Multi-component chemical defense in seahares (Gastropoda: Opisthobranchia): Antipredator compounds act as both honest and deceptive signals to multiple predator species

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Abstract

Multi-component chemical defense in seahares (Gastropoda: Opisthobranchia): Antipredator compounds act as both honest and deceptive signals to multiple predator species

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Many organisms produce chemical defenses to deter predation, yet the behavioral, neurophysiological, and cellular mechanisms of chemical defenses are largely unexplored. Animals do not face a single hypothetical predator, but rather a diversity of predators whose behaviors and sensory systems can be highly divergent. Do prey chemical defenses function differently versus different predator species? Can chemical defenses be honest signals to some species and deceitful to others? These issues were examined using seahares (*Aplysia* spp. and *Stylocheilus* spp.) and their cnidarian, crustacean, and vertebrate predators. Seahares release defensive secretions called ink and opaline from independent glands. In behavioral assays, seahares with full glands versus those with depleted glands had a significant survival advantage against sea anemones, crabs, and lobsters, but not against fishes. Isolated secretions and their components were used to explore mechanisms of defense against sea anemones and

lobsters. A 60 kDa glycoprotein from ink was isolated, cloned, sequenced, and expressed. This protein ("escapin") was responsible for the aversive reaction of sea anemones to ink via lysis of anemone cells. It also had antibacterial effects against Gram positive and negative bacteria. Escapin is the first reported antipredator protein of any organism.

Ink and opaline protected seahares against crustaceans in a different and unusual way – by stimulating them to feed. Opaline was as stimulatory as homogenates of squid and shrimp in behavioral studies and electrophysiological assays of chemosensory neurons of lobsters. The attractiveness of opaline and ink is likely due to extremely high levels of free amino acids. In particular the highly stimulatory amino acid, taurine, is three orders of magnitude more concentrated in opaline than seahare haemolymph. These secretions are a supernormal feeding stimulus that can act as a sensory trap, exploiting the chemosensory systems of crustaceans. Via this novel chemical defense "phagomimicry", crustaceans are deceived into attending to a false food stimulus from the secretions while dropping the seahare and thereby incurring a nutritional cost. Thus chemical defenses can function differently versus different predator species, acting as honest signals (as escapin does to sea anemones) to some and deceitful signals (as phagomimicry does to crustaceans) to others.

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Dedication

To Dr. Thomas G. Nolen
for inspiring me to become a scientist

Chapter I. Introduction

Research Problem

The literature of chemical ecology contains many examples of prey species that use chemical defenses to protect themselves from predation (Eisner and Meinwald 1966; Brodie 1977; Tachibana 1988; Dumbacher *et al.* 1992; Faulkner 1992; Tomaschko 1994; Daly 1995; Pawlik *et al.* 1995; Lindquist and Hay 1996). Natural products chemists working alongside chemical ecologists have isolated and identified numerous active compounds from these organisms (Tachibana *et al.* 1984; Paul 1992; Avila 1995; Bryan *et al.* 1995; Lindquist *et al.* 2000; Maia *et al.* 2000; Gronquist and Meinwald 2001). Yet little is known about how chemical defenses function against any single predator, let alone the diversity of predators that prey species encounter (Endler 1986; Eisner and Meinwald 1995; Hay 1996). Endler (1986) makes the point that "There are major gaps in our knowledge of most of the defenses, especially where multiple functions and predators exist, but the gaps are especially great for non-visual sensory modes." Since that statement was made few of these gaps have been filled.

It is usually assumed that most defensive chemicals act as poisons that harm the predator directly (Berenbaum 1995). However, studies of direct effects of chemical defenses at the physiological, neurological, or molecular levels are scarce (but seeLindquist and Hay 1995; Schnitzler *et al.* 2001; Wee and Tan 2001; Zhu *et al.* 2001). One can ask if chemical defenses always cause some form of harm to the predator, thereby functioning as honest signals (Dawkins and Krebs 1978; Johnstone and Grafen 1993; Krebs and Davies 1993; Hasson 1994)? Or can they also function as deceitful signals, exploiting predator sensory systems in a manner similar to that described in the context of sexual selection (Ryan and Rand 1990; Stowe *et al.* 1995) or prey capture (Christy 1995)? In this way, could the prey make its chemical defenses seem more harmful than they are? Hay (1996) suggests that "...prey might mimic more potent metabolites by making non-toxic compounds with structural groups that bind to prey receptors for toxic compounds." To investigate such issues, one can examine responses of predators and their sensory systems to chemical defenses at behavioral, neurophysiological, and molecular levels. Knowing that a predator stops an attack or avoids a prey species after a chemical defense is employed does not reveal the mechanism involved. It could be direct damage, simple distastefulness, or deceitful manipulation of a predator's sensory systems.

Furthermore, although chemical defenses may be limited in variety, a prey species is likely to face predation from multiple predator species - often from several different phyla (Endler 1986; Sih *et al.* 1998). Do prey chemical defenses function in the same way versus bird or beetle, or versus fish or crustacean? Can they be honest signals to one species, and deceitful to another? Answering these questions requires a combination of behavioral, neurophysiological, biochemical, and molecular approaches, using a range of predator species.

Statement of Purpose

My objective is to build on the wealth of data provided by natural historians, behavioral and chemical ecologists, and natural products chemists about chemically defended animals by investigating *mechanisms* of chemical defense for a group of organisms that face multiple predators from several different phyla. My ultimate goal is to understand how chemical defenses function against predators at behavioral, physiological, and neurophysiological levels. This research also addresses two fundamental questions in the study of predator-prey interactions. The first is how individual prey defend against multiple predator species with a limited defensive repertoire. By taking a comparative approach involving predators from three different phyla, we can begin to understand how at least one prey species can accomplish such a task. This comparative approach will also be useful in addressing the second question: can chemical defenses function as both honest and deceitful signals? Chemical defenses are usually thought of as posing direct harm to the attacker or as chemical aposematic warnings that the prey is harmful. Both such examples represent chemical defenses functioning as honest signals from prey to predator. It is also likely that sensory exploitation may be used to deceive predators chemically into reacting as if harmless species are harmful, or by deceiving them in some other manner. This research will clarify how a single prey organism can use both honest and dishonest signals to defend against very different types of predators. The results may generate new hypotheses about chemical defense that can then be tested by ecologists in field settings. The project lays a foundation for further investigation of these processes at biochemical and molecular levels.

I have chosen to study the marine molluscs of the genus *Aplysia* and their relatives, commonly known as seahares, because they are abundant, easily observed and obtained, intertidal and subtidal herbivores throughout the world's temperate and tropical oceans (Carefoot 1987). They are well known for their chemical defenses (of which they have at least three) and their predators have been identified both in the field and laboratory (Johnson and Willows 1999). Additionally, due to their use in neurobiological research, one species *(Aplysia californica)* is available year round from the NIH National Resource for *Aplysia* Mariculture Facility.

Aplysia, like all but one of the ten genera of seahares, possesses two active chemical defenses as well as the passive chemical defense of distasteful skin (Johnson and Willows 1999). The two active defenses are secretions released into the mantle cavity from two separate glands. The better known of the two is ink, generally a bright purple fluid released in large quantity. Some species including another California seahare, *A. vaccaria*, release a white fluid only from the ink gland; while others such as the Atlantic seahare *A. dactylomela* release both purple and white (see Chapter II). The second defensive secretion comes from the opaline gland which lies ventral to the mantle cavity and releases into it. Opaline is a whitish and extremely viscous substance that is often, but not always, released with ink. Both secretions are directed outward toward the site of predatory attack via the siphon (Walters and Erickson 1986).

Though many species of predator will occasionally prey on seahares, no organism is known to make seahares a primary part of the diet – perhaps due to their effective chemical defenses (Johnson and Willows 1999). For this reason, I have chosen from among the known predators of seahares those that are particularly useful in addressing questions of chemical defense mechanisms at physiological and neurobiological levels – sea anemones, crustaceans, and fish. Sea anemones have been used in many studies of feeding behavior and chemosensory neurophysiology (Lawn 1976; McFarlane 1984; Boothby and McFarlane 1986; Cho and McFarlane 1995) and are perhaps the best-documented predators of *Aplysia* (Winkler and Tilton 1962; Nolen *et al.* 1995; Johnson and Willows 1999). It has also been shown behaviorally that *Aplysia* chemical defenses are an effective deterrent against sea anemone predation (Nolen *et al.* 1995). Spiny lobsters of the genus *Panulirus* have been used for decades as a model system for understanding chemosensory neurobiology (Ache and Macmillan 1980; Ache and Derby 1985; Zimmer-Faust 1987; Trapido-Rosenthal 1995; Derby 2000). Like many crustaceans, they will also prey on seahares in both the lab and field (Pennings 1990a; Walters *et al.* 1993). Fishes are the most widely used predator organisms in marine chemical ecology (Hay *et al.* 1998) and have been used in several studies of seahare chemical defense (Russell 1966; Kinnel *et al.* 1979; Pennings 1990a, 1994; Alves-Gomes *et al.* 1995; Carefoot *et al.* 1999; Pennings *et al.* 1999; Ginsburg and Paul 2001; Pennings *et al.* 2001). None of these studies, however, addressed the effects of the defensive secretions, ink and opaline, on acceptance or rejection of live seahares by fishes. Fish also represent a good future model for exploring the chemosensory side of chemical defenses. Using fish, crustaceans, and cnidarians, I can address both the issue of how prey species cope with multiple predators using limited defensive repertoires and the issue of honest and dishonest signaling in the context of chemical defenses.

Research Questions

Research Question #1: Are seahare chemical defenses effective against multiple predators from different phyla?

Few studies have addressed how chemical defenses may function differently against different predators in the marine environment (Pennings 1990a) and none have done so explicitly. Most the work that has been done on multiple predators examines their effects on prey species and has been conducted primarily in freshwater and terrestrial environments (Gonzalez and Tessier 1997; Peckarsky and McIntosh 1998; Sih *et al.* 1998; McIntosh and Peckarsky 1999; DeWitt *et al.* 2000; Eklov 2000; Nystrom *et al.* 2001; Van Buskirk 2001; Schmitz and Sokol-Hessner 2002). I examine here how prey affect differently multiple predators from three different phyla. Though many behavioral studies have already been conducted with seahares and their predators it is important to establish behavioral evidence first on the potential roles of the seahare active chemical defenses of ink and opaline before investigating mechanisms at physiological levels. It is quite likely that the defenses will show differences in effectiveness depending on the predator as well as differences in behavioral mechanism.

Objective 1. Are the ink and opaline secretions attractive, repulsive, or neutral to predators of three different phyla?

Three possible behavioral responses to the defensive secretions of seahares are repulsion (most likely), attraction (unlikely), and no response (a possibility).

Research Question #2: If seahare chemical defenses are effective, what are the likely active components of their defensive secretions?

Having determined whether seahare ink and opaline are effective against different predators, I can begin to address what the active components may be in cases where the defenses were found to be effective. The behavioral studies will also point toward certain potential components by the nature of the predator's response. I will isolate, purify, and identify components of the secretions in order to test further their roles.

Objective 2a. Isolate proteins from the secretions.

Ink from several different species of sea hares has been shown to contain proteins with antibacterial and cytolytic effects (Yamazaki 1993; Melo *et al.* 2000; Petzelt *et al.* 2002). Such proteins may be active components of a repulsive defense system.

Objective 2b. Determine the free amino acid content of the secretions.

Free amino acids are highly effective feeding stimulants for marine carnivores and scavengers, including sea anemones, crustaceans, and fish (Carr 1988; Caprio 1988; Sorensen and Caprio 1998; Derby 2000). It is also known that at least a few free amino acids have been found in the ink of *Aplysia* (Troxler *et al.* 1981). It is possible that these amino acids could play a role in the responses of predators. For this reason, I will analyze both ink and opaline secretions for free amino acids.

Research Question #3: How do the active components function to deter predation?

Once active components have been identified, isolated, and quantified, I can begin to assess their particular roles in physiological studies with predators.

<u>Objective 3a. Can the active components cause any physical damage to predators</u>?

Proteins that are found in the ink of other species of seahares have been shown to have cytolytic effects against other cell types (Yamazaki 1993; Melo *et al.* 2000; Petzelt *et al.* 2002). I hypothesize that such cytolytic activity is responsible for the rejection behavior of sea anemones to *Aplysia californica* ink (Nolen *et al.* 1995).

<u>Objective 3b. Can the active components stimulate the sensory system in some way?</u>

Of the three predators used in my study, crustaceans are the best choice to begin a detailed investigation of mechanisms of chemical defense at the neurophysiological and molecular level. Spiny lobsters are already an established model system in chemosensory biology and offer both established techniques and a wealth of published data with which to compare my findings. Understanding how *Aplysia californica* chemical defenses function at a neuronal level in spiny lobsters will be the first step in understanding how chemical defenses function against a variety of organisms. I hypothesize that the secretions from the opaline gland and possibly also the ink gland mimic food and thus attract and initiate feeding by lobsters. I further hypothesize that the secretions excite the lobster's food-detecting chemosensory neurons (CNs), by containing either the same components as in food (amino acids, amines, nucleotides, ammonia: Carr 1988) or mimics of these compounds that serve as competitive agonists for the food-detecting receptor sites on these CNs.

Research Question #4: Can chemical defenses represent both honest and dishonest signals, depending on the predator receiving the signals?

Chemical defenses could function honestly, either by causing direct harm to the predator or by warning the predator that ingestion of the prey would directly reduce its fitness. Chemical defenses could also function dishonestly (as aposematism in palatable species often does) by convincing a predator that a harmless prey is harmful to eat. They could also function dishonestly by activating receptors for harmful substances while not actually doing damage themselves or by fooling a predator to respond in some other inappropriate way that would allow the prey to escape. By comparing the different secretions of seahares and their effects on predators from 3 different phyla, I will uncover such differences and address this larger question of animal signaling. For example, if I find that the secretions are indeed attractive to lobsters but are mimics of feeding stimulants or have little or no nutritional value compared to the seahare itself, I can say that the chemical defenses function as deceitful signals to the predator. If I find, for example, that the protein in the ink is causing cytolysis of sea anemone tissue, then I can say that ink function as an honest signal from seahare to anemone.

Objective 4. Do the secretions offer greater nutritional value than the seahare itself?

I will measure nutritional value by quantifying ash-free dry weight as well as carbon and nitrogen ratios (Cruz-Rivera and Hay 2000). I will compare these values for ink and opaline as well as body tissue in order to compare the nutritional value to the predator of eating the secretions alone or of eating the seahare body.

Chapter II. Literature Review

The seahares are an order of gastropod molluscs that, like most of the subclass Opisthobranchia, have largely given up the physical protection of a hard outer shell and have replaced it with an array of chemical and behavioral defenses (Faulkner and Ghiselin 1983). The seahares comprise the order Anaspidea that contains two families (Akeridae and Aplysiidae) that are composed of nine common genera: *Akera*, *Aplysia*, *Dolabella*, *Dolabrifera*, *Petalifera*, *Phyllaplysia*, *Notarchus*, *Stylocheilus*, and *Bursatella* (Beeman 1968; Thompson and Seaward 1989). The phylogenetic relationships of these genera have recently been established using multiple molecular markers (Medina and Walsh 2000). The seahares share (among many other characters) a pair of defensive glands that are found only in the order Anaspidea. I will first review the literature on seahare predation, followed by a brief overview of their passive defenses and non-chemical active defenses. The bulk of the review then covers seahare active chemical defenses – the subject of this dissertation.

PREDATORS

It is common for researchers who study learning and memory in the seahare *Aplysia* to joke that neurobiologists are the primary predators of seahares. Though it is true that few organisms make seahares a major part of their diet, predators of these animals certainly do exist (for a complete list of reported seahare predators, see Johnson and Willows 1999). Such predation, limited though it may be, is of extreme importance to the prey species regardless of its importance to the feeding ecology of the predator

(Nolen *et al.* 1995). The assumed lack of major predators of seahares, in fact, points to the success of their many defense systems.

Sea anemones are perhaps the best-known predators of seahares (Johnson and Willows 1999). They share the same intertidal habitats and in such close proximity that seahares often blunder into their tentacles (pers. obs.). The following sea anemones have been shown to ingest seahares in field observations or experiments: *Actinia olivacea* (Willan 1979), *Aiptasia tagetes* and *Phymanthus crucifer* (Tobach *et al.* 1989), *Anthopleura xanthogrammica* (Winkler and Tilton 1962), and *Cerianthus* sp. (Schuhmacher 1973). In addition, Thompson (1960b) showed that both *Anemonia sulcata* and *Tealia felina* eat seahares in the lab. The chemical defense of ink has been shown to play an important role in seahare escape from sea anemones. The purple colored ink of *Aplysia californica* enhances survival against the giant green sea anemone *Anthopleura xanthogrammica* (Nolen *et al.* 1995). When an anemone ingests a seahare, the seahare releases ink, which causes the anemone's tentacles to contract and its gastrovascular cavity to evert, effectively expelling the seahare. Even normally palatable pieces of fish can be made unpalatable to sea anemones by coating them with *A. californica* ink.

Arthropods have also been identified as predators of seahares. These include a pycnogonid *Anoplodactylus evansi* (Rogers *et al.* 2000b) and several crustaceans: the spiny lobster *Panulirus interruptus* (Pennings 1990a), the shore crab *Carcinus mediterraneus* and another Mediterranean crab (Susswein *et al.* 1984), the crab *Calappa* sp. and the hermit crab *Dardanus* sp. (Sarver 1978). Just as seahares are model organisms in both chemical ecology and neurobiology, one of their crustacean predators, the spiny lobster, is a prominent model organism in chemosensory biology. Spiny lobsters have been used in numerous behavioral, neurophysiological, and molecular studies of chemical senses. For example, mechanisms of chemical sensing, including binding of chemical stimuli to receptor proteins, transduction and coding by receptor neurons, central processing, and perception and behavioral responses, have been extensively detailed in spiny lobsters (Carr *et al.* 1990; Ache and Zhainazarov 1995; Atema 1995; Beltz 1999; Xu and McClintock 1999; Derby 2000; Zimmer and Butman 2000; Munger *et al.* 2000). Most work in chemical sensing by lobsters has involved feeding attractants, which include small nitrogenous compounds such as amino acids, amines, and nucleotides (Carr and Derby 1986; Carr 1988; Sorensen and Caprio 1998). This well-defined crustacean chemosensory system is a useful model for seahare chemical defenses against a major group of predators.

Most fishes avoid eating seahares (see Introduction to Chapter V); however, there are a few species (e.g. wrasses) that do so consistently. These include the wrasses *Thalassoma duperei* and another unidentified wrasse seen feeding on *Aplysia juliana* in the field in Hawaii (Sarver 1978). They also include the wrasses *Thalassoma lutescens*, *T. quinquevittatum*, *Cheilinus fasciatus*, and the bream, *Pentapodus macrurus* that fed on *Stylocheilus striatus* in Guam (Pennings *et al.* 2001) as well as the wrasse *Halichoeres semicinctus* and the garibaldi *Hypsypops rubicundus* that fed on *Aplysia californica* off the coast of California (Pennings 1990a). The seahare *Akera* has also been found in the stomachs of flounder *Limanda limanda* (Morton and Holme 1955; Thompson 1960a). Fishes are not only interesting seahare predators but tend to be the primary behavioral assay organisms in marine chemical ecology as a whole (for many examples see Hay *et al.* 1998).

DEFENSE IN POST-METAMORPHIC SEAHARES

The defenses of post-metamorphic seahares (for review of larval and egg defenses see Johnson and Willows, 1999) can most conveniently be divided into either passive or active categories. Passive defenses are those for which direct action of the seahare's nervous system is not required. These are always operating and therefore are first lines of defense. Active defenses, on the other hand, are usually latent and must be activated – usually by a predatory attack. They represent a final line of defense.

Passive Defenses

In a review paper we extensively covered the passive defenses of seahares (Johnson and Willows 1999). Since they are not a focus of this dissertation, I review them only briefly here. Many seahares are known to possess three passive defenses, including cryptic coloration, large size, and distasteful skin. Many seahares are quite cryptic as juveniles living in and among the macroalgae that they eat (pers. obs.). Large adults, though not cryptic due to their size alone, are still not aposematic in coloration as are many other opisthobranch molluscs, though they appear to be just as distasteful (Thompson 1960a). The large size of some adult seahares, (they are the largest gastropod molluscs and may weigh several kilograms), is a deterrent to many smaller potential predators. Examples include the seahare-eating opisthobranch, *Navanax* (Leonard and Lukowiak 1986; Pennings 1990b) and the predatory pycnogonids (Rogers *et al.* 2000b). Many studies of seahare palatability have shown that seahares or their tissues are often rejected after being sampled by predators (Thompson 1960a; Martin 1966; Russell 1966; Ambrose *et al.* 1979; Kinnel *et al.* 1979; Pennings 1990a; Ginsburg and Paul 2001; Pennings *et al.* 2001). Though this is not the case for all seahare/predator pairings (Walters *et al.* 1993; Nolen *et al.* 1995; Pennings *et al.* 2001), it does suggest that unpalatability may be a passive defense of seahares. This is further supported by the fact that known algal secondary metabolites with antifeedent properties have been found in the skin of some seahares (Winkler 1969; Kinnel *et al.* 1979; Faulkner 1992; Rogers *et al.* 2000a). A problem with most palatability studies that utilized live seahares is that they don't control for confounding effects of the active chemical defenses of ink and opaline which are often released during predatory attack (Thompson 1960a; Martin 1966; Pennings 1990a; Ginsburg and Paul 2001). To date only the study by Nolen *et al.* (1995) controls for active chemical defenses by comparing animals that have had the secretions removed prior to presentation to the predator.

Active Defenses

Active defenses of seahares have also been reviewed in Johnson and Willows (1999), but because this dissertation focuses on active chemical defenses, I here provide a more complete discussion. First I briefly review the active defenses of seahares which are not chemical in nature.

Non-chemical Active Defenses

The active, non-chemical defenses of seahares consist of withdrawal of the siphon, withdrawal and turning of the body, and escape locomotion, including both galloping and swimming. The siphon withdrawal reflex of the seahare *Aplysia californica* has been the center of much basic cellular neurobiology research since the late 1960s (Kupfermann and Kandel 1969; Carew and Kupfermann 1974; Kandel 1979; Advokat 1980; Rankin 1987; Frost and Kandel 1995; Wright and Carew 1995; Fischer *et al.* 2000). It has been suggested as serving a defensive function in seahares by most of the authors above, but this has never been documented experimentally or even published anecdotally. It has been shown, however, that the siphon is crucial for directing defensive secretions of ink and opaline toward the site of predatory attack (Walters and Erickson 1986) and that siphon withdrawal is inhibited by the presence of ink (Stopfer *et al.* 1993; Illich *et al.* 1994). Seahares are also capable of withdrawing the head and tail and turning the body away from the site of attack (Lederhendler *et al.* 1975; Walters and Erickson 1986; Nolen *et al.* 1995). This type of withdrawal response, as opposed to siphon withdrawal, has been observed in both laboratory and field encounters with predators (DiMatteo 1982a; Nolen *et al.* 1995). After withdrawal and possibly turning of the body, the seahare begins escape locomotion, either galloping or swimming. Galloping was first distinguished from normal crawling in both its speed and saltatory style by Jahan-Parwar and Fredman (1979). The increased speed and the lack of continuous slime trail left by this type of locomotion increase the chances of escape from predators. Only the genera *Akera*, *Aplysia*, and *Notarchus* contain member species that are able to swim. Which species are capable of swimming is somewhat controversial (Johnson and Willows 1999), but for those that certainly do, swimming has been shown to be elicited by predatory attack (Martin 1966; Willan 1979; Nolen and Johnson 2001).

