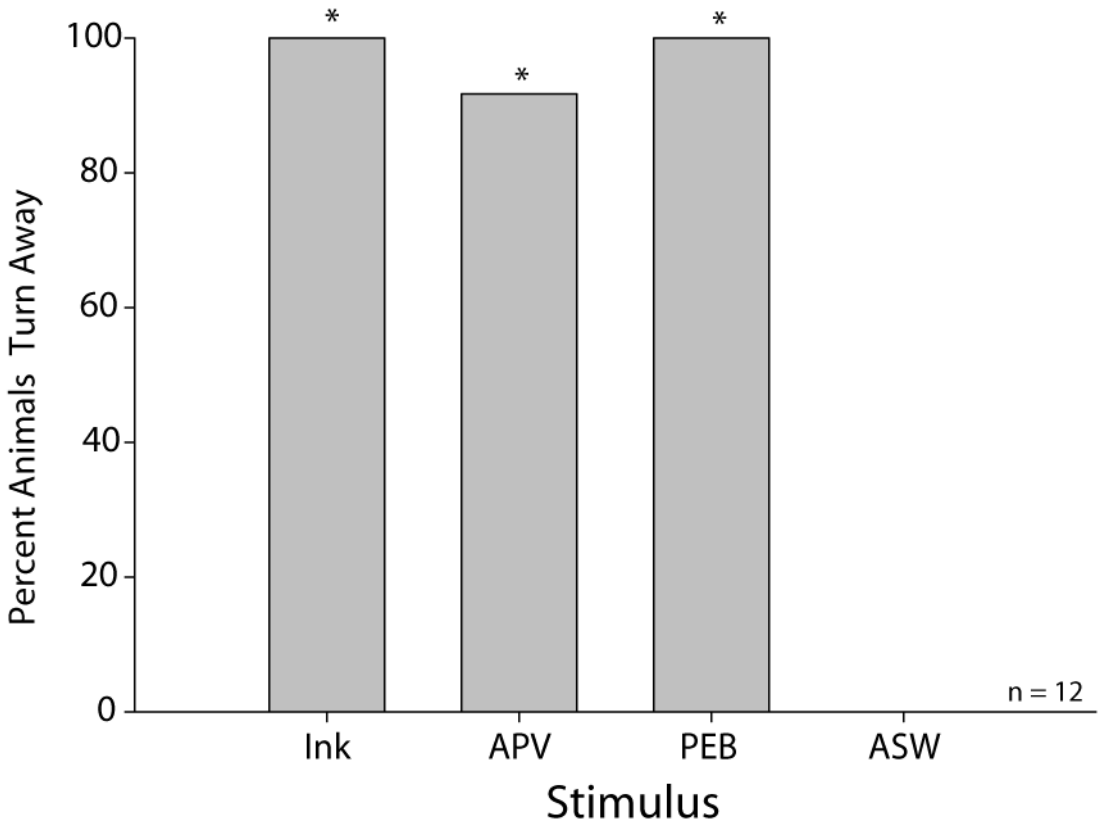

Figure 4.3 Behavioral responses in the barbel assay demonstrate that 100% full strength ink, APV, and PEB are detected by the barbels. All 12 fish tested turned away from freeze dried shrimp soaked in ink and PEB, and 11/12 turned away from APV. * - response is significantly different from ASW.