Active Chemical Defenses

The most dramatic behavior of the seahares is the release of bright purple fluid from the mantle cavity. This inking, is one of two active chemical defenses of seahares. Though these defenses are reviewed elsewhere (Johnson and Willows 1999), it is relevant to include that material here and to add additional information reported since that publication. Three key misconceptions in the literature are: 1) not all seahares have an ink gland or release ink; 2) inking is a high threshold all-or-none fixed act; and 3) opaline function and chemistry are described. I show below that these assumptions are false.

Ink Secretion

All seahares have ink glands (compiled from Eales 1921; Marcus and Marcus 1955; Morton and Holme 1955; Winkler 1957; Eales 1960; McCauley 1960; Kay 1964; Russell 1966; Bebbington 1969; Marcus 1972; Morton 1972; Bebbington 1974; Thompson 1976; Bebbington 1977; Willan and Morton 1984; Kamiya *et al.* 1989; Gosliner 1994). Both of the major reviews on *Aplysia* (Kandel 1979; Carefoot 1987) as well as research articles (e.g. Carew and Kandel 1977a) give the impression that some species do not possess ink glands. Seahares are capable of releasing either purple ink, white ink, or a combination of the two from different vesicles in the same gland (unpubl. data) and the release of black ink has also been reported (Eales 1960). One genus of seahare, *Dolabrifera*, seems to have lost the ability to produce ink of any kind, though it still possesses a vestigial ink gland (unpubl. data). The ink gland has been called by several different names, including "mantle gland," "purple gland," and "Blochmann's gland." This has caused some confusion in the literature but the primary reason that some species were thought not to release ink is because the opaline secretion, which is often white, was confused with white ink. Species that commonly release only white ink, such as *Aplysia vaccaria*, *A. juliana*, and *A. depilans*, were thus described as only capable of releasing opaline. It was not until 1989 that Kamiya *et al.* demonstrated that collection of white opaline and white ink was possible from the same species.

Ink gland

The ink gland is located near the mantle edge, partially beneath the tiny internal shell and suspended directly over the gill. The structure of the ink glands of *Aplysia californica* and *A. brasiliana* has been described by Prince *et al.* (1998). The glands are composed of three vesicle types (red-purple, amber, and clear) and two other cell types (rough endoplasmic reticulum (RER) cells and granulate cells) within a matrix of collagen and muscle (Prince *et al.* 1998). Most vesicles appear to have pores leading to the ventral surface of the gland. The contents of the vesicles are released when muscles around individual vesicles contract, squeezing the contents out and into the mantle cavity (Prince *et al.* 1998) where they are then directed by the siphon and pumped out of the mantle (Walters and Erickson 1986). The white ink gland of *Aplysia juliana* appears to be homologous to the purple ink gland (Kamiya *et al.* 1989) and it seems to have similar cell types to those found in the purple glands of other species (Prince and Johnson in prep).

Ink Chemistry and Chemical Ecology

The composition of purple ink has been examined by many authors (Christamanos 1955; Winkler 1959; Nishibori 1960; Chapman and Fox 1969; Troxler *et al.* 1981; MacColl *et al.* 1990; Paul and Pennings 1991; Prince *et al.* 1998). It is largely composed of red-algal derived pigments with the largest component being primarily phycoerythrobilin, a chromophore of the algal photosynthetic pigment phycoerythrin (Chapman and Fox 1969; MacColl *et al.* 1990; Prince *et al.* 1998). Purple ink of *Aplysia californica* was confirmed to be composed of 65% (dry mass) r-phycoerythrobilin (Troxler *et al.* 1981) but a large amount of proteinaceous material of unknown function has also found (Troxler *et al.* 1981; MacColl *et al.* 1990). Several biomedical studies have identified 60-70 kDa proteins in the ink of various seahares that have antibacterial (Kamiya *et al.* 1988; Kamiya *et al.* 1989; Yamazaki *et al.* 1990; Nistratova *et al.* 1993; Melo *et al.* 2000), cytolytic (Yamazaki *et al.* 1986; Yamazaki *et al.* 1989a; Yamazaki *et al.* 1989b; Yamazaki *et al.* 1990), haemagglutinating (Melo *et al.* 2000), and anti-heparin (Rajaganapathi and Kathiresan 2002) activities. Another biomedical study describes the cloning of a cytotoxic protein from the purple fluid of *Aplysia punctata*, but unfortunately misidentifies its origin as from the albumin gland

(Petzelt *et al.* 2002). Prince and colleagues (1998) have suggested that the protein component of ink may be produced in the RER cells of the ink gland and may be stored in the amber vesicles; more evidence from Prince and Johnson (in prep) suggests that this is the case. However, functions of these proteins for the seahares themselves have yet to be identified.

No study has shown algal secondary metabolites, a component of the passive defense of distasteful skin (Winkler 1969; Kinnel *et al.* 1979; Faulkner 1992; Rogers *et al.* 2000a), to be a component of ink. Winkler (1969) found no brominated compounds in either ink or opaline of *Aplysia californica*. Paul and Pennings (1991) also found no trace of malyngamides (secondary metabolites of cyanobacteria) in the ink of *Stylocheilus*. Additionally, Pennings *et al.* (1999) were unable to show any secondary metabolites in *Dolabella auricularia* ink though the ink was found to be unpalatable to some reef fishes. Though there is nothing yet published, Dr. Valerie Paul and I identified (using GC-mass spectroscopic analysis) algal secondary metabolites in the ink of *A. parvula*. Though secondary metabolites in the ink could play an independent or synergistic defensive role with other ink components, this has yet to be experimentally determined.

Only two studies have examined the composition of white ink. Schreiber (1932 cited in Winkler 1959) stated that the white ink of *Aplysia depilans* was a urobilinogen, closely related to purple ink, and that it was chemically distinct from opaline. The only other study to examine white ink found that it contained 0.211 mg/ml of protein (Kamiya *et al.* 1989).

Function(s) of Purple Ink

Many hypotheses have been proposed, and a few tested, for the roles of purple ink in the biology of (most) seahares. These include a) camouflage (Eales 1921, 1960); b) alarm signal (Walters *et al.* 1993); c) pheromone (Tobach *et al.* 1965; Fiorito and Gherardi 1990; Stopfer *et al.* 1993); d) aposematism (Ambrose *et al.* 1979); e) startle (Nolen *et al.* 1995); f) bile excretion (Chapman and Fox 1969; Tobach *et al.* 1989); g) anti-feedant (DiMatteo 1981, 1982b; Walters *et al.* 1993; Nolen *et al.* 1995); h) sensory irritant (Carefoot *et al.* 1999); and i) adventitious cue of danger (Johnson and Nolen 1991; Nolen *et al.* 1995). I will concentrate on (f-i) because they may hold most explanatory power for the functions of inking. For a general review of many of the proposed functions of purple ink, including an analysis of their potential likeliness, see Nolen *et al.* 1995.

Bile excretion

I include bile excretion, a non-defensive hypothesis, in my discussion not because this is a likely explanation for the current utility of the ink gland – it is not (Nolen *et al.* 1995), but because of its explanatory power in the evolution of the gland. Both Chapman and Fox (1969) and Tobach *et al.* (1989) suggest that the ink gland is primarily for processing and excretion of unwanted algal pigments. Though this hypothesis holds no experimental support for the current function of the ink gland, it is possible that it was the ancestral condition. The ink gland could have evolved first as an organ of bile excretion, a method of ridding the body of unwanted red algal pigments. If an animal happened to release its ink "waste" while under attack, and the predator found the ink aversive, there might be a strong and rapid selective pressure toward the co-option of this gland from an excretory organ to a defensive one. Such a change would involve evolution of neural control of ink release (Carew and Kandel 1977a) as well as mechanisms for directing it toward the site of attack (Walters and Erickson 1986).

Anti-feedant

There is a great deal of anecdotal and experimental evidence to support an anti-feedant function of ink. Willan (1979) found that the inking response of *Aplysia parvula* to attack by the seastar *Coscinasterias calamaria* caused the seastar to reduce its crawling rate while the seahare escaped. In a feeding experiment with gulls, pieces of baitfish injected with *A. dactylomela* ink were rejected at a rate of 94.9% (vs. 0.0% in controls), suggesting the ink to be highly distasteful (DiMatteo 1981). DiMatteo (1982) and Walters *et al.* (1993) also found that *Aplysia* ink (perhaps mixed with opaline) was aversive to crabs that would otherwise feed on the seahare tissue. Paul and Pennings (1991) showed that extracts from the ink of *Stylocheilus longicauda* at natural concentrations deterred feeding in reef fishes. Pennings (1994) found the ink of *A. kurodai* to deter feeding in the intertidal crab, *Hemigrapsis sanguineus*, while the ink of *Dolabella auricularia* did not deter feeding in portunid swimming crabs. Pennings and colleagues also found extracts of *D. auricularia* ink to deter most fishes at high concentrations and certain fishes at lower concentrations (Pennings *et al.* 1999).

To date, only one direct test of the survival value of ink has been performed (Nolen *et al.* 1995). In one part of this study, green-seaweed-fed *Aplysia californica* (they contained no red-algal derived passive or active defenses) were offered to the sympatric predator *Anthopleura xanthogrammica*, the great green sea anemone. The chemically depauperate seahares were placed on the oral disc of the anemones and then ink or a seawater control was applied to the tentacles. Seventy-one percent of the ink group survived the encounter while only 7% of the controls survived. The anemones also rejected their normal diet of whitefish when ink was present (50% ink vs. 10% seawater). Ink caused the anemone to recoil its tentacles and expel the contents of its gut.

Some authors provide anecdotal evidence that ink has no anti-feedant function (Leonard and Lukowiak 1986; Tobach *et al.* 1989). In both of these cases, however, direct tests of survival with and without ink were not conducted (e.g. Nolen *et al.*, 1995). Although these examples show that ink does not appear to be used against all predators (Leonard and Lukowiak 1986) nor is its use always effective (Tobach *et al.* 1989), they do not override evidence that ink functions as an anti-feedant (Willan 1979; DiMatteo 1981, 1982b; Paul and Pennings 1991; Walters *et al.* 1993; Pennings 1994; Nolen *et al.* 1995; Pennings *et al.* 1999).

Sensory irritant

The most recent hypothesis for a function for seahare ink is that it is a sensory irritant (Carefoot *et al.* 1999). As the authors state, this is not incompatible with other hypotheses of ink function, but rather begins to address mechanisms for the deterrent properties that have already been shown. Though this hypothesis has merit, the article in which it is raised actually presents little evidence to support it. All the studies are behavioral and thus could not directly show if the sensory system suffered some form of physical irritation. Though the work suggests that animals in a confined space respond to ink by changing their behavior, and in some cases even by dying after ink exposure, no sensory systems were actually investigated. It is also reasonable to suppose that some component in the ink is causing direct damage to cells of an organism as a mechanism of "sensory irritation," a term that the authors did not adequately define. With further work at the physiological, neuronal, and cellular levels, however, this hypothesis may be evaluated.

Adventitious cue of danger

Since seahares release ink in response to attack by predators, if a nearby conspecific had the ability to detect that ink and its direction, the detecting seahare might be able to protect itself from predation. Evidence suggests that juvenile (Johnson and Nolen 1991) and adult (Walters *et al.* 1993) *Aplysia californica* respond to ink by head withdrawal, turning, and locomoting away from the ink. If the head was completely covered in ink the animal remained stationary while waving its head as if to remove it from the ink (Johnson and Nolen 1991). Both behaviors could be advantageous to the seahare because a) in the first case it would be best to move away from the site of nearby predation and b) in the second case (particularly with anemone predation) it would be best to stay put until a safe direction was determined (Nolen *et al.* 1995). Similar results were found by Stopfer *et al.* (1993) with adult *A. californica* and by

Johnson (pers. obs.) with adult *A. brasiliana*. Though this ink avoidance response cannot be considered one of ink's functions, because the inking animal derives no fitness benefit (unless it can be shown that nearby conspecifics are also close relatives), it does add support to the hypothesis that ink serves an anti-predator role.

Function(s) of White Ink

To date, no research has been conducted on the function of white ink in the species that release it. The classic work by Flury (1915) on the "opaline" of *Aplysia depilans* and that of Pennings (1994) with *A. juliana*, both likely describe white ink instead, though they may have been contaminated by real opaline. It is likely that both authors referred to white ink because the white secretions, which they called opaline, were obtained simply by "irritating the animals" (Pennings 1994). According to Kamiya *et al.* (1989), who extracted both white ink and opaline from *A. juliana*, white ink is released more readily than opaline and would, therefore, have been a primary component in both the Flury and Pennings studies. Pennings (1994) found the white secretions to deter feeding in crabs and to cause avoidance behavior as well. Flury (1915) found that the white secretions when added dropwise to aquaria caused paralysis in a variety of organisms including cnidarians, annelids, echinoderms, molluscs, arthropods, and some fishes. It also caused paralysis followed by death when injected into frogs. When tasted by the author (!) it was bitter, burned, and later caused a sore scratchy throat (Flury 1915). Though both of these studies are suggestive of the capabilities of white ink, controlled experiments without possible opaline contamination are needed.

Neurophysiology and Neuroethology of Inking

The first papers on the neurophysiology of inking (Carew and Kandel 1977a, 1977b, 1977c) concluded that inking was a high-threshold, all-or-none fixed act under the control of three electrically coupled motoneurons. Recent work suggests that inking is not necessarily high threshold (Nolen and Johnson 2001), that it is not an all-or-none fixed act (Nolen and Johnson 2001) and that the identified cells may not in fact be ink motoneurons (Ross *et al.* 1995; Prince *et al.* 1998).

The results of Carew and Kandel (1977a; 1977b; 1977c) are more subtle and interesting than the phrase "high threshold, all-or-none fixed act" imply and it is surprising that these conclusions were made based on the data. Confusion begins with the term "threshold" and the fact that it can refer independently to both behavioral and cellular processes. Behavioral observations and experiments by Carew and Kandel (1977a) as well others (Carew and Kupfermann 1974; Leonard and Lukowiak 1986; Johnson 1994; Nolen and Johnson 2001) have shown that the behavioral threshold for ink release is not high, but in fact varies greatly. It may depend largely on fullness of the gland, which is red-seaweed diet dependent in the case of purple inking species (Nolen and Johnson 2001), or it may depend on other environmental factors such as calm vs. rough habitat (Kupfermann and Carew 1974). As it seems to be established that threshold for inking is variable, how did it become codified that inking was "high threshold"?

There are two answers to this question. The first, alluded to above, concerns a confusion of behavioral threshold with cellular threshold. Carew and Kandel (1977a) identified three putative motoneurons for ink release (L14A, L14B and L14C) within the abdominal ganglion of *Aplysia californica.* Byrne (1980) identified two more putative motoneurons (R19A and R19B) within the same ganglion, bringing the presently known total to five. These cells all show high resting potentials and high threshold for spike initiation (Carew and Kandel 1977a; Byrne 1980). So in this sense the *cells* may be called high threshold but this says nothing about the behavior. The second reason for calling inking high threshold concerns the type of stimulus used. In these neurophysiological experiments, focal electric shock was applied to the head or tail of the animal to induce inking. With this unnatural stimulus (see Nolen *et al.* 1995 for discussion), behavioral thresholds may sometimes be high. However, with more natural stimuli, e.g. anemone tentacles (Nolen *et al.* 1995), or weak stimuli distributed over a larger area of the body (Nolen and Johnson 2001), thresholds are often quite low.

The second characteristic of inking, its all-or-none property, is also ambiguous. In the original paper of Carew and Kandel (1977a), a behavioral experiment with 20 *A. californica* found that after a strong electric shock, 12 released most of their available ink (mean = 86%) while the other 8 only released a small amount of their available ink (mean = 2.8%). Thus 40% percent of the animals were capable of releasing a small amount of ink, i.e. not all-or-none, and yet only the behavior of the 12 that released all their ink were considered. In another experiment three additional animals were found to release small amounts of ink (less than 10% of available) and did so multiple times, i.e. not all-or-none (Carew and Kandel 1977a). For answers as to why "all-or-none" became fixed in the literature, we should again look to the nervous system.

None of the putative inking motoneurons show spontaneous spiking activity and all are electrically coupled to each other, though with very different coupling strengths (Carew and Kandel 1977a; Byrne 1980). The cells appear to innervate overlapping areas within the ink gland. Because of the electrical coupling, when one of the cells is artificially induced to fire, the others usually follow, causing most of the ink stores to be released. This electrical coupling of the neurons might suggest an all-or-none fixed act, but since *A. californica* is capable of releasing variable amounts of ink, then we must infer that the nervous system is capable of functioning in a more subtle way than an electrically coupled circuit might imply. Carew and Kandel (1977a, c) reported evidence of this. First, when the individual putative motoneurons were stimulated intracellularly, ink release occurred as a *graded* function of the number of spikes in the cell (Carew and Kandel 1977a). Though the cells often function together (because of the coupling), they can have different thresholds and can receive asynchronous synaptic input, which can result in independent activation of the cells (Carew and Kandel 1977a). Once again, the observed electrical coupling may offer an alternative interpretation. As shown by Getting (1974) and Getting and Willows (1973), electrical coupling in an interneuronal network results in inhibition of asynchronous inputs and promotes burst generation when inputs are spatially and temporally synchronous. Thus, this network feature may relate to sensory filtering for spatio-temporal synchrony rather than threshold.

Another way that the circuit may change is by altering the strength of neuron coupling. The fast and slow excitatory postsynaptic potentials (EPSPs) have opposite effects on the electrical coupling of the cells, with the fast EPSPs reducing coupling and the slow EPSPs increasing coupling. The amount of modulation also varies depending on the cells under consideration. For example, coupling between L14A and L14B may be varied only by about 10%; however, coupling between either of those cells and L14C can vary by two to four times (Carew and Kandel 1977c). All these possibilities for circuit modulation unfortunately seem overlooked in the summarizing phrase "all-or-none fixed act".

In all, a seahare's ability to vary the amount of defensive chemicals released depending on the severity of the predatory threat may have adaptive value. Nolen and Johnson (2001) suggest that since ink is a valuable resource (e.g. an effective antifeedant against anemone predation) that is only capable of being replenished from empty after three days of feeding on red-seaweeds, the animals are likely to use it "wisely." This would explain the increased thresholds in rough environments (Carew and Kupfermann 1974) (to prevent accidental release), the often high thresholds seen to unnatural stimuli (focal electric shock), and the low thresholds seen toward a natural predator (Winkler and Tilton 1962) to which ink is an effective deterrent (Nolen *et al.* 1995).

As for the five identified cells implicated in the release of ink, it appears that at least three of them (and maybe the other two) may not actually be motoneurons. Prince and colleagues (1998) found that the intact gland, as well as individually isolated ink vesicles, release in response to the neurotransmitter acetylcholine. The L14 cells, however, are not cholinergic (Koester and Kandel 1977). This suggests that there may be unidentified ink motoneurons either in the central nervous system or in the periphery (Prince *et al.* 1998). Furthermore, inking in response to mechanical stimuli is still possible after lesioning of the peripheral nerves containing the L14 axons (Ross *et al.* 1995). As for the two additional putative motoneurons (Byrne 1980), the case is still open.

Opaline Secretion

The opaline gland is found only in the seahares and is found in every species within the order (compiled from MacFarland 1918; Eales 1944; Macnae 1955; Morton and Holme 1955; Eales 1960; McCauley 1960; Kay 1964; Beeman 1968; Bebbington 1969; Marcus 1972; Morton 1972; Bebbington 1974; Thompson 1976; Bebbington 1977; Willan and Morton 1984; Gosliner 1994). Some confusion has arisen, however, because members of the family Runcinidae (Opisthobranchia: Cephalaspidea) have also been reported to have an opaline gland (Burn 1963; Ghiselin 1963; Miller and Rudman 1968). It is now clear that the runcinid gland is not homologous to the opaline gland of the Anaspidea (Hoffmann 1935; Marcus and Marcus 1970) and it has therefore been proposed by Gosliner (1994) that the runcinid gland be referred to as the "pallial gland." Like the ink gland, the true opaline gland has also been called by several different names (e.g. gland of Bohadsch, poison gland, grape-shaped gland (Eales 1921), and hypobranchial gland (Marcus 1972)). However, "opaline gland" is the accepted term today (Gosliner 1994).

Opaline gland

The opaline gland is located beneath the floor of the mantle cavity and releases into the mantle cavity by one to many openings (Eales 1960). Opaline is released less readily and in smaller quantities than either white or purple ink (Johnston 1850; Eales 1921; Kamiya *et al.* 1989). Furthermore, unlike the ink gland, which is capable of releasing at least two colors of secretion (depending on the species), little color variation has been reported for the opaline gland. The gland morphology, however, can be quite different between species. Most seahares, including all the genera other than *Aplysia* (*Akera, Dolabella, Dolabrifera, Petalifera, Phyllaplysia, Notarchus, Stylocheilus,* and *Bursatella*), have only one opaline gland morphology. It consists of many large vesicles, each with individual openings to the floor of the mantle cavity (Gosliner 1994). This form has been called "simple-multiporous" (Eales 1960). It is also found within most members of the genus *Aplysia*, however, two other morphologies have been described. In 10 "compound-uniporous" species, all of vesicle ducts have fused and release through one large, common opening in the floor of the mantle cavity (Eales 1960). This type of opaline gland looks very much like a (very small) bunch of grapes suspended from their common duct leading to the floor of the mantle cavity. The other morphology appears to be transitional between the first two forms. Some of the vesicles have fused together and release through a common opening, while others still release individually. For this reason this morphological form is called "transitional" (Eales 1960).

Opaline Chemistry and Chemical Ecology

Aside from the fact that little is known of opaline chemistry and chemical ecology, some of what is in "known" in the literature is likely erroneous. As mentioned in the Ink section, white ink (released by only a few species) has often been confused with opaline because of the similarity in color. In Carefoot's 1987 review of the genus *Aplysia*, Flury (1915) and Ando (1952) are cited as the only authors to have investigated the toxicity of opaline. Unfortunately, neither of them can be said to have done so with confidence. As mentioned in the ink section, Flury likely studied white ink since he only collected the white secretion released after irritating *Aplysia depilans*. Since white ink is released more readily than opaline (Kamiya *et al.* 1989), the "opaline" studies of Flury (1915) as well as those in Pennings (1994), where the animals were simply irritated to release their white secretions, cannot be properly evaluated. The secretions were not collected directly from the intended glands. The statement in Carefoot (1987) about Ando must be from a mistranslation from the original Japanese, for though Ando mentioned opaline, his only experiments were on an oily extract of the red seaweed *Laurencia nipponica* which he injected into various organisms (Ando 1952). Because Ando's seaweed extraction caused symptoms similar to those described by Flury's "opaline" injections, he hypothesized that opaline was derived from red-algal components (Ando 1952).

The first work on opaline appears to be by Schreiber (1932 cited in Winkler 1959). Using paper chromatography he found the mucilaginous secretion to be very proteinaceous and did not see that it was related to either purple or white ink, which did appear to be related to each other (Schreiber 1932 cited in Winkler 1959). Winkler (1957) also found opaline so viscous that he was able to stretch a thread of it for approximately 5 m through the air. In a later study, Winkler (1969) found that the opaline gland, like the ink gland, was free from any red-algal brominated compounds that had been detected in the skin. This however seems to be in dispute. When opaline glands of *Aplysia punctata* were extracted with methanol followed by hexane, three major and 4 minor peaks were found - all of which were brominated (Quiñoa *et al.* 1989). Secondary metabolites palisadin A and palisadin B, derived from a diet of *Laurencia obtusa*, have also been found in the opaline of *Aplysia parvula* (Rogers *et al.* 2000a). This is the first published report of secondary metabolites from either seahare defensive secretion. Though not a natural method of delivery, when injected into crabs, the opaline secretion of *Aplysia juliana* causes paralysis followed by death (Kamiya *et al.* 1989), suggesting that it may contain some neurotoxic substances.

Function(s) of Opaline

Few hypotheses have been proposed (and none tested) for the role of opaline in the biology of seahares. Unlike purple ink, diet does not appear to be a factor in opaline production (Nolen *et al.* 1995). Kupfermann and Carew (1974) saw *Aplysia californica* release opaline in response to a sea anemone in the field after it was placed on the tentacles by the experimenters, but the seahare was ultimately ingested. The only other reference relating to the natural function of opaline appears to be an unpublished observation by Kittredge (Kandel 1979) that opaline inhibits feeding in crabs. This is an area of seahare biology in need of further research.

Neurophysiology and Neuroethology of Opaline Release

MacFarland described innervation of the opaline gland for several species of *Aplysia* (MacFarland 1909) and for *Dolabella* (MacFarland 1918). It is clear from his work (and earlier work that he cites) that in some species the opaline gland is innervated by both the pedal and abdominal ganglia and in other species the pedal ganglion only. Hoffman (Kandel 1979) suggests that the pedal innervation is ancestral, however no relevant comparative studies have been reported. Ironically the motoneurons responsible for release of opaline reside in neither the pedal nor the abdominal ganglion but are instead found in the right pleural ganglion and send processes through pedal nerve P5 (Tritt and Byrne 1980).

Tritt and Byrne (1980) identified three motoneurons in the right pleural ganglion of *Aplysia californica*, designated PLR1, PLR2, and PLR3. Like the putative ink motoneurons, they were found to have high resting potentials, no spontaneous spiking, and to be electrically coupled to one another (Tritt and Byrne 1980). The gland showed a graded release of opaline when the cells were firing at a low rate and the authors made no claim that opaline release was an all-or-none fixed act. This and the subsequent paper (Tritt and Byrne 1982) that showed dopamine to be the most likely transmitter of the motoneurons are the only neurophysiological studies to date on mechanisms of opaline release. No studies have been conducted on behavioral implications of opaline release. Like most areas of the opaline system, this is also ripe for experimental investigation.

In summary, the seahares show a range of both passive and active chemical defenses in addition to non-chemical strategies. With such a multi-component arsenal at their disposal, the anaspids are capable of defending themselves against a variety of predatory threats that they encounter. The mechanisms by which these defenses function against the various predators as well as which defenses are chosen depending on context are areas largely unexplored. This dissertation aims to address these issues.

Chapter III. Experiments with Cnidarian Predators

Introduction

Large, carnivorous sea anemones are perhaps the best-known predator of seahares (Johnson and Willows 1999). They often share the same intertidal habitats and it is not uncommon for seahares to blunder into their tentacles (pers. obs.). It has also been established that ink functions as an effective anti-feedant with at least one seahare/sea anemone pair. Nolen et. al (1995) showed that the ink of *Aplysia californica* caused the tentacles of *Anthopleura xanthogrammica* to shrivel and its gastrovascular cavity to evert, expelling its contents. This rejection behavior was shown both with live seahares present and with palatable pieces of fish after both had been coated with ink. The active component of ink that produces this response has not been determined. A survey of the biomedical literature on seahares suggests a possibility.

A number of antibacterial and cytolytic proteins have been isolated from seahares (Kamiya and Shimizu 1981; Yamazaki *et al.* 1984; 1985; Kamiya *et al.* 1986; Kisugi *et al.* 1987; Kamiya and Yamazaki 1989; 1989), including several specifically from the ink of various species (Yamazaki *et al.* 1986; Kamiya *et al.* 1988; 1989; 1989a; 1989b; 1990; Melo *et al.* 1998; 2000). For this reason I decided to analyze the ink of *Aplysia californica* for similar proteins and activity. Upon finding a dominant protein in the size range from other species in the literature, I proceeded to determine its origin within the gland, isolate and purify it for further study, clone the gene responsible, analyze it for antibacterial effects, and evaluate its effect on sea anemone predators.

Materials and Methods

Animals and Animal Care. All species used are sympatric, normally living in intertidal and subtidal waters off the west coast of the USA. Using sympatric species is important to identify ecologically relevant predator-prey interactions. Seahares (*Aplysia californica*) and their primary red-algal diet of *Gracilaria tikvahiae* were provided by the NIH National Resource for *Aplysia* in Key Biscayne, Florida. Sea anemones, *Anthopleura xanthogrammica* and *A. eligantissima*, were provided by Marinus Inc. and Russell Wyeth of Friday Harbor Laboratories, respectively. Animals were maintained at Georgia State University in separate re-circulating, artificial seawater tanks.

Collection of Pure Secretions. Pure ink and opaline secretions from *A. californica* were collected and processed using established procedures (Nolen *et al.* 1995) along with modifications described below. Seahares were anesthetized by chilling in seawater at 4°C for 1 hr. They were then injected with an isotonic solution of magnesium chloride to arrest neural conduction and thus prevent inking. Glands were removed by dissection and transferred either to a Petri dish in the case of the ink gland or an ultracentrifuge tube in the case of the opaline gland.

Ink gland processing. The ink glands were gently squeezed in a Petri dish with the blunt end of a scalpel handle to release most of the ink secretion from the individual vesicles contained in the gland. The secretion was then pipetted to a microfuge tube and frozen at –80°C until needed.

Opaline gland processing. The opaline glands were gently blotted dry on a Kimwipe® to remove seawater (since water causes opaline secretion to polymerize) and transferred to an ultracentrifuge tube. This was centrifuged at 30,000 g for 30 min at 4°C (Beckman Optima TLX Ultracentrifuge). The pure opaline was then pipetted away from the gland tissue, transferred to a microfuge tube, and frozen at –80°C until needed.

Whole Sea Anemone Assay. Carnivorous sea anemones, *Anthopleura elegantissima*, were used to assay secretions for activity. Ink samples were divided into two equal fractions and one fraction heated to 80°C for 15 min and then allowed to return to room temperature. Both fractions were relabeled by another lab member so that the experimenter was blind to the treatment. Each sea anemone (n=18) received one fraction presented in random order. The opposite treatment was present during a second trial in order to examine within individual variation in the sea anemones. 50 µl of test solution was delivered by micropipette to the tentacles of sea anemones and aversive or feeding responses noted. Aversive responses involve contraction and shriveling of the tentacles while feeding responses involve movement of the tentacles toward the mouth and opening of the mouth (Nolen *et al.* 1995; unpubl. data). Tentacle contraction and shriveling was scored on a scale of 0 to 3 with 0 being no contraction, 1 weak, 2 medium, and 3 strong contraction. A substance was scored as stimulatory if tentacles moved toward the mouth and the mouth opened.

Sea Anemone Tentacle Assay. *Anthopleura xanthogrammica* was used in all tentacle assays to examine potential cytolytic effects of ink and ink proteins. The first assay involved removing the tentacle tips from *A. xanthogrammica* with scissors and incubating those tips at 18°C for 18 hr in several different solutions. The tips were placed in individual wells of a 96-well plate with 40 μl of the following solutions: unheated pure ink, ink that had been heated (15 min @ 80°C), pure opaline, and artificial seawater (as a control). There were eight replicates for each treatment. The next day, tentacle tips were removed from the wells, rinsed in artificial seawater, placed on a slide with coverslip, and examined under an Axoplan 2 compound microscope (Zeiss) with Nomarsky optics. Images were captured at low and high magnification. The second tentacle assay was the same as the first except that the experimental treatments were different. They included purified native ink protein, over-expressed ink protein from *E. coli*, bovine serum albumin (BSA) as a protein control, and artificial seawater as a standard control. Additionally, these tentacles were examined under green fluorescence with an Axoscop compound microscope (Zeiss).

Isolation of Proteins from Secretions. Proteins were isolated and purified using an ÄKTA 100 Automated FPLC (Amersham Pharmacia Biotech). A preparative grade Hi-Load Superdex 200 16/60 column (Pharmacia) or an in-house made Sephacryl 300 HR 26/60 column was used for initial size separation with fractions collected in an automated fraction collector. Fractions identified to have activity by bacterial assay (see below) were concentrated using a Biomax 5K NMWL membrane Ultrafree Centrifugal Filter Device (Millipore). Active fractions were further purified on a cation exchange Mono S column, and fractions were collected, assayed, concentrated, and frozen at –80°C.

Antibacterial Assay. *Escherichia coli* (strain JM109) was used to assay antibacterial activity throughout the protein separation process. Bacteria were plated on Petri dishes containing LB media (1 L = 950 ml H_20, 10 g bacto-tryptone, 5 g bacto-yeast extract, 10 g NaCl). Microliter quantities of the sample fractions and negative (buffer alone) and positive (pure ink) controls were placed on the bacterial lawn, and the dishes were incubated overnight at 37°C. If bacterial growth was inhibited, it was expressed as a clear spot on the plate while the rest of the plate had a bacterial lawn. Plates were scanned with a Calibrated Imaging Densitometer GS-710 scanner (Biorad) for data storage.

Gene Cloning

Protein Sequencing. Pure ink samples from *Aplysia californica* were analyzed for dominant protein bands using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 12% gels. A single dominant protein band was found at about 61,000 Daltons. This band was blotted from the polyacrylamide gel to a PVDF membrane and sent to Dr. John Leszyk at the University of Massachusetts Medical School Laboratory for protein microsequencing and proteomic mass spectrometry. After identifying peptide fragments from the ink protein, a BLAST search was conducted to find homologous protein fragments.

mRNA Extraction and RT-PCR. One hour prior to dissection, the seahares were handled to induce release of ink in order to stimulate potential upregulation of mRNA following depletion of the ink protein. In order to isolate mRNA from *A. californica ink* glands without degradation of mRNA, the animals were dissected in MgCl/ DEPC solution. The dissections were conducted in a 4°C cold room and the glands were immediately transferred to liquid N_2. Glands were ground with a mortar and pestle while still in liquid N_2. This material was transferred to an RNAse-free tube and mRNA isolated following the manufacturer's protocol (Roche mRNA Isolation Kit, Cat. No.1-741-985). Primers were designed based on the results of homologous sequences recovered following the BLAST search (listed in the results). Primers were synthesized at the Georgia State University Core Facility. Primers were used for RT-PCR following the manufacturer's protocol (Roche Titan One Tube RT-PCR System, Best Nr.1-888-382) using an Eppendorf Mastercylcer Gradient thermocycler. The resultant RT-PCR product was then cloned in a pGEM T-vector (Promega), amplified, and sequenced. Sequencing was conducted at the Georgia State University Core Facility. This sequence was verified by alignment with homologous sequences from GenBank using MacVector 6.5.3 (Oxford Molecular).

5'/3' Rapid Amplification of cDNA Ends (RACE) PCR. 5'/3' RACE PCR was conducted to complete the cDNA clone of the ink protein after RT-PCR. 1 μg mRNA was used with the 5'/3' RACE Kit (Roche Cat. No. 1-734-792) and the manufacturer's protocol was followed. Three specific primers were designed from the original RT-PCR isolated fragment and used in 5'RACE: one for first strand cDNA synthesis (SP1=GTTCACGTCGGGTGTGTTGGGCAGC), one for dA-tailed cDNA amplification (SP2=TGGTAGGTGAACAGACGGCC), and one for nesting PCR (SP3=CCCGGTCGCAGAACTCGAAA). A final primer was needed for the 3' RACE reaction (SP6=ATCTACACCCTGGAGGAAGG). All PCR products were analyzed on a 1% agarose gel. PCR products that appear to be of appropriate size were subcloned into pGEM-T vector (Promega) and sequenced as described above.

Over-Expression of the Ink Protein. Primers were designed to amplify the whole coding sequence so that the protein could be over-expressed in an *E. coli* expression system. The 5' primer included a *Bam*HI restriction site to allow in-frame insertion into the amplification and expression vectors (5'GGATCCCATGTCGTCTGCTTTCCTTC3'). The 3' end included an extra *Hin*dIII restriction site (5'AAGCTTGAGGAAGTAGTCGTTGATGA3'). PCR was conducted using Expand High Fidelity PCR System (Roche), the resultant whole gene fragment of expected size was cloned into pGEM-T vector (Promega), and the plasmids were amplified. The plasmids were then cut with *Bam*HI and *Hin*dIII and the gene subcloned into the pET-20b expression vector (Invitrogen) using the same enzymes. The sequence was confirmed before beginning over-expression.

For over-expression, the plasmid was transformed in to *E. coli* strain BL21λDE3. Twenty-six liters of these cells were grown in LB media in a Pilot Plant Fermentor (New Brunswick Scientific) at 37°C until reaching an OD_{600} of 0.5 at which point they were induced with 0.5 mM IPTG for 2 hr. The cells were harvested, concentrated by centrifugation (5,000g @ 4°C), resuspended in 0.1 M PPB containing 1mM PMSF protease inhibitor and broken on a French pressure cell (Sim-Aminco) at 16,000 psi. The resultant mixture was centrifuged at 40,000 rpm for 1 hr in a Beckman Coulter Optima XL-100K ultracentrifuge. SDS-PAGE was used to confirm the location of the ink protein. Since the protein formed an inclusion body and was found in the pellet, it was first dissolved in denaturing buffer (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-Cl, pH 8.0) and loaded onto an anion exchange column, Mono Q 10/10 (Pharmacia) using 8 M Urea, 20 mM PPB, and 1 mM DTT as the A buffer and the same buffer plus 1 M NaCl (B buffer) to elute the protein. The protein was again confirmed by size using SDS-PAGE and the resultant band sent to Emory University School of Medicine Microchemical Facility for MALDI-TOF MS analysis to verify the identity of the expressed protein.

Ink Protein Concentration. In pilot studies, the purple pigment in pure ink interfered with spectrophotometric readings used in the standard Bradford assay of protein concentration. For this reason, ink protein content was approximated by SDS-PAGE of ink samples. Three known quantities of BSA were used to calibrate a Calibrated Imaging Densitometer GS-710 (Biorad). Protein concentration measurements established ecologically acceptable levels of escapin for the sea anemone tentacle assay.

Results

Does a Protein in the Ink of *Aplysia californica* Cause Aversive Reactions in Sea Anemones?

The ink *of Aplysia californica* has a powerful effect on carnivorous sea anemones (Nolen *et al.* 1995). Ink causes tentacles to shrivel and the gastrovascular cavity to evert, expelling its contents. I hypothesized that if a protein was responsible for these reactions, then heating the ink and thus denaturing any proteins should eliminate the response. The results of the whole sea anemone assay suggest that significantly fewer anemones showed strong tentacle contractions in response to heated ink (Fig. 3.1). This suggested but did not confirm the possibility that a protein was responsible because other compounds in the ink could also have been affected by heating. If, however, I had seen no difference in the two groups, I would have been able to safely eliminate a protein as the causative agent. At this point a protein was still a likely suspect.

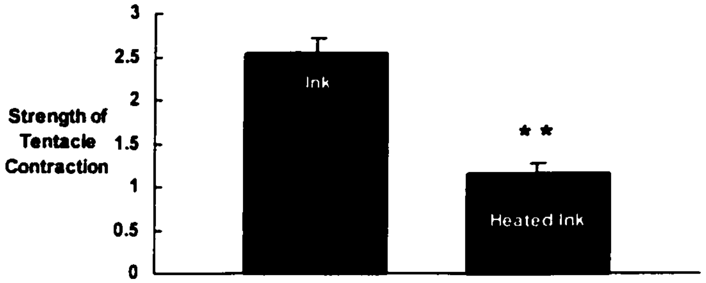

Figure 3.1 Heated Ink is Less Aversive to Sea Anemones. In a blind paired test, sea anemones, *Anthopleura eligantissima*, responded with significantly lower amounts of tentacle contraction to ink that had been heated to 80°C for 15 min than to unheated ink (Wilcoxon Matched Pairs, z=3.1798, p=0.0015).

What Proteins are Found in the Ink of *Aplysia californica*?

With a protein now as a possible candidate, I used SDS-PAGE to identify any dominant bands that might correspond to those already known from different species of seahares. The results showed that indeed there was one dominant protein of about 60 kDa (Fig. 3.2). This was in the size range of proteins reported in the ink of other seahares (Yamazaki 1993). I wanted then to determine where in the ink gland this protein might be packaged.

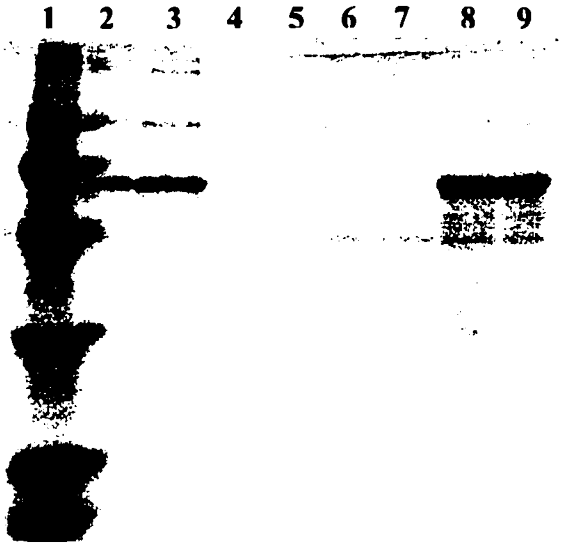

Figure 3.2 Dominant Ink Protein Localized in Amber Vesicles. SDS-PAGE showing the dominant ink protein of *Aplysia californica* and its location within the gland. **Lane 1**: Standards – 97, 68, 29, 18, & 14 kDa; **Lanes 2-3**: pure ink showing major band at ~ 60 kDa; **Lanes 4-7**: purple vesicles showing no major band; **Lanes 8-9**: amber vesicles showing major band at ~ 60 kDa.

In *Aplysia californica* the ink gland contains mostly purple vesicles but also a large population of amber-colored vesicles (Prince *et al.* 1998). The function of these amber colored vesicles had not been determined, though Prince and colleagues hypothesized that they might be for protein storage. I used microdissection to remove intact purple and amber vesicles, and was able to test pure vesicles on SDS-PAGE. The protein appeared only in the amber vesicles (Fig. 3.2). TEM micrographs taken at about the same time by my collaborator, Jeff Prince of the University of Miami, showed what looked like protein crystals in the amber vesicles of *A. californica*.

Since all of the identified *Aplysia* ink proteins to date show antibacterial properties, I conducted a bacterial growth assay to look for antibacterial effects. The results (Fig. 3.3) showed that pure ink of *A. californica* inhibited growth. This appears as a clear circle on the bacterial lawn, but is difficult to see in the figure because the ink pigment obscures the lack of bacterial growth. The pure opaline secretion, on the other hand, enhanced bacterial growth as shown in Fig. 3.3 by a much thicker lawn beneath the opaline spot. The inhibitory effect was also seen from the contents of the amber vesicles of the ink gland but not the purple vesicles, confirming the results from SDS-PAGE and further suggesting that the dominant 60 kDa protein was likely responsible. The ink of *Aplysia californica* inhibited the growth of several types of bacteria including both Gram-negative and Gram-positive species. In addition to *E. coli*, the growth of Gram-negatives *Pseudomonas* sp. and *Salmonella* sp. as well as Gram-positives *Bacillus subtilus* and *Streptococcus* sp. was inhibited by the ink. This antibacterial effect has also been shown with the ink proteins from other species of seahare (Yamazaki 1993).

Purification of the Ink Protein

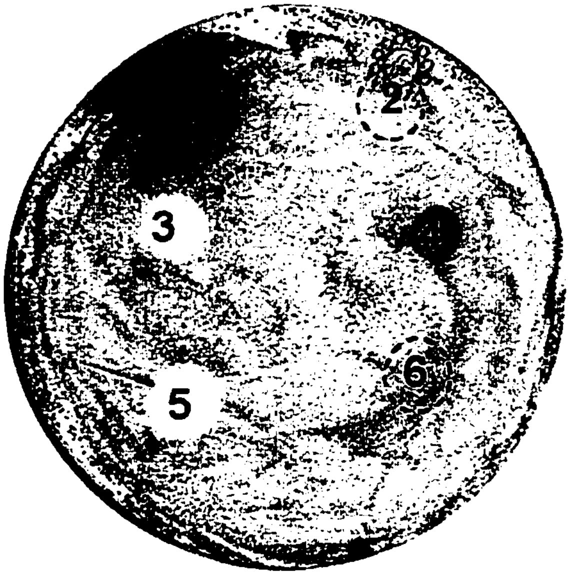

Figure 3.3 Antibacterial Assay. After applying a bacterial lawn to an LB coated plate, 5 µl samples were spotted on the plate and the plate was incubated overnight at 37°C. Clear spots on the plate represent antibacterial activity and are seen only for 1, 3, and 5. Spot **1**: pure ink; **2**: buffer control; **3**: purple & amber vesicles together; **4**: opaline; **5**: amber vesicles alone; **6**: purple vesicles alone.

To determine if the dominant protein was indeed responsible for the antibacterial activity as well as the response of sea anemones, I isolated and purified the protein from the crude ink secretion. Early attempts to separate the protein from the algal pigments in the ink, including salting-out the protein with ammonium sulfate and spin filtration through molecular weight cut-off membranes, failed. The algal pigments, though only a few hundred Daltons in size, seemed to show a strong affinity for the protein and were not easily separated. I began modifying a published 25-step separation protocol (Melo *et al.* 2000) that had been used for the protein in the ink of *A. dactylomela* and was ultimately able to reduce the number of steps to five using two Fast Protein Liquid Chromatography (FPLC) columns. Pure ink, collected as described above, was loaded directly onto either a Hi-load Superdex 200 16/60 (Pharmacia) or a Sephacryl 300 HR column made in the lab for size separation. This larger Sephacryl column could support the loading of 13ml of ink in a single run. The proteins eluted before most of the pigments and thus permitted separation of the two. The fractions were analyzed both with SDS-PAGE and the antibacterial assay to determine which peaks contained the protein. Once the appropriate peak from the FPLC had been identified it could be isolated in all subsequent runs. The active fractions were mixed together and concentrated using Biomax 5K Centrifugal Filter Devices (Millipore) and then re-diluted in buffer and loaded onto the second column, the Mono S 5/5 (Pharmacia). Mono S is a cation exchange column that has a high affinity for proteins with low pKa values. The pKa for *A. californica* ink protein was estimated to be 5.08 (described below in the gene cloning section) and thus tightly bound to the column. This produced a clean peak on the FPLC and a clean single band on SDS-PAGE. Antibacterial assays confirmed that this was indeed the active protein in *A. californica* ink. After purification, the native protein appeared to be pigmented. It had the same amber color of the vesicles from which it was located in the ink gland.