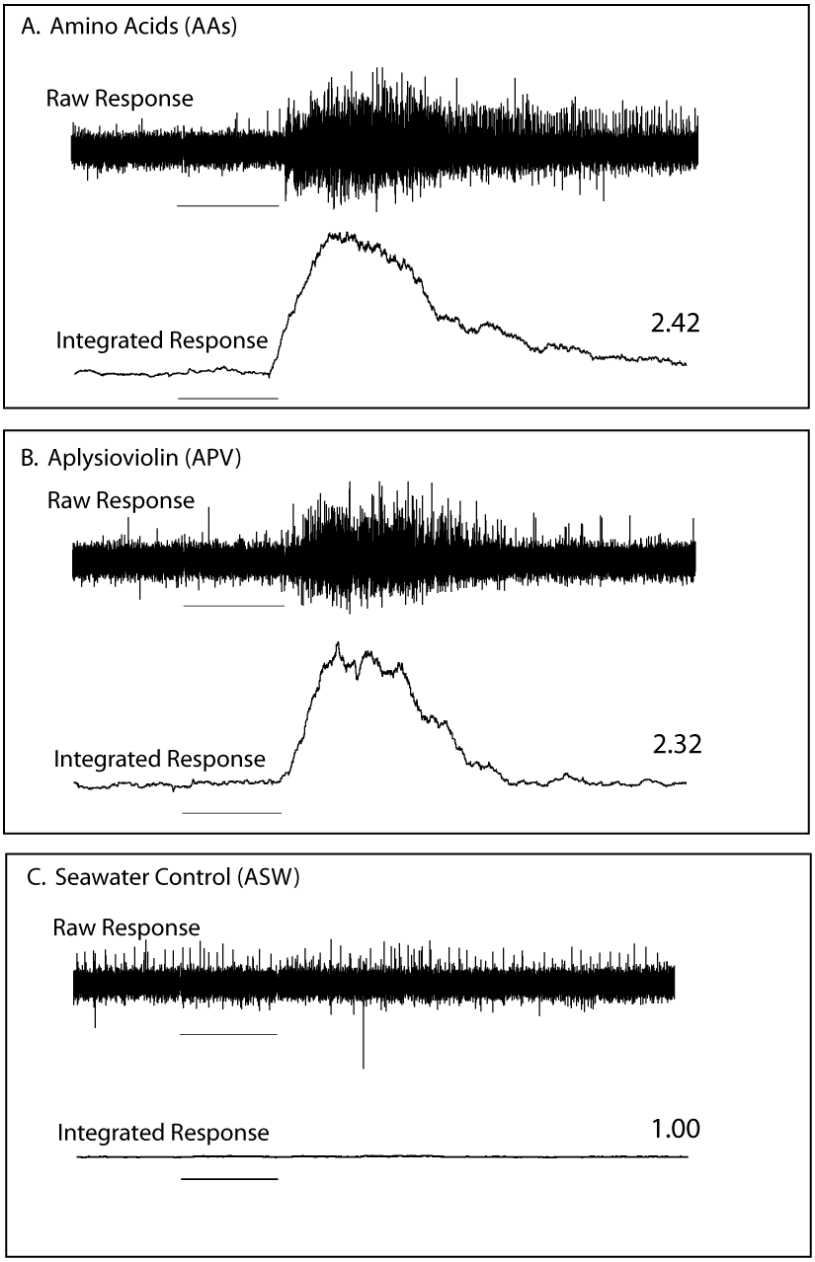

Figure 4.4 Electrophysiological recordings from a branch of the facial-trigeminal nerve complex. Each box represents a single representative recording from one branch of this nerve following presentation of a stimulus (A: AAs, B: APV, C: ASW). The upper trace depicts raw recordings for each stimulus, and the lower trace shows the same data transformed through integration with a 0.1 sec time constant. The numbers represent the maximum amplitude above the baseline for each integrated recording. These data were used to analyze relative responses of each nerve branch to each stimulus. Bars represent 2-sec stimulation period.