Gene Cloning

Purification requires a large number of animals because one adult *A. californica* (~200 g) produces about 0.5 – 0.7 ml of ink. Additionally, since this protein may underlie defense of seahares and could have biomedical relevance, I tried to clone it. I sent ink protein isolated from *A. californica* ink to Dr. John Leszyk at the University of Massachusetts for sequencing. One internal sequence appeared to be novel (ESGLDIAVFEYSDR), but another internal sequence of 15 amino acids (VFMTFDQPWWLQNER) matched exactly the first 13 of an internal sequence of cyplasin L and S that were submitted to NCBI in December 2000 (Accession numbers:

CAC19362 and AJ304801). The cyplasins, isolated from a European species of seahare, *Aplysia punctata*, were reported to be antibacterial and cytolytic. At the time, only the sequence had been released and the publication did not come out until February 2002. I contacted the author and found the cyplasin proteins had come from the purple ink of *A. punctata*. With this information I designed six primers for RT-PCR from the Cyplasin L sequence, including four 5' primers: 1) TTCGAGTTCTGCGACCGGGT, 2) TCATGSAAGTGGACTGGCCC, 3) TGACCTTTGACCAGCCTTGG, 4) AGACGGTGGTTGCACGTTGT, and two 3' primers: 1) CCAAGGCTGGTCAAAGGTCA and 2) AGTCCCCAGAAACGTTGGAC. I also designed two degenerate 5' primers from the *A. californica* ink protein sequence: CGIAAC/TCGIGAC/TCAG and GAG/ATCIGGICTIGAC/TATA/C/TGC. The RT-PCR product after cloning, amplification, and sequencing produced one fragment of 955 bp that was aligned with cyplasin L. 5'/3' Rapid Amplification of cDNA Ends (RACE) PCR produced 5' and 3' fragments that completed the gene.

Aplysia Ink Protein Sequence

The complete sequence contains 1605 base pairs, coding for 535 amino acids with a total molecular weight of 60,300 Daltons (Fig. 3.4). A BLAST search found that amino acid identity was 61% for cyplasin L, and 58% for cyplasin S. The new *A. californica* ink protein also showed 61% identity with another antibacterial protein from a seahare, this one from aplysianin-A precursor of *Aplysia kurodai* (Accession # Q17043). It also had a 48% identity with achacin precursor, an antibacterial protein from the giant African land snail *Achatina fulica* (Accession # P35903). Since I believed that this newly discovered protein from the ink of *Aplysia californica* was likely responsible for the defensive features of ink, I chose to call it "escapin."

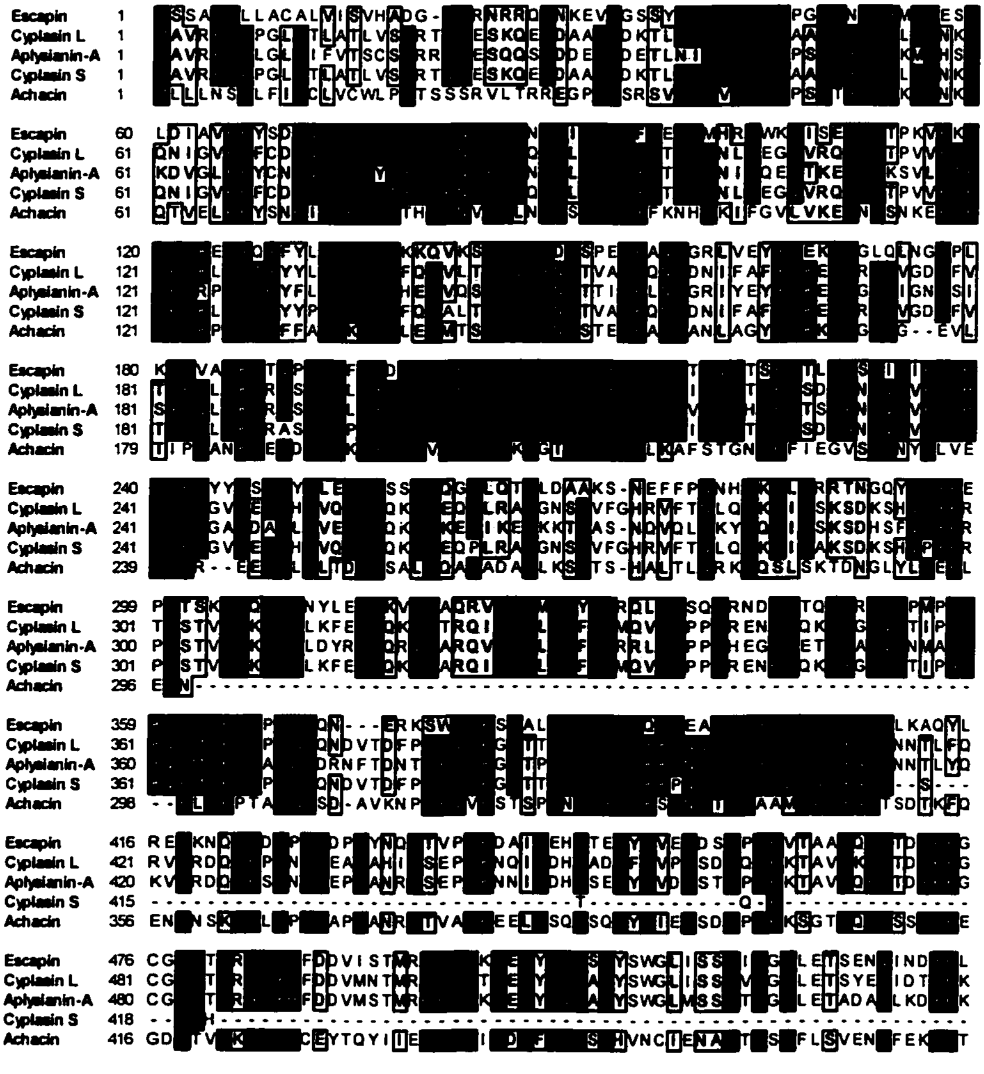

Figure 3.4 Amino Acid Alignment of Escapin and Related Sequences. Escapin, the newly sequenced protein from ink gland of *Aplysia californica*, was aligned using Clustal W with four sequences with which it shares greatest sequence identity. They are cyplasin L (CAC19362), from *Aplysia punctata*; aplysianin-A precursor (Q17043), from *A. kurodai*; cyplasin S (CAC19361), from *A. punctata*; and achacin precursor (P35903), an antibacterial protein from the giant African land snail, *Achatina fulica*.

Over-Expressing Escapin in a Prokaryotic System

Escapin was over-expressed in *E. coli* (BL21λDE3) and isolated by Mono Q column (Fig. 3.5). The molecular weight of expressed escapin was smaller than native escapin, suggesting that the native protein might have post-translational modifications that would not be possible to produce in a prokaryotic expression system. This is perhaps why the expressed escapin appeared to have lost any antibacterial activity. It has been shown for other seahare antibacterial proteins that glycosylation is important in the functioning of the protein (Yamazaki 1993).

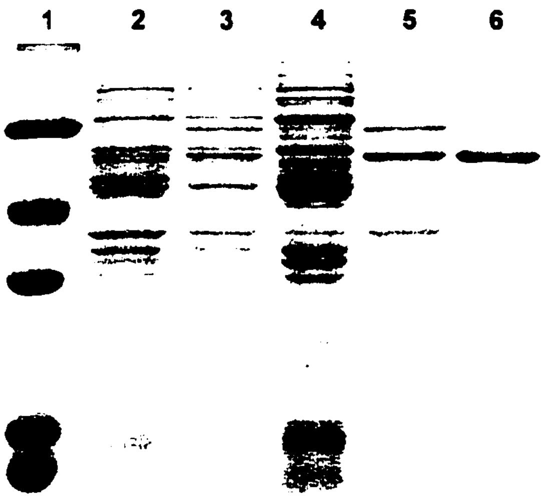

Figure 3.5 Over-Expression of Escapin in *E. coli*. Lane 1: Standards – 68, 45, 29, 18, & 14 kDa; 2: uninduced; 3: IPTG induced; 4: cell-free extract of 3; 5: pellet of 3; 6: after Mono Q purification – showing predominant 60 kDa protein.

Is Escapin Responsible for the Sea Anemone Responses?

While ink is denser than seawater and also slightly viscous, the isolated protein alone is not. Thus escapin can't be used directly in behavioral experiments with sea anemones. Mixing the protein into solutions of methylcellulose did not produce a consistency similar enough to ink. Since FPLC-purified escapin is valuable, I tried to examine its effects directly on isolated tentacles. If the protein had cytolytic effects, as had been reported for cyplasin and other seahare ink proteins used against tumor cells, then it was possible that it would cause direct damage to isolated anemone tentacles observed under a compound microscope. I incubated the tentacles for 18 hr with test solutions to maximize the chances of seeing any effect. The first set of experiments was conducted with 4 treatments: pure ink, heated ink (15 min @ 80°C), pure opaline, and artificial seawater (ASW). Samples that were incubated in ASW, opaline, and heated ink showed little damage (Fig. 3.6), while those in pure unheated ink showed two features that set them apart: 1) the outer layer of cells of the tentacles containing the nematocysts was largely gone (Fig. 3.7), and 2) most of the zooxanthellae were expelled (Fig. 3.7 and Fig. 3.8 C) with many appearing ruptured (Fig. 3.8 D). This experiment showed that ink did cause direct damages to tentacles if left in contact long enough and that heating of the ink destroyed this activity.

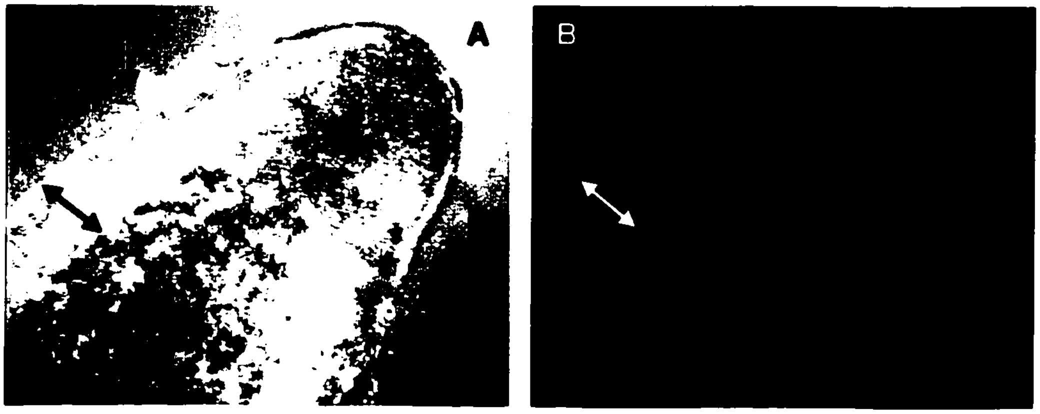

Figure 3.6 Tentacles Incubated in Artificial Seawater. Cut tentacle tip from *Anthopleura xanthogrammica*, incubated 18 hr in 40 µl of ASW as a control. **A**: Tentacle under Nomarsky optics showing a dark inner layer with symbiotic zooxanthellae and an outer layer (double arrow) of sea anemone cells. Some discharged nematocysts are also visible (single arrow). **B**: Tentacle under green fluorescence showing only the autofluorescent zooxanthellae. The red zooxanthellae give a clear outline of the inside of the tentacle.

The second experiment was only possible after I had expressed escapin in *E. coli*. Since this protein was identical in amino acid structure yet lacked the post-translational modifications of natural escapin, it made a useful control. In addition to the two escapins, I also used BSA as another protein control. Each protein was mixed into a solution of artificial seawater at a concentration near the natural concentration of escapin found in *A. californica* ink (1 mg/ml). Tentacle tips from *A. xanthogrammica* were again used (10 replicates for each treatment). The experiment was exactly as described above for heated/unheated ink treatments, but in addition the samples were analyzed with fluorescence microscopy since it was found that the symbiotic zooxanthellae were autofluorescent when excited by green light. All tentacles that had been incubated in native escapin showed moderate to severe damage to themselves and their symbionts (Figs.3.7 C and 3.8 D) as had those incubated in

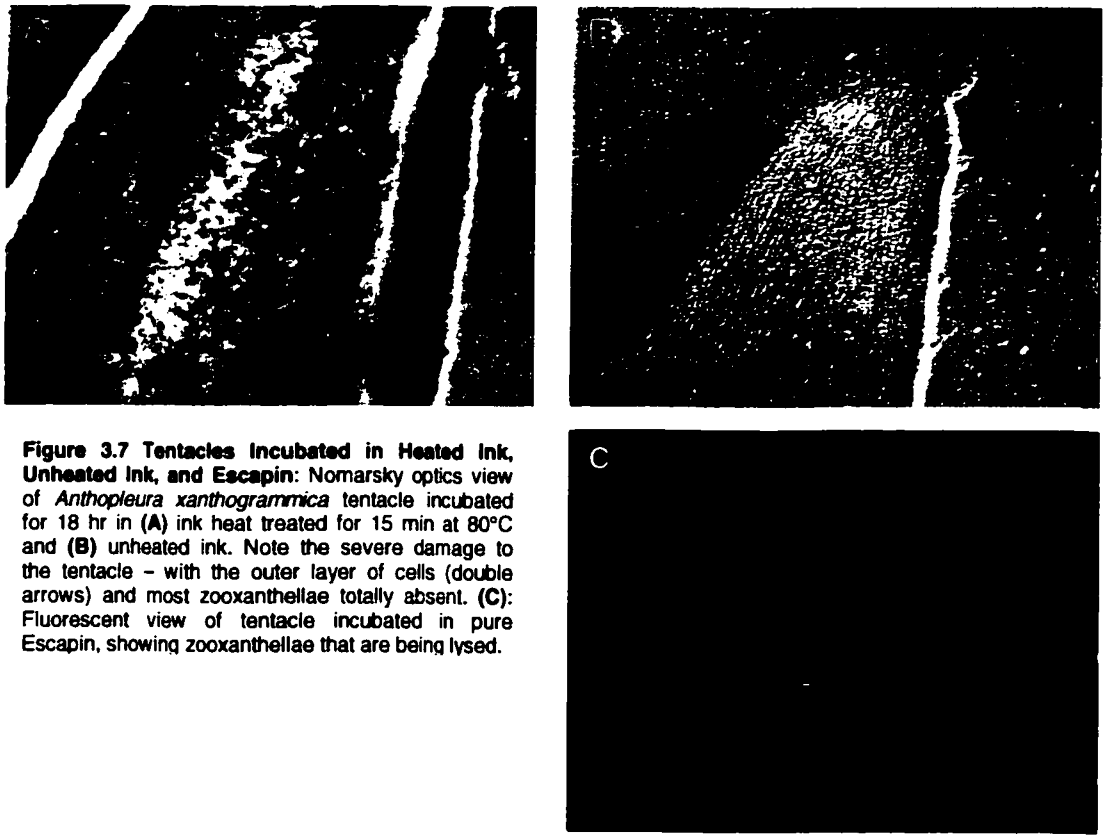

Figure 3.7 Tentacles Incubated in Heated Ink, Unheated Ink, and Escapin: Nomarsky optics view of *Anthopleura xanthogrammica* tentacle incubated for 18 hr in **(A)** ink heat treated for 15 min at 80°C and **(B)** unheated ink. Note the severe damage to the tentacle - with the outer layer of cells (double arrows) and most zooxanthellae totally absent. **(C)**: Fluorescent view of tentacle incubated in pure Escapin, showing zooxanthellae that are being lysed.

whole ink (Figs. 3.7 B and 3.8 C). None of the controls (BSA, ASW, and expressed escapin) showed any damage to tentacles or zooxanthellae (Figs. 3.6 and 3.8 A, B).

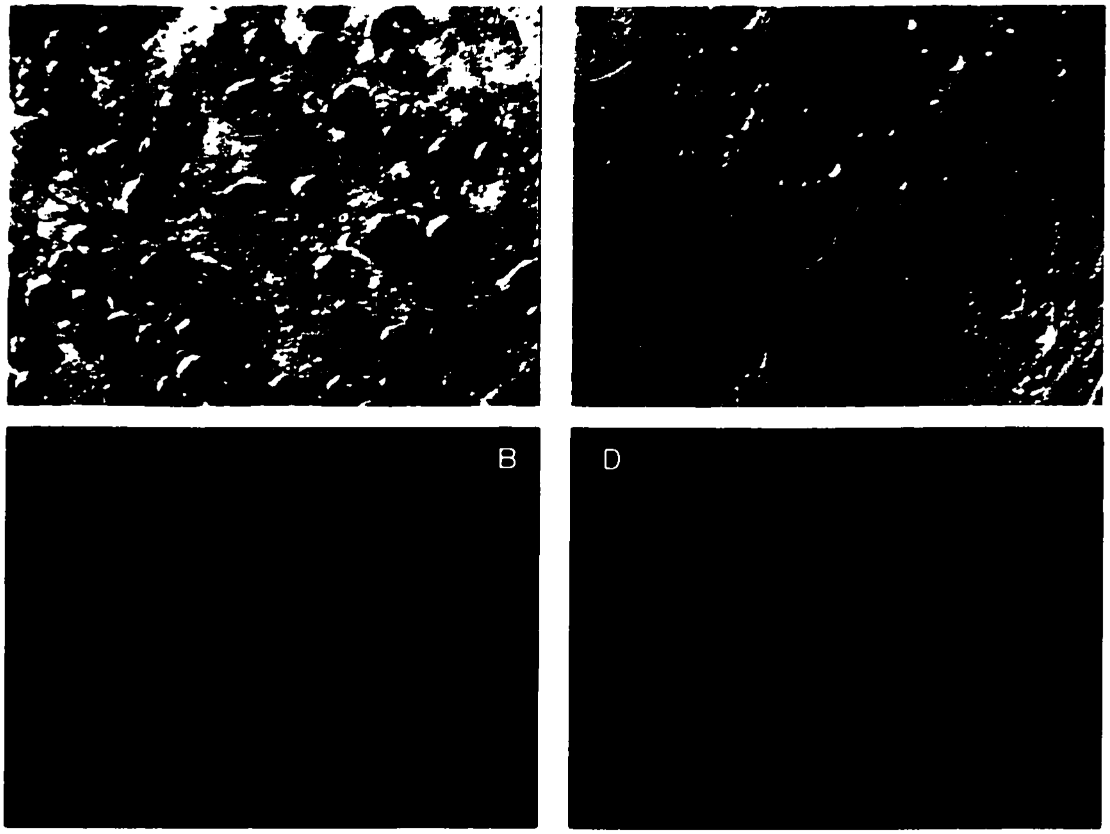

Figure 3.8 Higher Magnification of Control and Experimental Incubations. **A**: Nomarsky optics view of intact symbiotic zooxanthellae from a tentacle incubated in seawater; **B**: Fluorescent view of zooxanthellae from a tentacle incubated in BSA (1 mg/ml); **C**: Nomarsky optics view of lysed zooxanthellae from a tentacle incubated in pure ink; **D**: Fluorescent view of lysed zooxanthellae from a tentacle incubated in pure Escapin (1 mg/ml).

Discussion

It has been thought by most authors (this one included) that the anti-predator properties of seahare ink must be derived in some way from the chemically defended algae on which they feed. Both secondary algal metabolites and the algal pigments themselves have been considered likely candidates for the defensive properties of ink and, in fact, these two may still play a role with certain predator species. Biomedical researchers, though primarily involved with seahares for the purpose of drug discovery, have been highlighting alternative compounds that may also be of ecological relevance. The results of these experiments show that at least against sea anemone predators, it is a protein made by the seahare and not something derived from algae that is responsible for the defensive qualities of ink. This 60 kDa protein, which I have named escapin for its role in the escape of seahares from their most abundant predator, is very similar in sequence to the two other seahare proteins that have been sequenced. It is also similar in its antibacterial and cytolytic qualities to proteins found from the ink of several seahares, including in the white ink of *Aplysia juliana*.

The question becomes, with so many proteins with interesting properties known from seahares, why have they been overlooked for their ecological roles for so long? Perhaps one reason is that natural products chemists and chemical ecologists have focused almost exclusively on secondary metabolites, compounds that are not involved in the primary metabolism of organisms. Proteins such as escapin have been ignored for little reason other than that they did not fall into the class of compounds examined by those addressing questions in chemical ecology. The discovery of a natural function for escapin in the chemical defense of *Aplysia californica*, along with the knowledge of similar compounds in other seahares, suggests that there may be more to chemical defense than secondary metabolites.

As for escapin, though I have shown here that it is sufficient to cause direct damage to sea anemone tissues, it is also possible that there are other compounds in the ink with synergistic effects. Since I have not yet been able to remove only escapin from ink while leaving all other components in place, I cannot yet say that it is both necessary and sufficient for the anti-predatory effects of ink against sea anemones. Another issue not considered in this study was the effect of diet on deterrence. All *Aplysia californica* in these studies were raised on only *Gracilaria tikvahiae*, a red alga not known to harbor secondary metabolites. It would be interesting to raise the seahares on chemically defended algae, such as *Laurencia* sp. or *Plocammium* sp., as well to look for potential secondary metabolite effects in addition to the cytolytic activity of escapin.

Escapin, like the other identified seahare proteins, also has biomedical potential that should be explored. The next step in this process would be to over-express the protein in eukaryotic cells so that post-translational modification would be possible. Such a step was recently taken for the *A. punctata* ink protein, cyplasin L, with positive results that showed that when expressed in insect cells the protein retained its antibacterial and cytolytic properties (Petzelt *et al.* 2002). Eukaryotic over-expression of escapin would provide enough protein for both biomedical and ecological experiments.

Another future direction is using the technique of RNA inhibition (RNAi) using double stranded RNA (Fire *et al.* 1998) to block the translation of escapin mRNA in whole, free-living seahares. In this way, I could address its function in intact seahares paired with natural predators in both laboratory and field settings. The dsRNA-treated individuals would only differ from conspecifics in the ability to express escapin but would be capable of producing all of the other components of ink. Such an experimental design allows the survival value of bioactive proteins, such as escapin, to be determined in a more direct way than has been possible until now.

Chapter IV. Experiments with Crustacean Predators

Introduction

As discussed in Chapter II, crustaceans are common generalist predators that have been reported to attack and ingest seahares both in the laboratory and field. The literature on seahare interactions with crustaceans, though not extensive, has produced some very differing perspectives, depending on how the experiments (or observations) were performed and which crustaceans were involved. Many studies assert that crustaceans will eat seahares (Sarver 1978; Willan 1979; DiMatteo 1982b; Susswein *et al.* 1984; Pennings 1990a; Walters *et al.* 1993) while others suggest that at least some will not (Krakauer 1969; Tobach *et al.* 1989). Some studies suggest that crustaceans find the defensive secretions of seahares aversive (DiMatteo 1982b; Walters *et al.* 1993; Pennings 1994), while other studies suggest that at least some crustaceans do not (Krakauer 1969; Walters *et al.* 1993; Pennings 1994).