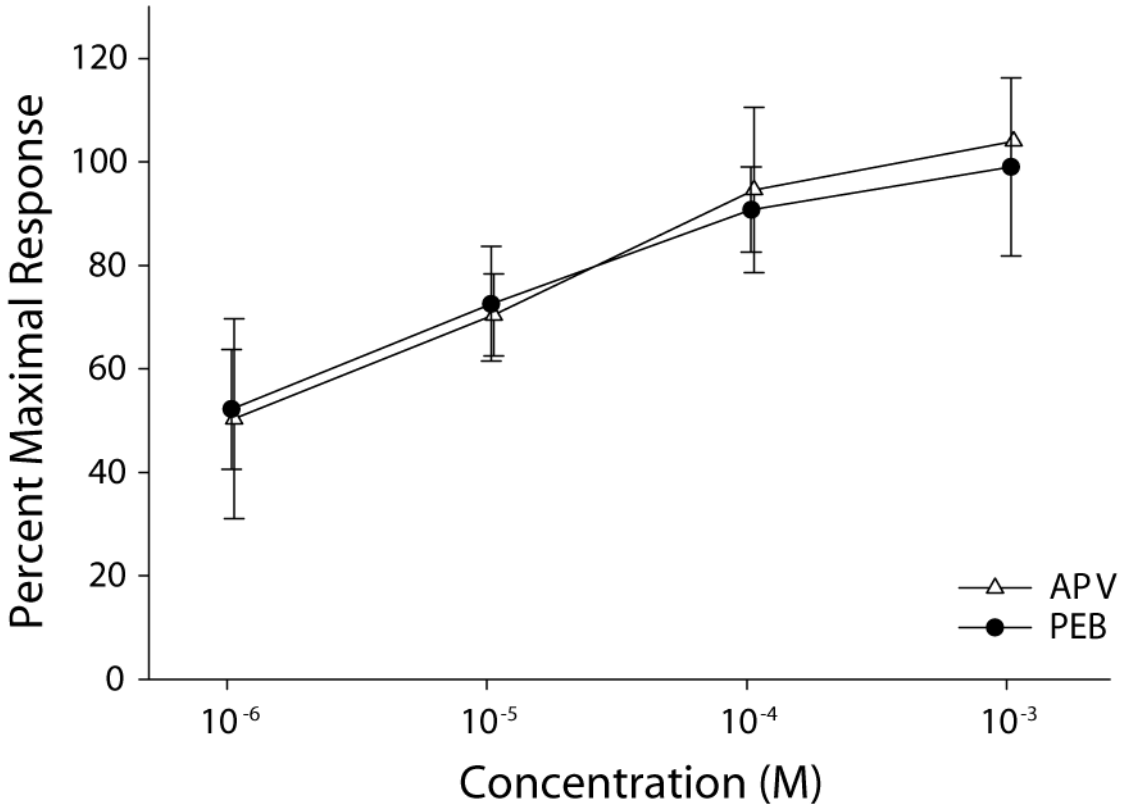

Figure 4.5 Electrophysiological concentration-response curve for APV and PEB. Integrated multi-unit recordings were taken from bundles of the facial-trigeminal nerve complex while presenting the maxillary barbel with different concentrations of APV and PEB. The integrated signal amplitudes (± SD) are displayed relative to the maximal response for APV. Response amplitude increased with concentration for APV and PEB, and the stimuli are not differentially stimulatory at similar concentrations. Concentration affected response magnitude for APV and PEB.