No study has attempted to evaluate the survival value of the secretions, by presenting seahares with and without chemical defenses to crustacean predators. Furthermore, the studies that described aversive responses to seahare ink and/or opaline did not examine the mechanism that might be involved. Carlson and Nolen (1997), however, did undertake a study of neurophysiological responses of a potential seahare predator, *Cancer anntenarius*. Their results suggest that ink stimulates dactyl chemoreceptors and that it is likely a small molecular weight algal pigment, phycoerythrobilin, that is responsible (Carlson 1997; Carlson and Nolen 1997).

Though this study is helpful in directing attention toward understanding neural mechanisms of ink function, since accompanying behavioral studies were not performed it is difficult to evaluate the meaning of the electrophysiological data.

In this chapter, I will present experiments that were conducted with three sympatric seahare / crustacean pairs in order to gain a comparative perspective. The first pair consisted of portunid crabs and *Stylocheilus striatus*, the second pair was the spiny lobster *Panulirus interruptus* and the seahare *Aplysia californica*, and the third pair *Panulirus argus* and *Aplysia dactylomela*. *Stylocheilus striatus* is a small seahare (adult size 1- 5 cm) that lives in and feeds on cyanobacterial mats. Around the island of Guam, where I collected these seahares, they are primarily found in mats of *Lyngbya majuscula*. Living in and among these mats are several species of predatory portunid crabs. These crabs are quite aggressive and are known to attack and eat seahares in the laboratory and in the field (Johnson and Cruz-Rivera, pers. obs.) and thus make a good model for evaluating the effects of ink and opaline on seahare survival. Spiny lobsters are another reported predator of seahares, both in the lab and field (Pennings 1990a; Walters *et al.* 1993). They are also a model system in chemosensory neurobiology (Derby 1995; Derby *et al.* 2001). This second feature and the fact that one species (*Panulirus interruptus*) is sympatric with the well-studied and commercially available *Aplysia californica*, make them well suited for this study. I first attempted to evaluate the survival value of ink/opaline for live seahares, then examined the behavioral effects of these isolated compounds on the crustaceans. Next

I attempted to determine the potential active components in the secretions, and last evaluate the effects of these compounds on chemosensory neurons of spiny lobsters.

Materials and Methods

Animals and Animal Care.

Guam: The seahares, *Stylocheilus striatus*, and sympatric portunid crabs were collected from cyanobacterial mats of *Lyngbya majuscula* from several sites around the island of Guam via snorkeling. Seahares and crabs observed in the field were placed in separate Ziploc bags and transported back to the University of Guam Marine Laboratory. *Lyngbya majuscula* was also collected in the field in Ziploc bags and returned to the laboratory where it was immediately sorted to recover both seahares and crabs. Once separated, crabs and seahares were maintained in fresh aerated seawater with a natural supply of *Lyngbya majuscula*. Since crabs were not kept for more than a few days in the laboratory additional food was not provided.

Bermuda: Adult spiny lobsters, *Panulirus argus*, were collected by scuba diving and were maintained in fresh aerated seawater and fed three times per week. Specimens of *Aplysia dactylomela* were collected at Tobacco Bay, Bermuda, by snorkeling and returned to Bermuda Biological Station for Research in buckets of seawater. They were maintained in the laboratory in fresh aerated seawater and fed red algae.

Atlanta: Seahares, *Aplysia californica*, and their primary red-algal diet of *Gracilaria tikvahiae* were provided by the NIH National Resource for *Aplysia* in Key Biscayne, Florida. Spiny lobsters, *Panulirus interruptus*, were provided by Coastal Catch, Inc.

Animals were maintained at Georgia State University in separate re-circulating, artificial seawater tanks. *A. californica* was fed *G. tikvahiae ad libitum* while *P. interruptus* was fed shrimp pieces, three times per week.

Behavioral Assay with Portunid Crabs. An experiment was designed to test the effects of ink/opaline secretion on seahare survivorship with portunid crabs. Eighteen *Stylocheilus* individuals raised only on their natural diet of *Lyngbya majuscula* were paired by size and then one of each pair was randomly chosen to be de-inked. Animals were de-inked by gently wrapping them in a Kimwipe®. This caused them to release both ink and opaline secretions. This procedure was conducted once more before the experiment began. Nine portunid crabs were transferred to individual plastic containers with fresh seawater and allowed to acclimate for at least 1 hr. In the first round of experiments, the crabs were presented randomly with one member of each seahare pair (delivered by widemouth pipette) and then the other member of the pair in the second round, which took place 2 days later. All trials lasted 5 min and were recorded with digital videotape on a Sony camcorder (DCR-TRV10). Seawater was changed after the experiment and again twice a day between trials. Small crabs were grouped with smaller pairs of *Stylocheilus* as well to help control for any size effects.

Behavioral Assay with Spiny Lobsters. An assay similar to the one described above was conducted with the spiny lobster *Panulirus interruptus* and the seahare *Aplysia californica*. For this experiment 20 lobsters housed in individual recirculating 80 L tanks (60 x 30 x 45 cm) were used. The lobsters were maintained without food for one week prior to the experiment. 20 seahares each weighing ca 90 g were selected and paired by size. One of each pair was randomly chosen to be in either the de-inked or the full gland group. Ten seahares were de-inked by handling once, about 1 hr before the experiments and again directly prior to the experiment. The other 10 were left with glands presumably full. No seahare was used twice for any experiment regardless of whether or not the lobster attacked it. Lobsters were initially tested for feeding responses by delivering 1 ml of water in which frozen shrimp had been soaked for 30 min to 2 hr. Shrimp was chosen as a feeding stimulus because it is the normal laboratory food of the lobsters. Shrimp has also been shown to be a highly effective behavioral and electrophysiological stimulus (Fine-Levy and Derby 1992; Derby 2000). The animal was observed for 1 min and if it did not respond to the shrimp stimulus by searching behavior (defined as rapid walking and probing with the legs), the trial was ended. If it did respond, then the seahare was dropped into the tank and the interaction captured on digital videotape for 5 min. This was adequate time as most behavior, including attacking, eating, and/or rejecting the seahare took place within the first two min. At the end of trials in which seahare was rejected or ignored a post-test was conducted with a piece of shrimp. The lobster had 5 min to capture the shrimp to show its level of feeding motivation. Lobsters that ignored the post-test shrimp were excluded from analysis even if they had attacked a seahare. The same experiment was repeated the following week with lobsters receiving the opposite member of the seahare pair so that a within individual comparison was possible. For this experiment, 20 new seahares of the same size as the first experiment were used.

Filter Paper Assay with Spiny Lobsters. When seahares are present, it is difficult to evaluate the lobster's response to the defensive secretions and impossible to tell if either ink or opaline is responsible since both are often released together. To understand the response of the lobsters to the isolated compounds, a second experiment was designed. Whatman #4 filter paper disks were used to present secretions to *Panulirus interruptus* (Derby *et al.* 1984). These individuals were different from those used in the behavioral assays. Filter paper disks were presented to the mouthparts of spiny lobsters at which point the lobsters either attacked the paper (i.e. grabbed it with walking legs and then mouthparts), moved away from it, or ignored it. Ink and opaline were compared separately with squid extract (a mix of macerated squid pieces soaked in ASW for 1 hr and then poured through a coffee filter) as a positive control, seawater as a neutral control, and tannic acid as a negative (aversive) control (Derby *et al.* 1984). Tannic acid was chosen to control for the possibility that lobsters would attack and chew anything they could detect chemically. Tannic acid is one of the only substances know to be a feeding deterrent to clawed lobsters (Derby *et al.* 1984). All lobsters received all of the disks. Only one disk was presented per trial and order of presentation was random for each lobster. I conducted the trials in the dark (during the lobster's normal nocturnal feeding cycle) using an IR illuminator and recorded using an IR-sensitive digital camcorder (Sony DCR-TRV10).

The scorer of the video was blind to the identity of the stimulus. Since IR video is black and white, even disks soaked in the purple ink did not appear different than other disks.

Amino Acid Analysis. After many previous tries at doing amino acid analysis in-house, with little success (problems with the equipment and columns), I sent samples of ink, opaline, and haemolymph out for analysis. The samples were collected from the dissected glands of fifteen individual *A. californica* in the case of ink and opaline and five individuals in the case of haemolymph. Haemolymph was collected by first de-inking the seahares so as to avoid ink/opaline contamination, then anaesthetizing the seahares for 2 hr at 4°C, washing an area of skin between the rhinophores with 70% EtOH, and then making a small incision in that region to release the haemolymph while holding the seahare over a sterile tube. Samples of ink were pooled as were samples of opaline and haemolymph because analysis of multiple replicates of each was prohibitively expensive. Since it was an equal composite from multiple individuals I am comfortable that this represents a fair analysis of amino acid content and concentration for these laboratory-reared seahares. Amino acid analysis of free amino acids was conducted by The Scientific Research Consortium, Inc. (www.aminoacids.com). Serial dilutions of the ink, opaline and haemolymph samples were run and the results presented in a dilution corrected format. Twenty-two free-amino acids plus ammonia were analyzed.

Ash-free Dry-weight. Ash-free dry-weights were measured for ink, opaline, and body wall of *Aplysia californica* to determine organic content as one measure of nutritional value. These experiments were conducted with the help of Cynthia Kicklighter at the Georgia Institute of Technology Marine Laboratory on Skidaway Island, Georgia. Ink samples from 5 individuals were pooled and opaline samples from 5 individuals were also pooled to increase the overall sample volumes to measurable sizes. All samples were weighed wet and then dried for three days in a drying oven at 50°C. Samples were reweighed to get their dry weights and then ashed in a muffle furnace and finally re-weighed.

CHN Analysis. Carbon, Hydrogen, Nitrogen analysis is another measure of nutritional value. The ratio of carbon to nitrogen reflects relative nutritional quality with increasing ratios indicating decreasing nutritional value (Cruz-Rivera and Hay 2001). CHN analysis was performed by the Chemical Analysis Laboratory of the University of Georgia. Three freeze-dried samples (Virtis benchtop lyophilizer) of each of following *Aplysia californica* samples were analyzed: ink, opaline, foot, parapodia, and internal organs.

Electrophysiological Recordings of Chemoreceptor Neuron Activity. Single-unit extracellular electrophysiological techniques were used to record from individual chemoreceptor neurons (CNs) of spiny lobsters, using established techniques (Derby 1995; Steullet and Derby 1997). This technique uses a perfused antennule preparation placed in a chamber that separates the recording environment (in lobster saline) from the stimulating environment (in artificial seawater). The chemical stimuli are introduced via electronically driven valves of an olfactometer. Recordings are made using fine-tipped glass electrodes placed against the side of the axons of the CNs. A major advantage of this preparation is that responses can be recorded from single units (i.e. individual CNs) for up to several hours. Single-unit responses were processed and quantified using DataPac2000 software (Run Technologies).

Experiments with *Panulirus argus*. Pilot studies were conducted in Bermuda using the spiny lobster *Panulirus argus* and the ink of sympatric seahare *Aplysia dactylomela*. The protocol for finding CNs involved attaching the electrode to a bundle of antennular nerve and then stimulating the antennule with either a shrimp odor stimulus (homogenized frozen shrimp 300 g/ml) diluted 1:1000 or a mixture of 8 compounds at 10^{-5}M (8mix): L-arginine, L-glutamate, glycine, taurine, betaine, ammonium chloride (NH_4), adenosine-5'-monophosphate (AMP), and DL-sucrose. When a single-unit recording was obtained, the CN's response to the ink of *Aplysia dactylomela* was tested with a series of dilutions. If the cell was still responsive, the single components of the mix were used to characterize the cell's response. Ink was then presented in dilutions starting with 1:1000 and working upward to 1:10 and downward to 1:10,000. Mixtures of ink and normal stimulants were presented to the CNs as well.

Experiments with *Panulirus interruptus*. After establishing the amino acid content of the ink and opaline of *Aplysia californica*, an artificial mixture of each was created. These mixtures were prepared in artificial seawater (ASW) that contained only the amino acids in the appropriate concentrations but did not contain any of the other components (e.g. algal pigments and proteins). This permitted testing whether amino acids and ammonia alone elicit responses from lobster CNs. The protocol for finding CNs was the same as described above except that either a dilute shrimp odor stimulus (300 g/L shrimp homogenate diluted 1:1000 with ASW) or an ink or opaline stimulus diluted 1:1000 in ASW was used. If a CN responded to any of these search stimuli, then it was tested again 3 times with the search stimulus (allowing a 3-min interval between all trials) to determine if a response was consistent. If so, then individual compounds at 10^{-5}M concentrations (ammonia, AMP, aspartate, cysteine, glutamate, histidine, and lysine) were tested in random order to identify the CN type. Next ink, opaline, artificial ink, and artificial opaline, all at 1:1000 dilution, and tannic acid at 10^{-5}M were tested.

Results

Do Seahare Secretions Play a Defensive Role in Encounters with Portunid Crabs?

While in the lab of Dr. Valerie Paul at the University of Guam Marine Laboratory I observed that after placing a filter paper disk with ink from the seahare *Dolabella auricularia* in a tank, that within a few moments a large portunid crab approached the ink-soaked disk and began feeding on it. This behavior was contrary to what I expected, and what was published about the deterrent properties of ink. To follow up, I induced the seahare *Stylocheilus striatus* to release ink in a container of 20+ small (~ 1 cm carapace width) portunid crabs and then removed the seahare. Many of the crabs moved into the cloud of ink and opaline and appeared to be eating. Most of the crabs that moved into the area stayed there for 10 min or more. This suggested that instead of being repellent, the seahares' secretions might be feeding stimulants. Thus I wanted to determine if portunids would attack and eat seahares and what behavioral role the seahare ink and opaline secretions played in such an attack.

To demonstrate a role for seahare defensive secretions, I compared crab responses to full gland and de-inked seahares. The results indicated a definite survival value for ink and opaline secretions (Fig. 4.1). Six of nine *S. striatus* individuals with full glands escaped while only one of nine escaped in the de-inked group, which is a statistically significant difference (Fig. 4.1). The crab attacked the seahare and if no ink/opaline was released the crab typically held the seahare up to its mouth with both claws and fed on it. If, however, ink/opaline was released, the behavior was quite different. The crab often held the seahare with one claw and used the other to sweep ink/opaline to the mouth. The seahare was eventually released as the crab switched to using both claws to feed on the secretions.

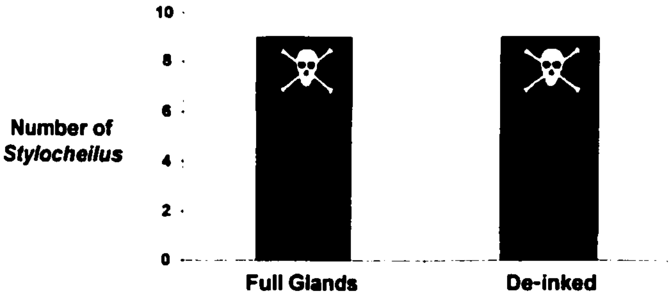

Figure 4.1 Survival of *Stylocheilus striatus* with Full and Depleted Glands in Encounters with Portunid Crabs. Seahares with full glands (n=9) were significantly more likely to escape than those without (n=9, Cochran's Q, Q=5.0, p<0.0253). Each of the portunids (n=9) was presented with both an experimental and a control seahare in random order and with at least 1 hr between presentations.

Do Seahare Secretions Play a Defensive Role in Encounters with Spiny Lobsters?

Pilot studies with *Aplysia* and lobsters were conducted first before any controlled experiments. The purpose of the pilot studies was to learn how lobsters and *Aplysia* interact in the laboratory, and in order to help plan the next experiments. Though an interaction between the Atlantic *P. argus* and the Pacific *A. californica* would not be ecologically meaningful, I wanted to observe how the two behaved together, as a comparison to more ecologically relevant pairings. Perhaps not surprisingly, in all pairings (n=12), *P. argus* ignored *A. californica* even after making physical contact.

Pilot studies with sympatric *P. interruptus* and *A. californica* had a different outcome. I used the four *P. interruptus* individuals that had been used in earlier pilot observations. Only one lobster (a pre-molt) did not attack the seahares. I observed several interesting behaviors from both the seahare and the lobsters. After an attack, the seahare would frequently release opaline a few seconds prior to releasing ink. This is contrary to my previous observations and those published in the literature that suggest opaline release has a higher threshold than inking (Johnston 1850; Eales 1921; Kandel 1979; Kamiya *et al.* 1989). After the seahares released opaline and ink, the lobsters sometimes backed up to the edge of the tank until they were almost oriented head down, tail up, while feeding by constantly rotating the *Aplysia* and taking bites. In three instances, the lobsters tail-flipped, in one case dropping the seahare. Lobsters would repeatedly eat seahares both on the same day and on different days. In a few cases with large seahares and large quantities of ink the lobsters behaved differently.

When ink was released via the seahare's siphon directly into the mouth of the lobster, the lobster dropped the seahare and seemed to be eating the ink. The seahare was able to escape. In another instance, a seahare was released, caught a second time, inked a second time, and was released again.

I conducted a controlled experiment similar to that done with portunid crabs in Guam with similar results. Though 20 lobsters were tested, 8 were eliminated from analysis because they either did not attack the seahares or failed to be attracted to shrimp in the post-test. Of the twelve lobsters that participated in the full experiment for two weeks, 4 dropped the seahares after they released ink, allowing the seahares to escape. All 12 seahares in the de-inked group were eaten (Fig. 4.2). The outcome of the tests with normal and de-inked seahares was statistically significant (Fig. 4.2). Additionally, 7 out of 12 lobsters exhibited a tail-flip response after encountering ink, while no lobsters exhibited tail-flip with the de-inked seahares (Cochran's Q, Q = 7.0, p <0.0081).

Figure 4.2 Survival of *Aplysia californica* with Full and Depleted Glands in Encounters with Spiny Lobsters, *Panulirus interruptus*. Seahares with full glands (n=12) were significantly more likely to escape than those without (n=12, Cochran's Q, Q=4.0, p<0.0455). Each of the lobsters (n=12) was presented with both an experimental and a control seahare in random order and with at least one week between presentations.

Do Lobsters Release Inking Seahares Because They Are Repelled?

The pilot studies, the experiments with portunid crabs, and those with spiny lobsters all suggested that when large quantities of ink and opaline were released, the crustaceans became more involved with the secretions and were either eating them or cleaning their mouthparts. It was difficult to tell if the crustaceans were repelled or attracted, yet the pilot studies in Guam suggested that attraction was (even if unusual) a possibility. To determine which was the case, I designed an experiment using filter paper disks coated in secretions and offered to lobsters. The results showed that both opaline and ink were attractive, but opaline more-so (Fig. 4.3). Opaline was as attractive as squid extract (Cochran Q test, $Q = 0.2$, $p < 0.6547$) and more attractive than ASW ($Q = 8.0$, $p < 0.0047$). Ink's intermediate attractiveness is indicated by it being of similar attractiveness to both squid extract ($Q = 3.6$, $p < 0.0588$) and ASW ($Q = 2.7$, $p < 0.1025$).

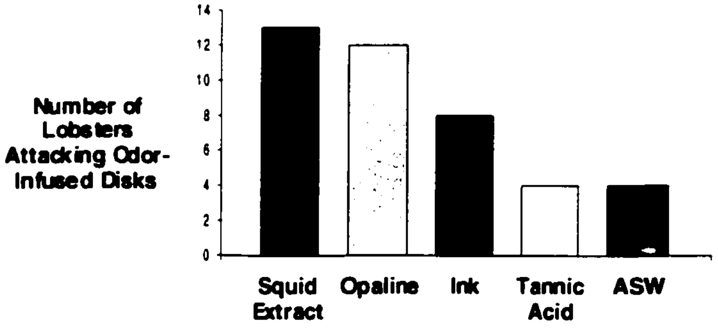

Figure 4.3 Filter Paper Behavioral Assay with *Panulirus interruptus*. Filter paper disks were infused with squid extract, opaline, ink, tannic acid, or artificial seawater (ASW). See text for explanation.

Amino Acid Analysis

Samples of ink, opaline, and haemolymph were analyzed for their free amino acid content (Table 4.1). Both ink and opaline contained high concentrations of the highly stimulatory amino acid, taurine. Taurine was 231 mM in opaline and made up approximately ¾ of the total free amino acid and ammonia concentration of opaline. In ink, taurine was much lower but still significant at 8 mM. Ink had higher concentrations of both ammonia (24 mM) and cysteine (15 mM). Additionally, opaline contained high concentrations of lysine (65 mM). The total concentration of free amino acids and ammonia in the haemolymph (0.990 mM) was more than 322 times lower than the concentration in opaline (319.063 mM) and more than 54 times lower

Table 4.1 Free Amino Acid and Ammonia Concentrations of Haemolymph, Ink, and Opaline (μM)

	Haemolymph	Ink	Opaline
Taurine	193.6	7,830.0	231,200.0
L-Lysine	20.8	0.0	65,190.0
Ammonia	271.6	24,360.0	6,810.0
L-Cysteine	0.0	14,830.0	1,844.0
L-Histidine	14.0	255.0	7,185.0
L-Aspartic acid	8.4	2,231.0	2,512.0
L-Glutamic acid	4.0	1166.0	1,616.0
L-Alanine	64.4	1,024.0	339.0
L-Glycine	31.2	181.0	791.0
L-Phenylalanine	8.0	130.0	648.0
L-Arginine	295.6	0.0	340.0
L-Leucine	6.4	327.0	10.0
L-Valine	10.8	301.0	56.0
L-Tyrosine	3.2	297.0	14.0
L-Threonine	10.0	193.0	236.0
L-Serine	12.8	214.0	68.0
L-Isoleucine	3.2	135.0	96.0
L-Proline	0.0	131.0	7.0
L-Methionine	4.4	122.0	35.0
L-Glutamine	17.2	121.0	9.0
L-Cystine	3.2	84.0	16.0
L-Asparagine	6.4	51.0	41.0
L-Tryptophan	0.8	5.0	0.0

than in ink (53.988 mM). Taurine levels in haemolymph were less than 0.194 mM and the most dominant component was arginine at 0.296 mM.

Are Ink and Opaline as Nutritionally Valuable as the Seahares Themselves?

I used two measures of nutritional value to determine if the defensive secretions offered a true food resource to predators that might ingest them. The values of ink and opaline were compared with various tissues from the seahares themselves including: foot, body wall, parapodia, and internal organs. The results of the ash-free dry weight show that neither ink or opaline were significantly different in % organic material per dry weight from the other *A. californica* body tissues (ANOVA, Tukey HSD test) suggesting that it has greater nutritional value. Results of CHN analysis show that carbon to nitrogen ratios of ink were significantly higher than those of opaline, parapodia, foot, and gut (ANOVA, Tukey HSD test, Fig. 4.4), suggesting that ink is lower in nutritional value than opaline and the body tissues.

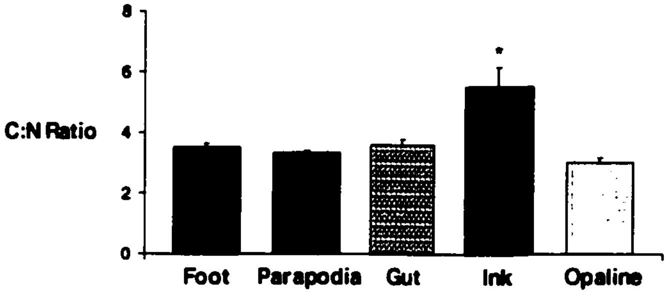

Figure 4.4 Carbon to Nitrogen Ratio for Ink, Opaline, and Tissues of *Aplysia californica*. Three freeze-dried samples of each type from *A. californica* were analyzed for the ratio of carbon to nitrogen. Values shown are means plus standard deviations. Ink was found to have a significantly higher C:N than any other samples suggesting that ink is lower in nutritional value (ANOVA F(4,10)=29.44562, p=0.000016; Tukey HSD ($p<0.05$).

Electrophysiology

Panulirus argus with *Aplysia dactylomela* ink

Ink diluted up to 10,000 times stimulated the majority of the chemosensory neurons (CNs) tested in the lateral flagellum of the antennules. Recordings were obtained from four different CNs representing 4 different cell types; a taurine-best cell, a glycine-best cell, an arginine-best cell and a broadly-tuned cell, but ink only elicited consistent results with taurine-best cells. The taurine-best cells gave similar responses to ink and taurine with approximately the same decrement in total number of action potentials when diluted (Fig. 4.5).

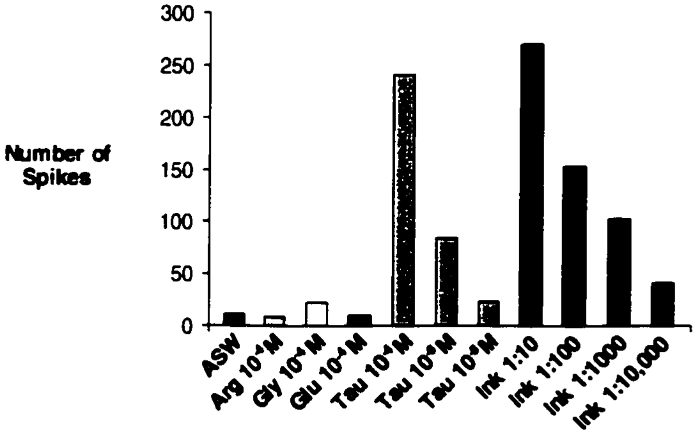

Figure 4.5 Characterization of an Antennular Chemosensory Neuron of *Panulirus argus*. This chemosensory neuron is excited in a dose-dependent fashion by a food stimulus (taurine) and ink of sympatric *Aplysia dactylomela*. Shown is neural activity (# of spikes in 3 sec. of stimulation) of a single neuron. Cell is unresponsive to seawater and 3 amino acids (L-arginine, glycine, and L-glutamic acid).

Panulirus interruptus with *Aplysia californica* natural and artificial ink and opaline

Aplysia dactylomela that were sympatric with *Panulirus argus*, were unavailable in Atlanta and accordingly, I switched to *Aplysia californica* and the California spiny lobster *P. interruptus*. Unfortunately, though electrophysiological recordings were straightforward when recording from *P. argus* in Bermuda, *P. interruptus* preparations produced very few recordable cells. The preparations seemed to die more quickly. Nonetheless the data obtained from a single taurine-best CN is consistent with that from *P. argus* and with what we now know about the free amino acid content of ink and opaline(Fig. 4.6). Opaline, ink, and artificial mixtures of the two (containing only the amino acids and ammonia in their appropriate

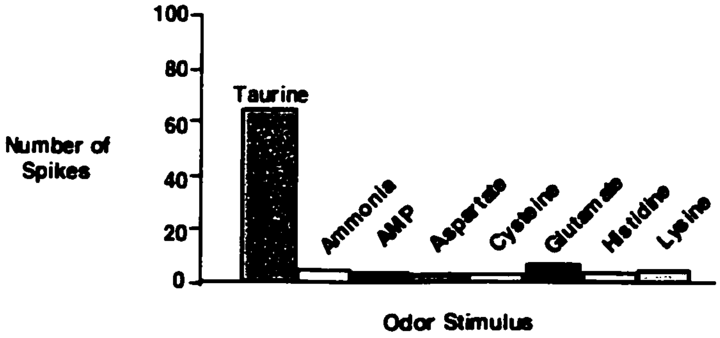

Figure 4.6 Characterization of a Taurine-Sensitive Cell of *Panulirus interruptus*. Recordings from a chemosensory neuron in the antennule of *P. interruptus* show that taurine is the only tested single compound that stimulated this cell. All test solutions were 10^{-5}M.

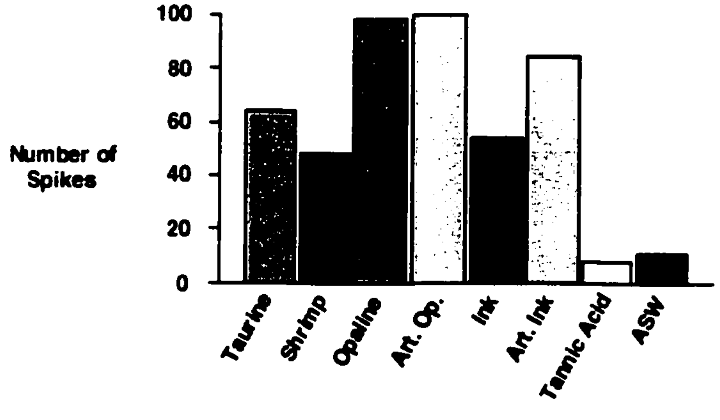

Figure 4.7 Ink, Opaline, and Artificial Ink and Opaline Stimulate Taurine Sensitive Cell of *Panulirus interruptus*. Recordings from a taurine-sensitive chemosensory neuron (characterized in Fig. 4.7) in the antennule of *Panulirus interruptus* show that ink and opaline, as well as artificial amino acid mixtures of the two, stimulated the cell while tannic acid and artificial seawater did not. T: taurine (10^{-5} M); S: shrimp (1:1000); O: opaline (1:1000); AO: artificial opaline (1:1000); I: ink (1:1000); AI: artificial ink (1:1000); TA: tannic acid (10^{-5} M); ASW: artificial seawater.

cause the lobster's behavioral response, but several more cells need to be recorded from before conclusions may be drawn about cellular mechanisms.

Discussion

A New Form of Chemical Defense: Phagomimicry

The chemical compositions of the defensive secretions, taken together with behavioral data that showed the secretions to be stimulatory to spiny lobster feeding and to be effective deterrents to portunid crabs, suggest seahares have an unusual and previously undescribed form of chemical defense. I call this defense "phagomimicry." Phagomimicry is defined as a chemical defense in which a secreted substance mimics the stimulatory properties of food to divert a predator. Behaviorally we knew that the secretions were effective because seahares with the defenses were significantly more likely to escape crustacean predators than those in which the secretions had been depleted. This was as true for crabs and *Stylocheilus* as it was for spiny lobsters and *Aplysia*. We also knew that opaline and ink were not significantly different from squid in their stimulatory properties. The free amino acid analysis, which revealed very high levels of amino acids, particularly taurine, in the secretions and very low levels in the haemolymph, suggests that amino acids are being concentrated in ink and opaline. There they might be used to stimulate crustacean chemosensory systems when the seahares are attacked. This 320 fold concentration difference in free amino acids and ammonia between haemolymph and opaline also suggests that the secretions function as a supernormal stimulus, defined as a stimulus that is more effective at eliciting a response than the typical stimulus the responder encounters (Tinbergen 1951). This would be necessary because since the prey itself *is* food to the predator and would release haemolymph when bitten, a supernormal stimulus of food would be required to direct the predator toward feeding on the secretions and not on the prey.

A phagomimetic substance is not only likely to be a supernormal stimulus, but is also likely to function as a form of sensory trap (Christy 1995). Sensory traps, as the name suggests, work because the sensory system of the target is "trapped" to respond in a certain way. In the vast majority of cases, detection of high concentrations of free amino acids means that a food source (with all of its protein, carbohydrate, and other nutritional components) is present and should be the focus of the predator's attention. The seahare exploits this property of its predator's nervous system by releasing secretions that mimic the stimulatory properties of food (high amino acid concentrations) and thereby divert the attention of its attacker. The results of the ash-free dry weight analysis suggest that the secretions are no more nutritious than the tissues of the seahare and the C:N ratio shows that ink is, in fact, less nutritious. In either case the whole seahare, without a doubt, has greater nutritional value than do the few hundred microliters of ink and opaline.

The electrophysiological data, though preliminary, is supportive of the phagomimicry hypothesis. Ink stimulates taurine-sensitive chemosensory neurons in the antennules of both Atlantic and Pacific spiny lobsters and opaline has been shown do so as well in the Pacific lobsters. Artificial ink and opaline were equally stimulatory suggesting that the amino acids in the secretions may be sufficient to elicit behavioral responses. Though the mechanism of phagomimicry is likely amino acid stimulation of the lobster chemosensory neurons, there may be more to it than that. The highly viscous nature of the opaline secretion may be important in keeping the stimulants in contact with the crustacean antennules and additionally its tactile features may be important in creating a false sensation of a real food item. It will also be important to determine what roles if any the cytolytic ink protein, escapin, and the algal pigments in the ink play in this process at the behavioral and neurophysiological levels. Lastly, it is of interest that 7 of 12 lobsters produced a tail-flip when in contact with ink. How ink or opaline triggers such a response and what its role may be for the seahare have yet to be determined.

Chapter V. Experiments with Vertebrate Predators

Introduction

Reef fishes have on rare occasion been observed to eat live seahares (Sarver 1978) and dead seahares (Kupfermann and Carew 1974). The seahare *Akera bullata* has been found in the stomachs of flounder (Morton and Holme 1955; Thompson 1960a) but no seahares have been found in the stomachs of thousands of fish examined off the coast of California by the California Fish and Game Laboratories (Winkler and Tilton 1962).

Most field and laboratory studies in which whole, live seahares were offered to fishes report that the seahares were routinely rejected (Thompson 1960a; Pennings 1990a; Ginsburg and Paul 2001; Pennings *et al.* 2001). Exceptions, however, have been observed. Pennings et al. (2001) found that various wrasses as well as the bream (*Pentapodus macrurus*) ate *Stylocheilus striatus*. Pennings (1990a) also found that the wrasse (*Halichoeres*) preferred green-seaweed-fed *Aplysia californica* to red-seaweed-fed and the garibaldi (*Hypsypops* sp.) ate both types of *A. californica* regardless of diet. Reef fishes were shown to make a distinction between frozen *Aplysia parvula* that had been raised on the secondary-metabolite-rich red alga *Portieria hornemannii* and those raised on the chemically depauperate red alga *Acanthophora spicifera* – choosing to eat the latter (Ginsburg and Paul 2001). In the same study, however, several reef fishes did consume frozen *P. hornemannii*-reared seahares (*Thalassoma* *lutescens*, *Halichoeres hortulanus*, *Balistapus undulates*, *Scares sp.*, and *Naso vlamingii*).

Two studies that examined laboratory responses of fishes to pieces of seahare tissue, including foot and mantle from three genera of seahares (*Aplysia*, *Dolabella*, and *Notarchus*) and body pieces from *Aplysia brasiliana*, found that all fishes rejected the tissues (Russell 1966; Kinnel *et al.* 1979). Extracts of seahare skin and ink have consistently been rejected by fishes in both field and laboratory settings, though ink extracts require higher concentration of material to be effective (Paul and Pennings 1991; Pennings *et al.* 1999). In the only study that examined the direct effects of seahare defensive secretions on fish feeding, it was shown that they were not effective deterrents (Pennings 1994). In one experiment krill and in a second experiment squid were soaked in the white ink (and perhaps opaline) of *Aplysia juliana* before the pieces were offered to fishes in lab and field settings respectively.

From the literature summary above, we can see that most fishes do reject seahares, their tissues, and extracts. We also see that some fishes will eat seahare regardless of diet and that wrasses are high on this list. And lastly we see from the two studies that manipulated the diet of seahares to include chemically poor algae that at least some fishes are capable of making a choice based on the diet of the seahare. Though these studies show that a diet rich in chemical compounds can play an important role in the defense of seahares, it is unclear whether it is through the *passive* defense alone of harboring distasteful algal toxins or other compounds in the skin or to the *active* defense of ink and opaline release after attack. It is also possible that some combination of the two is important. A few studies have suggested that seahare secretions can have direct and negative effects on fish, but all used unnatural conditions where the fishes were maintained in small tanks and exposed to the seahares secretions for long periods of time. In such cases, paralysis and death have been observed (Flury 1915; Willan 1979; Carefoot *et al.* 1999) and, alternatively, no ill effects have also been observed (Russell 1966; Krakauer 1969). The role that ink and opaline secretions may play in defending against fish predators in a more natural context has until now not been tested.

In this study, I also manipulate the diet of seahares as others have done, but additionally manipulate their active chemical defenses of ink and opaline and ask the following three questions: 1) do seahares with full defensive glands survive more predatory encounters with reef fishes than those with depleted glands? 2) are there differences in survivability between seahares of different species each fed on their natural, chemically-defended diets? and 3) do dietary differences, which affect composition of defensive secretions as well as skin, affect survivability?

Materials and Methods

Collection of *Aplysia parvula* and *Portieria hornemannii*. The red alga *Portieria hornemannii* (*Portieria*) with its accompanying *Aplysia parvula* (*Aplysia*) were collected by SCUBA and snorkeling at three sites on Guam: Apaca Point, Anae Island, and Double Reef. The algae were transported in coolers to the University of Guam Marine Laboratory and put it in holding tanks with aerated, fresh running seawater.

Aplysia is notoriously difficult to find on *Portieria*, therefore, the algae was left in the holding tanks until the seahares would leave and could be found crawling on the sides of the tanks. Each seahare was put in an individual Styrofoam cup filled with seawater and a supply of *Portieria* was also placed in the cup. Water was changed daily and food was added as it was eaten.

Collection of *Stylocheilus striatus* and *Lyngbya majuscula*. The cyanobacterium *Lyngbya majuscula* (*Lyngbya*) and its accompanying *Stylocheilus striatus* (*Stylocheilus*) were collected by snorkeling at Piti Bay, Guam. After transport in coolers to the marine lab, the *Stylocheilus* individuals were removed immediately from the *Lyngbya* and separated by size. One hundred individuals (2-3 mm in length) were each put into individual styrofoam cups with fresh seawater and one of four different diets discussed below. Water was changed daily and food was added as it was eaten.

***Stylocheilus* diets**. Four diets were used to raise the 100 individual *Stylocheilus*. They included two natural cyanobacterial diets, *Lyngbya* and *Hormothamnion enteromorphoides* (*Hormothamnion*), one artificial cyanobacterial diet made from *Spirulina* powder, and one green algal diet of *Enteromorpha clathrata* (*Enteromorpha*). *Lyngbya* was collected at Piti Bay, *Hormothamnion* at Asan Beach, and *Enteromorpha* at Tumon Bay. The artificial diet was made from 4 g *Spirulina* powder, 0.72 g water, and 36 ml de-ionized water. The *Spirulina* powder was mixed with half of the water and set aside. The agar was mixed with the remaining water and heated in a microwave on high until boiling (about 30 s). The two mixtures were combined and placed in a refrigerator to set. Small cubes of this gel food were cut to feed to individual seahares.

The four groups of 25 individual *Stylocheilus* were maintained on these four diets for 4 weeks. Most of the individuals in the natural and artificial cyanobacterial diets had reached a length of about 2 cm by this time. All but two of the individuals on the *Enteromorpha* diet showed no appreciable growth and none were used in feeding experiments. There was also mortality in each of the diet groups. Eleven animals died in the *Lyngbya* group, 7 in the *Hormothamnion* group, and 2 in the *Spirulina* group. The remaining animals were used in feeding assays with reef fishes.

First Palatability Assay with Reef Fishes. The night before the feeding assay was performed, the seahares were organized into seven groups of six animals each. The groups were arranged by size with two seahares closest in size being chosen from each of the three diet groups (*Lyngbya*, *Hormothamnion*, and *Spirulina*). Within these groups of six, three individuals (one from each diet group) were randomly selected for de-inking. De-inking consisted of gently wrapping the seahare in a Kimwipe®, which caused the animal to release ink and opaline from its two defensive glands. By this process I now had seahares from each of the diet groups with a full complement of their chemical defenses matched by size with those that were depleted of their chemical stores.

Just prior to the experiment, the seahares were placed in individual Ziploc sandwich bags with fresh seawater and the six bags of each size-group were then placed into a larger one-gallon Ziploc for transport to the reef. Feeding assays were conducted at Fingers Reef in Apra Harbor, Guam. Three SCUBA divers were needed for the experiment. One diver kept the bags of seahares and offered them to the fishes, one diver recorded eaten/rejected and fish species data on an underwater slate, and the third diver recorded the assay with an underwater video camera. Fishes were first presented control fish food pellets, then each seahare, one at a time, from the six seahares in the larger Ziploc bag (seahares that had been previously grouped by size), and lastly a second control food. This was done until all the seahares had been released into the water column and sampled by the fishes. The total number of seahares in the experiment was 50.

Second Palatability Assay. The following day the feeding assay was repeated at Fingers Reef with a new set of *Lyngbya*-fed *Stylocheilus* (n= 20) and *Aplysia* (n= 18) that had been maintained on their field diet of *Portieria*. The *Stylocheilus* individuals used in this experiment were all near maximum size (5 cm) while the *Aplysia* individuals were all 1.5-3 cm long. The *Aplysia* individuals had been paired by size and one de-inked as described earlier for *Stylocheilus*. The *Stylocheilus* individuals were also paired by size and one de-inked. We ran the assay first with *Aplysia* and then with *Stylocheilus*.

Tests for compounds in the skin. Six individuals of *Stylocheilus* and six of *Aplysia* were used to test for the presence of known secondary metabolites in the skin. Fresh skin samples from each individual *Aplysia* were extracted in ethyl acetate for gas chromatography-mass spectrometry (GC-MS). GC-MS was conducted using a

Hewlett-Packard 5980 Series II Plus gas chromatograph and an HP 5972 mass selective detector. A poly-imide-coated fused-silica capillary column (BP1, 50 m length, 0.22 mm i.d., 0.25 μm dimethyl siloxane stationary phase; SGE Pty Ltd) was used. Samples were injected in the splitless mode at an inlet pressure of 170 kPa at 70°C. The injection port temperature was maintained at 250°C with a ramp of 70 to 290°C. Helium was used as the carrier gas at a flow rate of 0.5 ml min^{-1}. Ion peaks were searched via the scan mode and analyzed with the software HP ChemStation (1993) to check for matches in the sample peaks with the known *Portieria* secondary metabolites, apakaochtodene A and B. The skin samples from the six *Stylocheilus* individuals were extracted in ethyl acetate to search for *Lyngbya* secondary metabolites, malyngamide A and B. Because malyngamides are not volatile enough for GC-MS analysis, the extracts were spotted on a silica TLC plate. Alongside the extract samples were samples of *Lyngbya* extract and extracted digestive glands from the six *Stylocheilus* individuals.

Results

First Palatability Assay (*Stylocheilus striatus* alone)

All *Stylocheilus* individuals in all diet groups and all defensive secretion groups were eaten (Fig. 5.1). Neither diet nor the presence of defensive secretions affected ultimate survivorship. Two fishes were observed to prey on the seahares: sergeant majors (*Abudefduf vaigiensis*) and wrasses (*Thalassoma hardwicke* and *T. lutescens*). Though all the *Stylocheilus* were ultimately eaten, we observed that sometimes fish delayed a second attack after ink release.

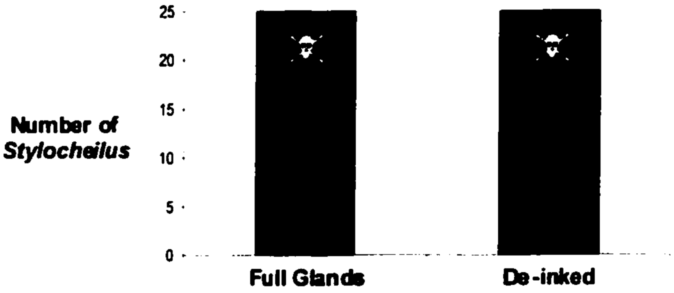

Figure 5.1 *Stylocheilus striatus* Survival with Full and Depleted Glands in Encounters with Reef Fishes. *Stylocheilus* individuals maintained on three different cyanobacterial diets were each divided into two groups: a full gland group and a de-inked group. All *Stylocheilus* individuals were eaten regardless of diet or experimental treatment.

Second Palatability Assay (*Stylocheilus striatus* and *Aplysia parvula*)

Individuals of *Aplysia* were offered first, and 8 of the 18 were ultimately eaten. There was no statistical difference between those that were able to release ink and opaline and those that had been de-inked (Fig. 5.2). Though the composition of fishes was qualitatively the same as the previous day, only wrasses ate any *Aplysia* individuals. Many fish approached within a centimeter or so of the seahare but did not attack. Many that did sample individual *Aplysia* rejected them after the first bite. Of those that were eaten all were rejected an average of 10 times before finally being consumed. The larger *Stylocheilus* individuals were offered after the *Aplysia* experiment to the same group of fishes and, as on the first day, all were quickly eaten regardless of whether they were able to release defensive secretions. Again with both *Aplysia* and

Stylocheilus, fish were sometimes observed to delay a second attack after the release of ink/opaline, but it did not affect the ultimate survival of the seahares.

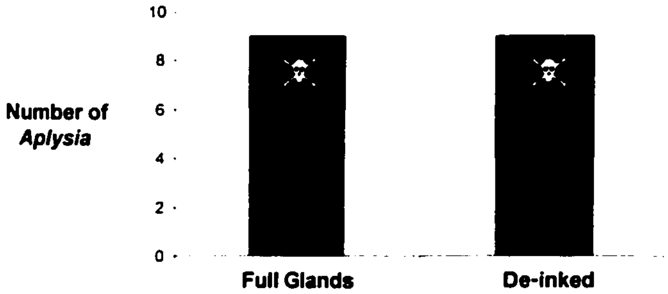

Figure 5.2 *Aplysia parvula* Survival with Full and Depleted Glands in Encounters with Reef Fishes. All *Aplysia parvula* individuals (n=18) had been raised on their normal chemically defended diet of *Portieria hornemannii* and then divided into two groups of nine, paired by size. One group was de-inked and the other allowed to maintain full ink and opaline glands. Though most *Aplysia* individuals were not eaten by the reef fishes, there was no statistically significant difference between the two groups (Fisher exact, one-tailed, p=0.3186).

Tests for compounds in the skin.

GC-MS analysis showed that the secondary metabolites of *Portieria*, apakaochtodenes A and B, were found in the skin samples of all six individuals of *Aplysia*. On the other hand the cyanobacterial secondary metabolites, malyngamides A and B, from *Lyngbya* were not found in any of the *Stylocheilus* skin samples though they were detectable in the digestive glands of the same individuals.