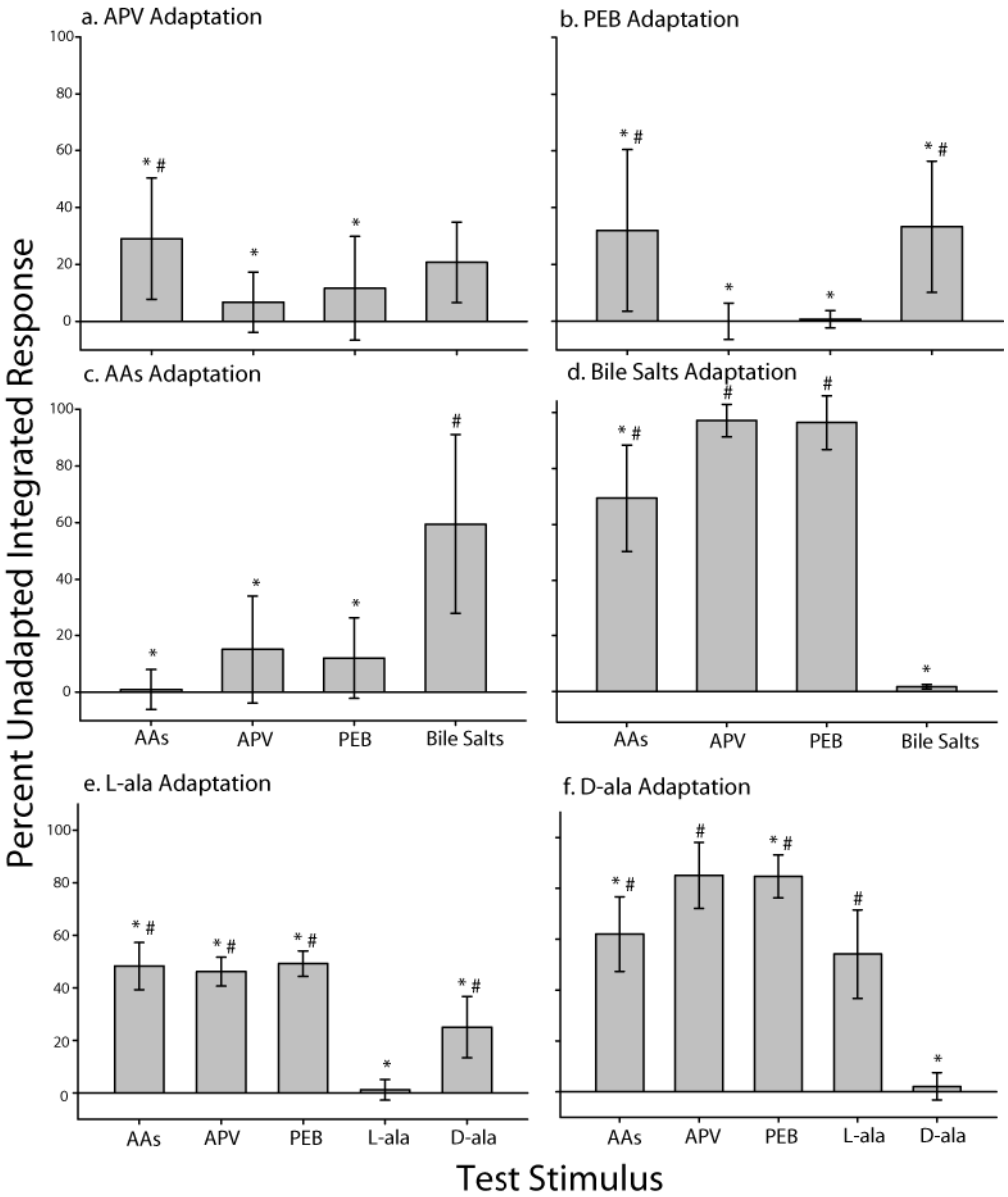

Figure 4.6 Cross-adaptation effects of different stimuli presented to the maxillary barbel. Results are depicted as the percent amplitude of the unadapted response, and values are mean ± SD. * - Adapted response is significantly different from 100% unadapted response. # - Cross-adapted response is significantly different from control (self-adaptation = the test response to the adapting stimulus). All adapting stimuli showed significant cross-adaptation of at least one test stimulus (ANOVA, APV: $n = 10$, $F = 3.161$, $df = 3$, $p < 0.05$; PEB: $n = 9$, $F = 6.402$, $p < 0.05$; AAs: $n = 10$, $F = 13.77$, $p < 0.05$; bile salts: $n = 4$, $F = 52.35$, $p < 0.05$; L-ala: $n = 4$, $F = 30.48$, $p < 0.05$; D-ala: $n = 4$, $F = 29.32$, $p < 0.05$). Statistical values for each figure are presented below. (a) Adaptation with APV reduced responses to APV, PEB, and AAs and showed a trend towards reduction of responses to bile salts. Adapted responses were reduced to control levels for APV and PEB, but not AAs. (b) Adaptation with PEB reduced responses to APV, PEB, AAs, and bile salts. Adapted responses were reduced to control levels for APV and PEB, but not AAs or bile salts. (c) Adaptation with AAs reduced responses to APV, PEB, and AAs. Adapted responses

were reduced to control levels for APV, PEB, and AAs. (d) Adaptation with bile salts was effective in self-adaptation and showed a low level of adaptation of AAs. Adapted responses were only reduced to control levels for bile salts. (e) Adaptation with L-ala reduced responses to APV, PEB, AAs, L-ala, and D-ala. Adapted responses were only reduced to control levels for L-ala. (f) Adaptation with D-ala reduced responses to AAs, L-ala, and D-ala. Adapted responses were only reduced to control levels for D-ala.