Discussion

In this study I addressed three questions in regard to the importance of seahare active chemical defenses against attack by reef fishes. The answer to the first question, (Do seahares with full defensive glands have a greater chance of surviving predatory encounters with reef fishes than those with depleted glands?), is no. Fullness of gland played no part in ultimate survival under these experimental conditions. All *Stylocheilus* individuals were eaten, and the majority of *Aplysia* individuals were not. The answer to the second question, (Are there differences in survivability between seahares of different species each fed on their natural, chemically-defended diets?), is yes. *Aplysia*, even when much smaller in size than *Stylocheilus*, was less likely to be eaten by reef fishes. The answer to the third question, (Do dietary differences within a seahare group affect survivability?), is no. Under these experimental conditions, *Stylocheilus* raised on three different diets, showed no difference in survivability. Pennings and Paul (2001), however, have shown such a differences in survivability of *Aplysia* with diet.

All of the answers above should be viewed within the context of the experimental conditions. One of the first issues that is often raised about these types of feeding assays with reef fishes is that they do not mimic the natural conditions that seahares face. This is true in that seahares are unlikely to be found falling through the water column. The strength of such is experiments is not in their ecological realism, but what they can tell us about the strength of defenses. Such a test puts the experimental organism through a worst-case scenario, such as if a seahare were knocked off the substrate and past hundreds of hungry fish. The test is one-sided in the sense that if seahares are eaten, they may still be defended – we do not know, but if they consistently survive such a gauntlet of mouths we can be certain that they are well defended against the fish species that are present. By this method, I confirmed the results of Ginsburg and Paul (2001) that *Aplysia parvula* raised on the chemically-rich red alga *Portieria hornemannii* is well defended against reef fishes. I also confirmed that the antifeedent compounds apakaochtodene A and B were present in the skin of *Aplysia*. For *Stylocheilus* on the other hand the question is still largely unanswered. I did not find appreciable malyngamide antifeedent compounds in their skin though they were present in the digestive glands. It is possible, nonetheless, that *Stylocheilus* is still defended against some fishes but not others. Of all of the fishes present, including goatfishes, parrot fishes, trigger fishes and many others, only two types consistently ate *Stylocheilus* - wrasses (24 eaten) and sergeant majors (43 eaten). Sergeant majors, however, never ate any *Aplysia*. The 8 that were eaten were all eaten by wrasses. It is unlikely that sergeant majors so completely out-competed wrasses against *Stylocheilus* and not against *Aplysia*. All types of fishes present attacked the food pellets.

A potentially interesting observation was made on both days and with both types of seahares when large amounts of ink were released. Many of the fish appeared to "jump back" and hesitate before resuming attack. Unfortunately the experiment was not designed to score a startle effect in any meaningful way and the video footage was not sharp enough to be able to do so after the fact. This does, however, suggest a potential startle function for seahare ink against fish predators (Edmunds 1974). Such a startle is unlikely to have much of an effect on survivability for seahares dropped through the water column, as in these experiments. Perhaps, however, for seahares attacked while on the surface of macroalgae or cyanobacterial mats, such a startle would allow enough time for the seahare to escape to the interior. This question is one that can be addressed in future experiments.

To date, all of the experiments examining seahare defenses against fishes (this one included) have focused on the issue from the perspective of the seahare. Such studies use the fish simply as a tool to tell something about seahare biology and for that purpose they are useful. Perhaps now it might be interesting to look at these issues from the perspective of the predators, or more appropriately through their nervous systems as a whole. Why do some fishes, and not others, always reject seahares? Why are wrasses much more likely to eat seahares yet still reject many? Is there something about the taste or odor of seahares that advertises their unpalatibility? Are fish that eat seahares filled with algal toxins aversely affected later, and if so do they learn to avoid eating seahares in the future? These are just a few of the questions that could be addressed as we try to understand the mechanisms of seahare chemical defense.

Chapter VI. General Conclusions

The objectives of this research are to address 1) how individual prey with limited defensive repertoires defend against multiple species of predators; and 2) whether all chemical defense function as honest signals of danger from prey to predator. In Chapter I, I established four research questions and here I attempt to answer those question and discuss their implications.

Research Question #1: Are seahare chemical defenses effective against multiple predators from different phyla?

The answer is a qualified yes. I considered the behavioral responses of generalist predators from three phyla. Nolen *et al.* 1995 established that ink was an effective deterrent against sea anemone predators. Ink enhances the survival of seahares engulfed by predatory sea anemones in comparison to those without ink.

My experiments with portunid crabs and *Stylocheilus striatus* as well as those with spiny lobsters (*Panulirus interruptus*) and *Aplysia californica* showed that ink/opaline secretions provided a significant survival advantage to the seahares in comparison with seahares whose secretions had been depleted. These experiments also suggest that the mechanism of defense may be effective against crustacean predators in general and is not specific to any particular species. Such a strategy, though perhaps not optimized for a particular predator, would allow seahares to defend against the wide range of crustacean generalist predators.

The results of my experiments with reef fishes and the seahares *Stylocheilus striatus* and *Aplysia parvula* suggest that the active defenses of ink and opaline are likely less important than the passive defense of distasteful skin against these vertebrate predators. Previous studies have established that seahares raised on algae that harbor many secondary metabolites are rejected more by fish than those raised on toxin-free algae (Pennings 1990a; Ginsburg and Paul 2001). Such a difference in the smell and taste of seahare skin is even discernable by the human nose and palate (Nolen *et al.* 1995). The role, if any, of ink and opaline in the rejection of seahares by most species of fish was unknown. My experiments suggest that the secretions do not increase the seahare's chances of survival with fish in the context of a water column feeding experiment (Hay *et al.* 1998) where seahares are dropped through the water column past tens to hundreds of hungry fish. It is still possible, however, that in a more realistic setting that ink could function as deimatic or startle display (Edmunds 1974), providing the seahare a few extra moments to disappear into a clump of algae. Hints of this were seen in the water column feeding experiments when fish hesitated after ink release, before resuming an attack. Experiments specifically designed to test this hypothesis will be necessary before concluding that ink and opaline secretion plays no defensive role against fish predators.

Objective 1. Are the ink and opaline secretions attractive, repulsive, or neutral to predators of three different phyla?

Experiments with sea anemones confirmed that ink was repulsive to sea anemones, causing tentacle contractions, as described by Nolen *et al.* (1995). Opaline, on the other hand, induced a feeding response in sea anemones – the mouth opened and tentacles that had contacted the opaline were brought to the mouth. Pilot experiments with portunid crabs and controlled experiments with spiny lobsters showed that both ink and opaline were feeding stimulants and that opaline was not significantly different from squid extract in its stimulatory qualities. Since the field studies with reef fishes did not suggest that seahare secretions provided a survival advantage, the secretions were not tested with fishes for their attractive or repulsive properties.

Research Question #2: If seahare chemical defenses are effective, what are the likely active components of their defensive secretions?

Having established that seahare secretions are effective against sea anemone and crustacean predators, in that they provide a survival advantage to the individuals that have the secretions, it was necessary to determine what the likely active components were for these two phyla.

Objective 2a. Isolate proteins from the secretions. Since proteins with cytolytic effects have been isolated from the ink of several species of seahares (Kamiya *et al.* 1989; Yamazaki *et al.* 1989a; 1989b; Melo *et al.* 2000; Petzelt *et al.* 2002), proteins were likely candidates for the deterrent properties of ink against sea anemones. This was further suggested by my experiments showing that heated ink, which would contain denatured proteins, was significantly less effective at causing tentacle contraction in sea anemones. Having found a likely candidate protein band by gel electrophoresis, I was able to purify this protein using Fast Protein Liquid Chromatography (FPLC).

This protein (along with crude ink) showed antibacterial activity as seen in the proteins isolated from the ink of other seahares. It thus continued to be a candidate for the active component of ink against sea anemones. I was able to clone and sequence this protein and over-express it in *E. coli*. For its potential role in seahare escape from anemone predation, I tentatively named the protein "escapin". I will discuss this role under question #3, below.

Objective 2b. Determine the free amino acid content of the secretions.

Having established that spiny lobsters and likely crabs were attracted to ink and opaline, I looked for the most likely candidates for that attractive response. Free amino acids are extremely stimulatory to crustaceans (Carr and Derby 1986; Zimmer-Faust 1987) and had been found in *Aplysia* ink (Troxler *et al.* 1981). Samples of ink and opaline analyzed by The Scientific Research Consortium, Inc. (www.aminoacids.com) contained high levels of amino acids, most notably taurine was found at 231 mM concentration in opaline. Since taurine is one of most stimulatory of amino acids to crustacean feeding (Johnson and Atema 1986; Daniel and Derby 1991; Fine-Levy and Derby 1992; Sung *et al.* 1996), such high concentrations in opaline were likely significant.

Research Question #3: How do the active components function to deter predation?

After establishing that the secretions provide a survival advantage in behavioral assays against different predators and then developing likely candidate compounds for the behavioral responses seen by the predators, I was ready to evaluate the possible mechanisms of their effects against predators.

Objective 3a. Can the active components cause any physical damage to predators?

Escapin, the protein that I had isolated from the ink and cloned from the ink gland of *Aplysia californica*, caused cytolytic activity under Nomarsky and fluorescence microscopy in studies of sea anemone tentacles. Tentacles incubated in control solutions (bovine serum albumin, artificial seawater alone, opaline) showed no damage. Escapin that had been over-expressed in *E. coli*, and thus lacked post-translational modifications, had neither antibacterial effects nor cytolytic activity against sea anemone tentacles. This inactivity of the bacterially expressed form was also expected from the results of Petzelt *et al.* (2002) on the expressed ink protein, cyplasin L, from *Aplysia punctata.* A next step will be to over-express the protein in a eukaryotic system, which should produce a functional polypeptide, as was shown in Petzelt *et al.* (2002). Though short peptides have been implicated as mediators of chemical interactions between and within species (Thompson *et al.* 1986; Ireland *et al.* 1989; Rittschof 1990), escapin is the first reported protein used for chemical defense in any organism. After attractin (Painter *et al.* 1998), a pheromonal protein also from *Aplysia californica*, escapin is only the second characterized protein with any known role in the chemical ecology of a marine organism.

With such a powerful effect of cytolytic escapin on sea anemones, one might expect that crustaceans would be affected as well. Though I have yet to try purified escapin alone on crustaceans in either a behavioral or physiological context it is unlikely that the protein will have a similar effect. Unlike, sea anemones, most cells of crustaceans are not directly exposed to sea water. Even sensory structures are covered with thin porous cuticle that allows seawater, but not all molecules, to pass (Derby *et al.* 1997). The porosity of most crustacean sensilla has yet to be determined, but we do know that the cuticle surrounding aesthetasc sensilla of Caribbean spiny lobsters is impermeable to molecules over 8 kDa (Derby *et al.* 1997). Escapin has a molecular weight of 60 kDa and thus would not permeate this sensillar type. Certainly if the lobsters eat the ink, then they would be exposed internally. What effect, if any, escapin has within the digestive tract of crustaceans is yet to be determined. It is interesting to note that in two of three reported medical cases of human poisoning following the ingestion of seahares, the liver was affected (Hino *et al.* 1994; Sakamoto *et al.* 1998). In both cases it appears that hepatocytes underwent cytolytic damage and one group of authors (Sakamoto *et al.* 1998) suggests that this may be due to the cytolytic proteins of seahares, including those found in ink. Perhaps the hepatopancreas of crustaceans would be a good place to look for negative post-ingestive effects.

Objective 3b. Can the active components stimulate the sensory system in some way?

To address this question, crustaceans were the best choice from the three phyla of seahare predators under study. Spiny lobsters in particular, are a well established model system in chemosensory neurobiology (Ache and Derby 1985; Zimmer-Faust 1987; Derby 1995; Trapido-Rosenthal 1995; Derby 2000), and were well suited for investigating the sensory mechanisms of seahare defense. The results of experiments with *Aplysia dactylomela* ink and sympatric *Panulirus argus* as well as those with *A. californica* and sympatric *P. interruptus* suggest that taurine-sensitive chemosensory neurons in the lobster antennules were activated by opaline and ink. These same neurons were also stimulated by artificial mixtures of ink and opaline that contained only amino acids and ammonia in the appropriate concentrations (determined by the analysis of free amino acids, discussed above). This suggests that free amino acids and ammonia found in opaline and ink in very high concentrations may be sufficient to stimulate the feeding behavior of the lobsters. This feeding behavior, directed toward the secretions and not the seahare, seems to provide for a very unusual form of chemical defense, phagomimicry – discussed below.

Research Question #4: Can chemical defenses represent both honest and dishonest signals, depending on the predator receiving the signals?

The answer appears to be, yes. Though discussions of chemical defenses often treat them all as toxic compounds (Edmunds 1974; Berenbaum 1995) and thus honest signals (Harper 1991), some authors have suggested that chemical defenses could function in a myriad of deceptive ways to fool predators into rejecting an otherwise palatable organism (Hay 1996). Such deception is the basis of Batesian mimicry and is also used by predators to lure prey, as with angler fish (Wooton 1990) and fireflies (Lloyd 1975), and is used by males to lure females with "empty" nuptial gifts (Sadowski *et al.* 1999). It is just as likely that prey would be able to play the same game with their chemical defenses. I am unaware of any such experimentally documented cases in either a terrestrial or aquatic system.

The results of this study show that seahares concentrate stimulatory amino acids and ammonia in their opaline and ink secretions, more than 320-fold and 54-fold higher, respectively, than in haemolymph. These compounds are held together in the sticky opaline, a way of preventing the allelochemicals from rapidly dispersing (Sullivan and Webb 1983). This opaline/ink mixture coats the antennules and mouth parts of crustaceans, keeping the stimuli in close contact. Such a supernormal stimulus (Tinbergen 1951), with amino acid concentrations much higher than in real food, can function as a sensory trap (Christy 1995) to the attacking crustacean. In the majority of situations that a crustacean will face normally, such a high concentration of amino acids indicates a truthful signal of an available nutritional item. Responding in this way to amino acid stimuli increases the fitness of crustaceans by leading them to locate and exploit valuable food sources, but it is this stimulatory drive that seahares may exploit with their chemical defenses. This phagomimicry, of ink and opaline containing the stimulatory properties of food minus much nutritional value, allows the seahare to escape predators that may be impervious to the cytolytic escapin in ink and are certainly stronger and swifter than the seahares. Since crabs and lobsters would both ingest seahares repeatedly, when they release a seahare and eat the secretions alone they are losing a much larger source of nutrients.

Apparently seahares use the same chemical defensive secretions to signal two completetly different messages to two different target predators. The soft-bodied sea anemones receive the honest signal of cytolytic escapin – to sea anemones the ink of seahares is toxic and good reason for rejecting the otherwise palatable prey. The voracious crustacean predators receive the dishonest signal of false food – to crustaceans the ink and opaline are too attractive to ignore, even if it means losing a more nutritious meal. Honest and dishonest signals within the same defensive package allow seahares to address radically different predators from different phyla on their own sensory terms. A multi-component approach to multiple predatory threats permits organisms to navigate more safely through a world of different, but equally threatening, mouths.

Problems, Issues, and Future Directions

Some unresolved questions: (1) All of the *Aplysia californica* individuals used in this study were raised on relatively palatable *Gracilaria* spp. Might the results be different if they were raised on a more toxic alga such as *Laurencia* spp.? (2) Would the mouthpart chemoreceptor neurons of crustaceans be more relevant than the antennules, for monitoring feeding responses? (3) If opaline is stimulatory, might its release send a mixed signal to sea anemones and fish? (4) Has this kind of defensive appeasement of predators with chemicals been described in insect systems? I address these and other questions below.

(1) All of the *Aplysia californica* individuals used in these experiments were raised on the relatively palatable red alga *Gracilaria tikvahiae*. *A. californica* will feed on a wide variety of macroalgae, but given a choice will choose reds that contain

many secondary metabolites such as *Laurencia* spp. and *Plocamium* spp. (Carefoot 1987; Pennings 1990c). Other seahares species feed almost exclusively on undefended green algae such as *Ulva* spp. (Sarver 1978; Carefoot 1987). No experiments have directly compared the two groups of seahares in predatory encounters, but diet manipulation experiments have shown that a diet of algae rich in secondary metabolites does confer a survival advantage against at least some predators (Pennings 1990a; Ginsburg and Paul 2001). Additionally, using GC-MS I have found known algal secondary metabolites in the ink of *Aplysia parvula* that had been raised on algae containing the metabolites. Other authors, however, have not found significant quantities of algal secondary metabolites in ink of other seahares (Pennings and Paul 1993; de Nys *et al.* 1996; Pennings *et al.* 1999; Rogers *et al.* 2000a). At this point, it cannot be determined if such secondary metabolites are primarily for the deterrence of fish predators or would play a role with other species. A next step would be to conduct similar behavioral and electrophysiological experiments to those described here using seahares reared on algae that contain known secondary metabolites. Perhaps if secondary metabolites were at detectable levels in the skin of the seahare, then perhaps crustaceans and even sea anemones would be less likely to ingest seahares. Or, such ingestion of either the seahares or their secretions might have more negative effects on the fitness of the attacker. All of these options are possibilities.

(2) Why record from antennules of spiny lobsters rather than mouthparts? This choice was made for two reasons. First, antennules were observed to be coated with ink and opaline after a lobster attack on a seahare and thus would likely play a role in

the lobster's response. Second, the majority of literature on lobster chemosenses concerns the antennules. This second reason for choosing the antennules was to utilize established techniques and to compare my results with known responses. At this point, however, a logical next step will be to attempt recordings from the chemosensory neurons of the mouth parts, particularly the 3rd maxillipeds which are used to tear food and bring it to the mouth (Garm and Høeg 2001).

(3) Opaline is stimulatory to sea anemones as well as lobsters and would likely be stimulatory to fish as well. Do seahares release opaline more readily to crustaceans than to the other predators? I do not have a definitive answer to this yet, but have made some observations. Opaline is considered to have a much higher threshold of release than ink. This has been described in both neurophysiological terms (Kandel 1979; Tritt and Byrne 1980) and behavioral terms (Kamiya *et al.* 1989; MacColl *et al.* 1990; Nolen *et al.* 1995). I have handled and dissected hundreds of seahares from different parts of the world but I have never seen opaline release prior to ink release – except when observing attacks by spiny lobsters on *Aplysia californica*. I have observed at least 6 clear cases were opaline was released before ink after a lobster attack. Six may seem like a small number (out of less than 40 attack observations) but such low-threshold opaline release has never been reported for animals known to have full ink glands. Is this an issue of seahares "recognizing" their predators and acting accordingly, as has recently been shown for anurans (Van Buskirk 2001), or is it a more simple gradient response that opaline release is only stimulated by a flesh piercing attack such as that by a crustacean? If either is true, then seahares may

prevent an inappropriate opaline release to fish or sea anemone predators. Changing thresholds is interesting from a neuroethological perspective and reasonably tractable since putative motoneurons for ink and opaline release have already been identified (Carew and Kandel 1977a, 1977b, 1977c; Tritt and Byrne 1980, 1982).

(4) The phagomimicry of opaline and ink described as a defense against crustacean predators sounds similar to defenses against other arthropod predators in terrestrial systems. An appeasement secretion has been described in a quasi-symbiotic relationship between beetles and ants. The larvae of the staphylinid beetle *Pella funesta* live along the foraging trails and particularly in the garbage mounds of the ant *Lasius fuliginosus* where the beetle larvae feed on dead ants. When ants discover the beetle larvae they usually attack. At this point a larva may release a white secretion from its abdominal tip which it directs towards the head of the attacking ant. When this occurs the ant stops the attack and "licks up" the secretion (Hölldobler *et al.* 1982). This interesting description is a good candidate for a terrestrial example of phagomimicry. The *Pella* secretion has never been analyzed and no experiments have been conducted to determine the mechanism of appeasement. There are several other examples from the arthropod literature that may be worth further study. Phagomimicry may be in play, particularly in cases were a predator is reported to stop an attack and clean its mouthparts (but is not harmed in any noticeable way) after the secretion of a chemical defense. This mouth part "cleaning" was initialing observed by Walters *et al.* 1993 and later by me, after crustacean attacks on seahares. In many cases, more careful experiments are needed to tease apart cleaning the legs and antennae, which

usually involves bringing them across the mouth, and actual feeding on the secretions. Two likely phagomimicry candidates that have been described in this way are the anal defensive secretions of larval chrysopidae which the larva carefully places on the mouth of an attacking ant (LaMunyan and Adams 1987) and the foaming defense of *Pachycondyla* ants (Maschwitz *et al.* 1981). There are likely many others in both terrestrial and aquatic systems. Fooling predators with false scents of food, having evolved so many times for attracting mates, prey, and pollinators (for many examples see Stowe 1988), is likely to be a strategy used by many different organisms. Phagomimicry is probably not just for seahares.

List of References

Ache, B. W. and D. L. Macmillan (1980). Neurobiology. In: *The biology and management of lobsters*. J. S. Cobb and B. F. Phillips, Eds. New York, Academic Press: 165-173.

Ache, B. W. and C. D. Derby (1985). "Functional organization of olfaction in crustaceans." *Trends in the Neurosciences* **8**(8): 356-360.

Advokat, C. (1980). "Modulation of defensive reflexes in *Aplysia californica* by appetitive stimulation." *Behavioral and Neural Biology* **28**: 253-265.

Alves-Gomes, J., G. Ortí, M. Haygood, W. F. Heiligenberg and A. Meyer (1995). "Phylogenetic analysis of the South American electric fishes (Order Gymnotiformes) and the evolution of their electrogenic system: a synthesis based on morphology, electrophysiology, and mitochondrial sequence data." *Molecular Biology and Evolution* **12**(2): 298-318.

Ambrose, H. W., III, R. P. Givens, R. Chen and K. P. Ambrose (1979). "Distastefulness as a defense mechanism in *Aplysia brasiliana* (Mollusca: Gastropoda)." *Marine Behavior and Physiology* **6**: 57-64.

Ando, Y. (1952). "[The toxicity of *Tethys kurodai*]." *Kagaku (Tokyo)* **22**: 87-88.

Avila, C. (1995). "Natural products of opisthobranch molluscs: a biological review." *Oceanography and Marine Biology Annual Review* **33**: 487-559.

Bebbington, A. (1969). "*Bursatella leachi Guineensis* Subsp. Nov. (Gastropoda, Opisthobranchia) from Ghana." *Proceedings of the Malacological Society, London* **38**: 323-341.

Bebbington, A. (1974). "Aplysiid species from East Africa with notes on the Indian Ocean Aplysiomorpha (Gastropoda: Opisthobranchia)." *Zoological Journal of the Linnean Society* **54**: 63-99.

Bebbington, A. (1977). "Aplysiid species from eastern Australia with notes on the Pacific Ocean Aplysiomorpha (Gastropoda, Opisthobranchia)." *Transactions of the Zoological Society of London* **34**: 87-147.

Beeman, R. D. (1968). "The order Anaspidea." *Veliger* **3**(Supplement): 87-102.

Berenbaum, M. R. (1995). "The chemistry of defense: theory and practice." *Proceedings of the National Academy of Science USA* **92**(1): 2-8.

Boothby, K. M. and I. D. McFarlane (1986). "Chemoreception in sea anemones: betaine stimulates the pre-feeding response in *Urticina eques* and *U. felina.*" *Journal of Experimental Biology* **125**: 385-389.