(a) APV adaptation: t-test against 100% unadapted response, APV: $n = 10$, $t = 27.96$, $p < 0.001$; PEB: $n = 10$, $t = 15.36$, $p < 0.001$; AAs: $n = 10$, $t = 14.10$, $p < 0.001$; bile salts: $n = 2$, $t = 7.92$, $p > 0.05$. t-test against control levels, APV: $n = 10$, $t= 2.02$, $p > 0.05$; Dunnett's test, PEB: $n = 10$, $q = 0.64$, $p > 0.05$; AAs: $n = 10$, $q = 2.91$, $p < 0.05$. (b) PEB adaptation: t-test against 100% unadapted response, APV: $n = 9$, $t = 46.88$, $p < 0.001$; PEB: $n = 9$, $t = 98.61$, $p < 0.001$; AAs: $n = 10$, $t = 19.55$, $p < 0.001$; bile salts: $n = 8$, $t = 8.36$, $p < 0.001$. t-test against control levels, PEB: $n = 9$, $t = 0.77$, $p > 0.05$; Dunnett's test, APV: $n = 9$, $q = 0.08$, $p > 0.05$; AAs: $n = 9$, $q = 3.44$, $p < 0.05$. (c) AAs adaptation: t-test against 100% unadapted response, APV: $n = 10$, $t = 10.53$, $p < 0.001$; PEB: $n = 9$, $t = 7.174$, $p < 0.001$; AAs: $n = 10$, $t = 44.59$, $p < 0.001$. t-test against control levels, AAs: $n = 10$, $t = 0.44$, $p > 0.05$; Dunnett's test, APV: $n = 10$, $q = 1.74$, $p > 0.05$; PEB: $n = 10$, $q = 1.36$, $p > 0.05$. (d) Bile salts adaptation: t-test against 100% unadapted response, APV: $n = 4$, $t = 0.98$, $p > 0.05$; PEB: $n = 4$, $t = 0.75$, $p > 0.05$; AAs: $n = 4$, $t = 3.22$, $p < 0.05$; bile salts: $n = 4$, $t = 87.82$, $p < 0.001$. t-test against control level, bile salts: $n = 4$, $t = 0.04$, $p > 0.05$. (e) L-ala adaptation: t-test against 100% unadapted response, APV: $n = 4$, $t = 19.61$, $p < 0.001$; PEB: $n = 4$, $t = 21.05$, $p < 0.001$; AAs: $n = 4$, $t = 11.48$, $p < 0.05$; L-ala: $n = 4$, $t = 50.67$, $p < 0.001$; D-ala: $n = 4$, $t = 12.87$, $p < 0.001$. t-test against control level, L-ala: $n = 4$, $t = 0.63$, $p > 0.05$. (f) D-ala adaptation t-test against 100% unadapted response, APV: $n = 4$, $t = 2.29$, $p > 0.05$; PEB: $n = 4$, $t = 3.01$, $p > 0.05$; AAs: $n = 4$, $t = 5.19$, $p < 0.05$; L-ala: $n = 4$, $t = 5.27$, $p < 0.05$; D-ala: $n = 4$, $t = 36.39$, $p < 0.001$. t-test against control level, D-ala: $n = 4$, $t = 0.79$, $p > 0.05$.

CHAPTER 5

GENERAL DISCUSSION

5.1 Chemosensory mechanisms of chemical defenses

Many plants and animals use chemical defenses against herbivores and predators, which may act through those animals' sensory systems to produce aversion responses or by being toxic or harmful (Berenbaum 1995; McClintock and Baker 2001; Kelley et al. 2003; Cruz-Rivera and Villareal 2006; Derby 2007; Glendinning 2007). Plants often produce secondary metabolites that function in these ways against herbivores. As a result of our knowledge of these complex systems, most studies on the mechanisms of chemical defenses are concerned with herbivores and particularly insects (Stowe et al. 1995; Schar et al. 2001; Glendinning et al. 2002; Bernays and Singer 2005; Conner et al. 2007; Glendinning 2007). Many defensive chemicals in plants are known, as are their sensory mechanisms of action (Mustaparta 2002; Glendinning 2007). However, in aquatic environments, although many defensive chemical compounds are known, our knowledge of their mechanisms of action at the sensory level is limited. Chemical defenses can induce immediate aversion responses through olfactory and/or taste organs. Olfactory organs can detect low concentrations of deterrent compounds at a distance from the source (Glendinning 2007; Kobayakawa et al. 2007). Poisonous insects and plants sometimes generate unique odors that are detected through olfaction which may facilitate persistent memories in predators (Rothschild et al. 1994; Rowe and Guilford 1999). The effects of chemical defenses on behavior at the sensory level are better characterized for taste systems (Glendinning 2007). Taste organs play a major role in the decision as to whether to ingest or reject food. In vertebrates, taste organs mediate mostly reflexive behaviors (Lamb and Finger 1995; Scott 2005; Derby and

Sorensen 2008). Poisonous or toxic compounds are often deterrent (i.e. they are distasteful or unpalatable) even though there is considerable variation in the chemical structures and therefore the predicted shape of the active site of the receptor (Scott 2005; Chandrashekar et al. 2006). Cells that detect these aversive compounds are broadly-tuned and induce aversion responses (Meyerhof 2005). A family of approximately 30 types of G-protein coupled receptors (known as T2Rs) mediate bitter taste in mammals (Mueller et al. 2005). Taste cells frequently express more than one receptor type, allowing them to respond to all of the compounds that their suite of T2Rs detect (Ishimaru 2005). Thus, bitter taste cells detect a large number of aversive compounds without necessarily discriminating among them.

The use of chemical defenses by marine plants and animals is well documented by chemical ecologists, including demonstrations that defensive chemicals can have enormous impacts on communities and ecosystems (Hay 1996; Hay and Kubanek 2002; Kicklighter et al. 2004; Parker et al. 2005; Kicklighter and Hay 2006; Long and Hay 2006; Derby 2007; Pohnert at al. 2007). Chemical defenses of marine plants are often secondary metabolites. These metabolites are shown through feeding assays to affect palatability to herbivores and predators. However, limited studies in marine systems have revealed the sensory mechanisms through which these deterrent compounds function (Kem and Soti 2001). Chemical defenses of marine animals have been extensively studied in predatory fish and arthropods (Kicklighter et al. 2003; Long and Hay 2006; Ritson-Williams and Paul 2007). Usually chemical compounds are isolated from chemical defenses and tested for deterrence through feeding assays. Chemical compounds are mixed with known feeding stimulants and tested for feeding suppression (Cruz-Rivera and Hay 2003). These tests are highly effective in testing feeding suppression (Lindquist 2002), but the mechanisms responsible for the deterrence are generally unknown. To test how a compound

functions as a chemical defense, it must be isolated and tested on its own. I demonstrated that sea hare ink not only suppresses intake of normal feeds by fish, but that the ink is also rejected when presented alone (Chapter 3). Further behavioral and electrophysiological analyses allowed me to begin to understand the mechanisms by which ink deters feeding and may protect the sea hare from fish predators.

5.2 Mechanisms of action of the chemical defenses of *Aplysia californica*

The sea hare, *Aplysia californica*, employs both active and passive chemical defenses in its arsenal of anti-predatory adaptations (Faulkner and Ghiselin 1983; Pennings 1994; Frost and Kandel 1995; Wright and Carew 1995; de Nys et al. 1996; Painter et al. 1998; Gallimore and Scheuer 2000; Ginsburg and Paul 2001; Cummings et al. 2005). For my dissertation research, I focused on the actively released ink secretion, because its release and biological activity are well-studied in other predator-prey interactions (Nolen et al. 1995; Kicklighter et al. 2005; Kicklighter and Derby 2006) and because its active release is more amenable to studying behavioral effects. Sea hare ink is composed of the secretions of two separate glands which generally release their products simultaneously into the mantle cavity where they are mixed together before being expelled in the direction of the attacking predator. Both secretions are complex mixtures containing secondary metabolites, proteins, free amino acids, and other chemicals; however, the purple ink secretion also contains high concentrations of red algal derived pigments (MacColl 1990; Pennings and Paul 1993; Johnson and Willows 1999; Rogers et al. 2000; Petzelt et al. 2002, Kicklighter et al. 2005). As demonstrated by electrophysiological recordings, ink and opaline secretions stimulate both the gustatory and olfactory systems of spiny

lobsters (Kicklighter et al. 2005). These secretions are also highly stimulatory to the gustatory and olfactory systems of the sea catfish, and this stimulation is not solely explained by the free amino acid composition of the secretions (Sheybani et al. 2008). Results from my experiments (Chapter 2) demonstrated that the product of the ink gland, but not that of the opaline gland, is deterrent to fish predators, and release of the ink secretion reduces the intensity of predatory attacks on sea hares. Further study (Chapter 3) showed that both the gustatory and olfactory systems are affected by the ink secretion. Gustation is generally involved in the ingestion and acceptance phases of predation, so the behavioral implications of deterrent effects in this system are relatively clear. However, the role of olfactory detection of the ink secretion is an open question whose answer could address the complementary roles of the two chemical sensory systems in the marine environment.

The two major deterrent components of the ink secretion, aplysioviolin and phycoerythrobilin, were identified following bio-assay guided fractionation using the blue crab as a predator model (Kamio et al. 2010a). PEB is a light-harvesting phycobilin chromophore found in red algae and cyanobacteria. In the algae, PEB is covalently linked to a phycobiliprotein to form phycoerythrin (PE) (Rüdiger 1994; Adir 2005). The sea hare acquires PE from dietary red algae then cleaves PEB from PE in the digestive gland where it is then carried in the hemolymph to be stored in the ink gland (Coelho et al. 1998; Prince et al. 1998; Kamio et al. 2010b). APV is the monomethyl ester of PEB and has only been found in members of the genus *Aplysia* (Figure 4.1) (Kamio et al. 2010b). My demonstration of deterrent activity by APV and PEB, here and in other work in the lab, is some of the first research showing chemical defensive functions for pigmented molecules (McClintock and Baker 2001; Miyake et

al. 2001, 2004; Matz et al. 2008). It is also the first demonstration of an animal converting plant photosynthetic pigments into antipredatory chemical defenses.

My electrophysiological studies in sea catfish demonstrated that APV and PEB are similarly potent stimuli of the gustatory system and that they completely cross-adapt the responses to each other as would be expected from their structures and behavioral dose-response analyses (Chapter 4). Cross-adaptation with APV and PEB reduced, but did not eliminate, responses to AAs and bile salts, indicating that there are at least some amino acid and bile salt sensitive neural pathways that are relatively independent from that to APV and PEB; these relatively independent pathways likely mediate the aversive responses observed in behavioral assays. Adaptation with AAs greatly reduced responses to APV and PEB, demonstrating incomplete reciprocal cross-adaptation. Adaptation with bile salts also reduced responses to APV and PEB but to a lesser degree. These results indicate that APV and PEB share pathways with other stimuli at the molecular receptor, receptor cell, or primary afferent neuron level that affect the amplitude of the integrated gustatory response in primary gustatory fibers. Without further analyses with single fiber or receptor binding techniques, one cannot identify the level at which this interaction is occurring. The multiunit electrophysiological recordings I performed show that there are multiple independent pathways involved in the detection of APV and PEB, but for those pathways that do interact with both amino acids and APV/PEB my methods cannot identify where in the pathway the interaction occurs, the receptor molecule, receptor cell, or primary afferent fiber.

Since ink and opaline contain high concentrations of free amino acids and amino acids are potent feeding stimuli in fishes (Wegert and Caprio 1991; Kohbara et al. 1992; Kicklighter et al. 2005), it is interesting to consider the behavioral implications of cross-adaptations between

AAs and APV/PEB. From my results, I cannot determine whether APV and PEB function as activators or inactivators in the amino acid sensitive pathways. If they function as activators they would activate the shared pathways and possibly be perceived as AAs. If they are inactivators, then they would inhibit responses to AAs and reduce perception of AAs. If they function as inhibitors, then the high degree of cross-adaptation between APV/ PEB and AAs may enhance the behavioral aversion to APV and PEB by reducing gustatory fibers' ability to respond to AAs which are attractive stimuli (Michel and Caprio 1991; Caprio et al. 1993). Inhibiting the ability to detect AAs may reduce the sea hare's attractiveness by reducing the predators' ability to detect the stimuli it uses to identify prey. Presenting the stimuli in mixtures and recording from single fibers may help us to differentiate between these two potential mechanisms of action. In nature, feeding animals encounter complex mixtures of nutrients and other substances. The responses of the gustatory receptor cells are thus greatly affected by interactions between chemicals (Schoonhoven et al. 1992; Smith et al. 1994; Chapman 2003; Jørgensen et al. 2007). Complex stimulatory and inhibitory responses could be important in the sensory coding and behavioral response to mixtures of stimuli. The suppression of appetitive gustatory receptor cell activity by bitter substances, such as quinine, is a common phenomenon in several species (Dethier and Bowdan 1989, 1992; Chapman et al. 1991; Formaker et al. 1997; De Brito Sanchez et al. 2005). Similar interactions were seen in gustatory fiber studies testing quinine in channel catfish, where Group I fibers fire action potentials after quinine presentation and Group II fibers lose responsiveness to amino acids when they are mixed with the deterrent compound (Ogawa et al. 1997). These studies have not identified the mechanisms by which quinine affected responses to appetitive compounds. It is possible that the sensory interaction of deterrents with attractive

stimuli is a common principle in gustatory systems but further study, both at the cellular and molecular level with APV and PEB and with other isolated deterrent compounds, is needed.

5.3 Neurethology, neuroecology and the study of chemical defenses

Neuroethology is the evolutionary and comparative approach to the study of animal behavior and the underlying neural mechanisms. Neuroecology connects animal behaviors and the nervous mechanisms underlying those behaviors to the broader consequences to populations and communities. As examined by Derby and Zimmer (2007), the field of neuroecology seeks to allow researchers to connect the neural bases of behaviors to the study of how behavior affects population and species distributions in natural systems. Chemical ecology, and the study of chemical defenses, is a separate but overlapping lens through which to observe trophic interactions. The field of chemical ecology historically focused on the production of secondary metabolites, the behaviors in animals that detect these chemicals, and often the community level interactions and population dynamics that are consequences of the production of these compounds. Recent work exemplifies this approach which provides information about the identity, source, and effects of secondary metabolites, but stops short of examining the mechanisms by which the metabolites have their effects on the nervous system (Parker et al. 2006; Lane et al. 2009; Rasher and Hay 2010). Despite the crucial ecological importance of these secondary metabolites, the underlying mechanisms are lacking for most processes that utilize them and impact the structure of communities. Conversely, the field of chemosensory biology and most specifically deterrent taste has made use of bitter compounds from hedonic human experiments to study the responses in a variety of animals including fruit flies, fish, rats, and monkeys (Dahl et al. 1997; Ogawa et al. 1997; Scott et al. 1999; Chandreshekar et al. 2000;

Weiss et al. 2011). This approach has answered many questions about the transduction mechanisms involved in behavioral responses to deterrent compounds, but it does not allow the researcher to ask questions about the evolutionary or ecological relationships between the producers of these compounds and the behaviors that detection elicits in the consumers. To study chemical defenses, I tested *Aplysia californica* ink secretions to determine how they affected fish predators, and then used identified components from that ink to analyze the sensory perception of these deterrents in fish.

By first identifying compounds that the consumer encounters in its habitat and would need the capacity to detect and to respond to, one can ask a number of sequential questions that characterize the function of the chemical sensory system and also address interactions at many other levels of organization from chemical synthesis to population dynamics. The chemosensory systems of fish, for example, evolved to be sensitive to behaviorally relevant stimuli within its environment. It makes no more sense from an ecological perspective to test the chemoresponses of fishes to volatile odorants than it does to test their responses to quinine (Davenport and Caprio 1982; Kanwal and Caprio 1983; Lamb and Finger 1995; Ogawa et al. 1997; Yamashita et al. 2006) whose only known natural source is the bark of the cinchona tree. The study of chemical defenses is the ideal vehicle to pursue knowledge of chemosensory detection of relevant stimuli. One can study the production and costs of making the defensive compounds, the behavioral responses elicited by these compounds, the electrophysiological responses elicited by them, pathways involved in processing and responding to them, population dynamics resulting from possession, or lack, of them, and the ecological impact that these species have on each other and their environment. To fully understand the role of APV and PEB in the defense of *A. californica*, these compounds need to be tested against other common marine predators such as

the spiny lobster, which is a crustacean chemosensory model species with which a great deal of work has been done on sea hare chemical defenses (Johnson and Willows 1999; Nolen et al. 1995; Kicklighter et al. 2005; Shabani et al. 2007). My dissertation begins to examine the mechanisms by which APV and PEB stimulate the fish gustatory system in the periphery, but further questions must be asked to understand the coding of deterrent compounds in fish gustation. What is the identity of the receptors that detect APV and PEB, and what is the sensitivity and specificity of these receptors? Do APV and PEB interact with amino acid receptors directly or at some point in the signal transduction pathway? Further study is needed to understand the behavioral implications of simultaneous activation of deterrent receptors as well as the inhibition of attractive molecule receptors. The connection that deterrents make from molecule to individual to population to species and ecosystem provides a many tiered approach to understanding the natural world.

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