Brodie, E. D., Jr. (1977). "Hedgehogs use toad venom in their own defense." *Nature, Lond.* **268**: 627-628.

Bryan, P. J., W. Y. Yoshida, J. B. McClintock and B. J. Baker (1995). "Ecological role for pteroenone, a novel antifeedent from the conspicuous Antarctic pteropod *Clione antarctica* (Gymnosomata: Gastropoda)." *Marine Biology* **122**: 271-277.

Burn, R. (1963). "Australian Runcinacea." *Australian Journal of Zoology* **13**(1): 9-22.

Byrne, J. H. (1980). "Neural circuit for inking behavior in *Aplysia californica.*" *Journal of Neurophysiology* **43**: 896-911.

Carefoot, T. H. (1987). "*Aplysia*: its biology and ecology." *Oceanography and Marine Biology Annual Review* **25**: 167-284.

Carefoot, T. H., S. C. Pennings and J. P. Danko (1999). "A test of novel function(s) for the ink of sea hares." *Journal of Experimental Marine Biology and Ecology* **234**(2): 185-197.

Carew, T. J. and I. Kupfermann (1974). "The influence of different natural environments on habituation in *Aplysia californica.*" *Behavioral Biology* **12**: 339-345.

Carew, T. J. and E. R. Kandel (1977a). "Inking in *Aplysia californica.* I. Neural circuit of an all-or-none behavioral response." *Journal of Neurophysiology* **40**(3): 692-707.

Carew, T. J. and E. R. Kandel (1977b). "Inking in *Aplysia californica.* II. Central program for inking." *Journal of Neurophysiology* **40**: 708-720.

Carew, T. J. and E. R. Kandel (1977c). "Inking in *Aplysia californica.* III. Two different sympatric conductance mechanisms of triggering central program for inking." *Journal of Neurophysiology* **40**: 721-734.

Carlson, B. A. (1997).Analysis of chemoreceptive responses in the dactyls of *Cancer antennarius* to the aversive ink of *Aplysia californica.* B.S. Honors Thesis. Dept. of Biology, University of Miami.

Carlson, B. A. and T. G. Nolen (1997). "The effect of *Aplysia*'s defensive chemical ink on the dactyl chemoreceptors of predatory crabs (*Cancer anntenarius*)." *Society for Neuroscience Abstracts* **23**: 188.

Carr, W. E. S. and C. D. Derby (1986). "Chemically stimulated feeding behavior in marine animals: importance of chemical mixtures and involvement of mixture interactions." *Journal of Chemical Ecology* **12**(5): 989-1011.

Chapman, D. J. and D. L. Fox (1969). "Bile pigment metabolism in the sea-hare *Aplysia*." *Journal of Experimental Marine Biology and Ecology* **4**: 71-78.

Cho, K. and I. D. McFarlane (1995). "A method for recording electrical activity from the body wall nerve nets in sea anemones." *Journal of Experimental Biology* **198**: 817-820.

Christamanos, A. (1955). "Nature of the pigment of *Aplysia depilans*." *Nature, Lond.* **175**: 310.

Christy, J. H. (1995). "Mimicry, mate choice, and the sensory trap hypothesis." *American Naturalist* **146**(2): 171-181.

Cruz-Rivera, E. and M. E. Hay (2001). "Macroalgal traits and the feeding and fitness of an herbivorous amphipod: the roles of selectivity, mixing, and compensation." *Marine Ecology-Progress Series* **218**: 249-266.

Daly, J. W. (1995). "The chemistry of poisons in amphibian skin." *Proceedings of the National Academy of Science USA* **92**: 9-13.

Daniel, P. C. and C. D. Derby (1991). "Mixture suppression in behavior: the antennular flick response in the spiny lobster towards binary odorant mixtures." *Physiology and Behavior* **49**: 591-601.

Dawkins, R. and J. R. Krebs (1978). Animal signals: information or manipulation? In: *Behavioral Ecology: An Evolutionary Approach*. N. B. Davies, Ed. Oxford, Blackwell Scientific Publications: 282-309.

de Nys, R., P. D. Steinberg, C. N. Rogers, T. S. Charlton and M. W. Duncan (1996). "Quantitative variation of secondary metabolites in the sea hare *Aplysia parvula* and its host plant, *Delisea pulchra*." *Marine Ecology-Progress Series* **130**(1-3): 135-146.

Derby, C. D., P. M. Reilly and J. Atema (1984). "Chemosensitivity of lobster, *Homarus americanus*, to secondary plant compounds: unused receptor capabilities." *Journal of Chemical Ecology* **10**(6): 879-892.

Derby, C. D. (1995). Single unit electrophysiological recordings from crustacean chemoreceptor neurons. In: *Experimental cell biology of taste and olfaction: current techniques and protocols.* A. I. Spielman and J. G. Brand, Eds. Boca Raton, CRC Press: 241-251.

Derby, C. D., H. S. Cate and L. R. Gentilcore (1997). "Perireception in olfaction: molecular mass sieving by aesthetasc sensillar cuticle determines odorant access to receptor sites in the Caribbean spiny lobster *Panulirus argus.*" *Journal of Experimental Biology* **200**: 2073-2081.

Derby, C. D. (2000). "Learning from spiny lobsters about chemosensory coding of mixtures." *Physiology and Behavior* **69**: 203-209.

Derby, C. D., P. Steullet, A. J. Horner and H. S. Cate (2001). "The sensory basis to feeding behavior in the Caribbean spiny lobster *Panulirus argus.*" *Marine and Freshwater Research* **52**: 1339-1350.

DeWitt, T. J., B. W. Robinson and D. S. Wilson (2000). "Functional diversity among predators of a freshwater snail imposes an adaptive trade-off for shell morphology." *Evolutionary Ecology Research* **2**(2): 129-148.

DiMatteo, T. (1981). "The inking behavior of *Aplysia dactylomela* (Gastropoda: Opisthobranchia): evidence for distastefulness." *Marine Behaviour and Physiology* **7**: 285-290.

DiMatteo, T. (1982a). "Investigation into interspecific encounters of the sea hare *Aplysia dactylomela* Rang, 1828 (Gastropoda: Opisthobranchia)." *Veliger* **24**(1): 72-75.

DiMatteo, T. (1982b). "The ink of *Aplysia dactylomela* (Rang, 1828) (Gastropoda: Opisthobranchia) and its role as a defensive mechanism." *Journal of Experimental Marine Biology and Ecology* **57**: 169-180.

Dumbacher, J. P., B. M. Beehler, T. F. Spande, H. M. Garraffo and J. W. Daly (1992). "Homobatrachotoxin in the genus *Pitohui*: chemical defense in birds?" *Science* **258**(5083): 799-801.

Eales, N. B. (1921). "*Aplysia.*" *Transactions of the Liverpool Biological Society* **24**: 84.

Eales, N. B. (1944). "Aplysiids from the Indian Ocean, with a review of the family Aplysiidae." *Proceedings of the Malacological Society, London* **26**: 1-22.

Eales, N. B. (1960). "Revision of the world species of *Aplysia* (Gastropoda, Opisthobranchia)." *Zoology* **5**(10): 267-404.

Edmunds, M. (1974). *Defence in Animals: A survey of antipredator defences*. Harlow, Essex, Longman, Group.

Eisner, T. and J. Meinwald (1966). "Defensive secretions of arthropods." *Science* **153**: 1341-1350.

Eisner, T. and J. Meinwald (1995). "Chemical ecology." *Proceedings of the National Academy of Science USA* **92**: 1.

Eklov, P. (2000). "Chemical cues from multiple predator-prey interactions induce changes in behavior and growth of anuran larvae." *Oecologia* **123**(2): 192-199.

Endler, J. A. (1986). Defense against predators. In: *Predator-prey relationships: perspectives and approaches from the study of lower vertebrates*. G. V. Lauder, Ed. Chicago, Univ. of Chicago Press: 109-134.

Faulkner, D. J. and M. T. Ghiselin (1983). "Chemical defense and evolutionary ecology of dorid nudibranchs and some other opisthobranch gastropods." *Marine Ecology Progress Series* **13**: 295-301.

Faulkner, D. J. (1992). Chemical defenses in marine molluscs. In: *Ecological Roles of Marine Natural Products*. V. J. Paul, Ed. Ithaca, NY, Comstock: 119-163.

Fine-Levy, J. B. and C. D. Derby (1992). "Behavioral discrimination of binary mixtures and their components: effects of mixture interactions on coding of stimulus intensity and quality." *Chemical Senses* **17**(3): 307-323.

Fiorito, G. and F. Gherardi (1990). "Behavioral changes induced by ink in *Aplysia fasciata* (Mollusca, Gastropoda): evidence for a social signal role of inking." *Marine Behavior and Physiology* **17**: 129-135.

Fire, A., S. Xu, M. K. Montgomery, S. A. Kostas, S. E. Drive and C. C. Melo (1998). "Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*." *Nature* **391**: 806-811.

Fischer, T. M., J. W. Yuan and T. J. Carew (2000). "Dynamic regulation of the siphon withdrawal reflex of *Aplysia californica* in response to changes in the ambient tactile environment." *Behavioral Neuroscience* **114**(6): 1209-1222.

Flury, F. (1915). "Uber das Aplysiengift." *Archiv fur experimentelle Pathologie und Pharmakologie* **79**: 250-263.

Frost, W. and E. R. Kandel (1995). "Structure of the network mediating siphon-elicited siphon withdrawal in *Aplysia*." *Journal of Neurophysiology* **73**(6): 2413-2427.

Garm, A. and J. T. Høeg (2001). "Function and functional groupings of the complex mouth apparatus of the squat lobsters *Munida sarsi* Huus and *M. tenuimana* G.O. Sars (Crustacea: Decapoda)." *Biological Bulletin* **200**: 281-297.

Getting, P. A. and A. O. D. Willows (1973). "Burst formation by inhibition through electrically coupled neurons." *Brain Research* **61**: 13-18.

Getting, P. A. (1974). " Modification of neuron properties by electronic synapses. I. Input resistance, time constant, and integration." *Journal of Neurophysiology* **37**: 846-857.

Ghiselin, M. T. (1963). "On the functional and comparative anatomy of *Runcina setoensis* Baba, an opisthobranch gastropod." *Publications of the Seto Marine Biological Laboratory* **11**(2): 219-229.

Ginsburg, D. W. and V. J. Paul (2001). "Chemical defenses in the sea hare *Aplysia parvula*: importance of diet and sequestration of algal secondary metabolites." *Marine Ecology Progress Series* **215**: 261-274.

Gonzalez, M. J. and A. J. Tessier (1997). "Habitat segregation and interactive effects of multiple predators on a prey assemblage." *Freshwater Biology* **38**(1): 179-191.

Gosliner, T. M. (1994). Gastropoda: Opisthobranchia. In: *Microscopic Anatomy of Invertebrates Volume 5: Mollusca I*. F. Harrison, W. and A. J. Kohn, Eds. New York, Wiley-Liss Inc.: 253-355.

Gronquist, M. R. and J. Meinwald (2001). "Synthesis of unsaturated polyazamacrolides from the ladybird beetle *Subcoccinella vigintiquatuorpunctata*." *Journal of Organic Chemistry* **66**(4): 1075-1081.

Harper, D. G. C. (1991). Communication. In: *Behavioural ecology: an evolutionary approach*. J. R. Krebs and N. B. Davies, Eds. Oxford, Blackwell Scientific Publications: 374-397.

Hasson, O. (1994). "Cheating signals." *Journal of Theoretical Biology* **167**(3): 223-238.

Hay, M. E. (1996). "Marine chemical ecology: what's known and what's next?" *Journal of Experimental Marine Biology and Ecology* **200**: 103-134.

Hay, M. E., J. J. Stachowicz, E. Cruz-Rivera, S. Bullard, M. S. Deal and N. Lindquist (1998). Bioassays with marine and freshwater macroorganisms. In: *Methods in chemical ecology*. K. F. Haynes and J. G. Millar, Eds. Norwell, MA, Kluwer Academic Publishers: 39-141.

Hino, K., Y. Mitsui and Y. Hirano (1994). "Four cases of acute liver damage following ingestion of a sea hare." *Journal of Gastroenterology* **29**: 679.

Hoffmann, H. (1935). *Opisthobranchia*. Leipzig, Akademische Verlagagesellschaft.

Hölldobler, B., M. Moglich and U. Maschwitz (1982). "Myrmecophilic relationship of *Pella* (Coleoptera: Staphylinidae) to *Lasius fuliginosus* (Hymenoptera: Formicidae)." *Psyche* **88**: 347-374.

Illich, P. A., R. L. Joynes and E. T. Walters (1994). "Response-specific inhibition during general facilitation of defensive responses in *Aplysia*." *Behavioral Neuroscience* **108**: 614-623.

Ireland, C. M., T. F. Molinski, D. M. Roll, T. M. Zabriskie, T. C. McKee, J. C. Swersey and M. P. Foster (1989). Natural products peptides from marine organisms. In: *Biooganic marine chemistry*. P. J. Scheuer, Ed. Berlin, Springer-Verlag: 1-46.

Jahan-Parwar, B. and S. M. Fredman (1979). "Neural control of locomotion in *Aplysia*: Role of the central ganglia." *Behavioral and Neural Biology* **27**: 39-58.

Johnson, B. R. and J. Atema (1986). "Chemical stimulants for a component of feeding behavior in the common gulf-weed shrimp *Leander tenuicornis* (Say)." *Biological Bulletin* **170**: 1-10.

Johnson, P. M. and T. G. Nolen (1991). "Conspecific ink is aversive to juvenile *Aplysia californica*." *American Zoologist* **31**(5): 112A.

Johnson, P. M. (1994).Chemical defense in the gastropod mollusc *Aplysia*. B.S. Honors Thesis. Dept. of Biology, University of Miami.

Johnson, P. M. and A. O. D. Willows (1999). "Defense in sea hares (Gastropoda, Opisthobranchia, Anaspidea): multiple layers of protection from egg to adult." *Marine and Freshwater Behavior and Physiology* **32**: 147-180.

Johnston, G. (1850). *An introduction to conchology, or, elements of the natural history of molluscous animals*. London, John van Voorst.

Johnstone, R. A. and A. Grafen (1993). "Dishonesty and the handicap principle." *Animal Behaviour* **46**: 759-764.

Kamiya, H. and Y. Shimizu (1981). "A natural agglutinin inhibitable by D-galacturonic acid in the sea hare *Aplysia* eggs: characterization and purification." *Bulletin of the Japanese Society of Scientific Fisheries* **47**(2): 255-259.

Kamiya, H., K. Muramoto and M. Yamazaki (1986). "Aplysianin-A, an antibacterial and antineoplastic glycoprotein in the albumen gland of a sea hare, *Aplysia kurodai*." *Experientia* **42**: 1065-1067.

Kamiya, H., K. Muramoto, R. Goto and M. Yamazaki (1988). "Characterization of the antibacterial and antineoplastic glycoproteins in a sea hare *Aplysia juliana*." *Nippon Suisan Gakkaishi* **54**(5): 773-777.

Kamiya, H., K. Muramoto, R. Goto, M. Sakai, Y. Endo and M. Yamazaki (1989). "Purification and characterization of an antibacterial and antineoplastic protein secretion of a sea hare, *Aplysia juliana*." *Toxicon* **27**(12): 1269-1277.

Kamiya, H. and M. Yamazaki (1989). "Purification of dolabellanin-C, an antineoplastic glycoprotein in the body fluid of a sea hare, *Dolabella auricularia*." *Developmental and Comparative Immunology* **13**: 3-8.

Kandel, E. R. (1979). *Behavioral Biology of Aplysia*. San Francisco, CA, Freeman and Co.

Kay, E. A. (1964). "The Aplysiidae of the Hawaiian islands." *Proceedings of the Malacological Society, London* **36**: 173-190.

Kinnel, R. B., R. K. Dieter, J. Meinwald, D. C. J. Van Engen, T. Eisner, M. O. Stallard and W. H. Fenical (1979). "Brasilenyne and *cis*-dihydrorhodophytin: antifeedant medium-ring haloethers from a sea hare (*Aplysia brasiliana*)." *Proceedings of the National Academy of Science USA* **76**(8): 3576-3579.

Kisugi, J., H. Kamiya and M. Yamazaki (1987). "Purification and characterization of aplysianin-E, an antitumor factor from sea hare eggs." *Cancer Research* **47**: 5649-5653.

Kisugi, J., M. Yamazaki, Y. Ishii, S. Tansho, K. Muramoto and H. Kamiya (1989). "Purification of a novel cytolytic protein from albumen gland of the sea hare, *Dolabella auricularia*." *Chemical and Pharmaceutical Bulletin* **37**(10): 2773-2776.

Koester, J. and E. R. Kandel (1977). "Further identification of neurons in the abdominal ganglion of *Aplysia* using behavioral criteria." *Brain Research* **121**: 1-20.

Krakauer, J. M. (1969).The ecology of *Aplysia willcoxi* Heilprin at Cedar Key, Florida. M. Sc., University of Florida.

Krebs, J. R. and N. B. Davies (1993). The design of signals: ecology and evolution. In: *An introduction to behavioural ecology*. N. B. Davies, Ed. Boston, Blackwell Scientific: 349-374.

Kupfermann, I. and E. R. Kandel (1969). "Neuronal controls of a behavioral response mediated by the abdominal ganglion of *Aplysia*." *Science* **164**: 847-870.

Kupfermann, I. and T. J. Carew (1974). "Behavior patterns of *Aplysia californica* in its natural environment." *Behavioral Biology* **12**: 317-337.

LaMunyan, C. W. and P. A. Adams (1987). "Use and effect of an anal defensive secretion in larval chrysopidae (Neuroptera)." *Annals of the Entomological Society of America* **80**(6): 804-808.

Lawn, I. D. (1976). Chemoreception and conduction systems in sea anemones. In: *Coelenterate ecology and behavior*. G. O. Mackie, Ed. New York, Plenum: 581-590.

Lederhendler, I., L. Bell and E. Tobach (1975). "Preliminary observations of the behavior of *Aplysia dactylomela* (Rang, 1828) in Bimini waters." *Veliger* **17**(4): 347-353.

Leonard, J. L. and K. Lukowiak (1986). "The behavior of *Aplysia californica* Cooper (Gastropoda: Opisthobranchia): I. Ethogram." *Behaviour* **98**: 320-360.

Lindquist, N. and M. E. Hay (1995). "Can small rare prey be chemically defended? - the case for marine larvae." *Ecology* **76**(4): 1347-1358.

Lindquist, N. and M. E. Hay (1996). "Palatability and chemical defense of marine invertebrate larvae." *Ecological Monographs* **66**(4): 431-450.

Lindquist, N., N. Shigematsu and L. Pannell (2000). "Corydendramines A and B, defensive natural products of the marine hydroid *Corydendrium parasiticum*." *Journal of Natural Products* **63**(9): 1290-1291.

Lloyd, J. E. (1975). "Aggressive mimicry in *Photuris* fireflies: signal repertoires by *femmes fatales*." *Science* **187**: 452-453.

MacColl, R., J. Galivan, D. S. Berns, Z. Nimec, D. Guard-Friar and D. Wagoner (1990). "The chromophore and polypeptide composition of *Aplysia* ink." *Biological Bulletin* **179**(3): 326-331.

MacFarland, F. M. (1909). The opisthobranchiate Mollusca of the Branner-Agassiz expedition to Brazil, Stanford Junior University Publications.

MacFarland, F. M. (1918). "The Dolabellinae." *Harvard University Museum of Comparative Zoology Memoirs* **35**: 300-348.

Macnae, W. (1955). "On four species of the genus *Aplysia* common in South Africa." *Annals of the Natal Museum* **8**(2): 223-241.

Maia, L. F., R. D. Epifanio and W. H. Fenical (2000). "New cytotoxic sterol glycosides from the octocoral *Carijoa (Telesto) riisei*." *Journal of Natural Products* **63**(10): 1427-1430.

Marcus, E. and E. Marcus (1955). "Sea-hares and side-gilled slugs from Brazil." *Boletim do Instituto Oceanografico* **6**: 3-49.

Marcus, E. and E. Marcus (1970). Opisthobranchs from Curaçao and faunistically related regions. In: *Studies on the fauna of Curaçao and other Caribbean islands*. P. W. Hummelinck, Ed.: 167-188.

Marcus, E. D. B. R. (1972). "On the Anaspidea (Gastropoda: Opisthobranchia) of the warm waters of the western Atlantic." *Bulletin of Marine Science* **22**(4): 841-874.

Martin, R. (1966). "On the swimming behavior and biology of *Notarchus punctatus* Philippi (Gastropoda, Opisthobranchia)." *Pubblicazioni della Stazione Zoologica di Napoli I* **35**: 61-75.

Maschwitz, U., K. Jessen and E. Maschwitz (1981). "Foaming in *Pachycondyla*: a new defense mechanism in ants." *Behavioral Ecology and Sociobiology* **9**: 79-81.

McCauley, J. E. (1960). "The morphology of *Phyllaplysia zostericola*, new species." *Proceedings of the California Academy of Sciences* **29**(16): 549-576.

McFarlane, I. D. (1984). "Nerve nets and conducting systems in sea anemones: two pathways excite tentacle contractions in *Calliactis parasitica.*" *Journal of Experimental Biology* **108**: 137-149.

McIntosh, A. R. and B. L. Peckarsky (1999). "Criteria determining behavioural responses to multiple predators by a stream mayfly." *Oikos* **85**(3): 554-564.

Medina, M. and P. J. Walsh (2000). "Molecular systematics of the order anaspidea based on mitochondrial DNA sequence (12S, 16S, and CO1)." *Molecular Phylogenetics and Evolution* **15**(1): 41-58.

Melo, V. M. M., A. M. Fonesca, I. M. Vasconcelos and A. F. F. U. Carvalho (1998). "Toxic, antimicrobial and haemagglutinating activities of the purple fluid of the sea hare *Aplysia dactylomela* Rang, 1828." *Brazilian Journal of Medical and Biological Research* **31**: 785-791.

Melo, V. M. M., A. B. G. Duarte, A. F. F. U. Carvalho, E. A. Siebra and I. M. Vasconcelos (2000). "Purification of a novel antibacterial and haemagglutinating protein from the purple gland of the sea hare, *Aplysia dactylomela* Rang, 1828." *Toxicon* **38**: 1415-1427.

Miller, M. C. and W. B. Rudman (1968). "Two new genera and species of the superfamily Runcinoidea (Mollusca: Gastropoda: Opisthobranchia) from New Zealand." *Transactions of the Royal Society of New Zealand, Zoology* **10**(19): 183-189.

Morton, J. E. and N. A. Holme (1955). "The occurrence at Plymouth of the opisthobranch *Akera bullata,* with notes on its habits and relationships." *Journal of the Marine Biological Association U.K.* **34**: 101-112.

Morton, J. E. (1972). "The form and functioning of the pallial organs in the opisthobranch *Akera bullata* with a discussion on the nature of the gill in notaspida and other tectibranchs." *Veliger* **14**(4): 337-349.

Nishibori, K. (1960). "Studies on the pigments of marine animals IX. Pigments of the sea-slug *Aplysia kurodai.*" *Publications of the Seto Marine Biological Laboratory* **8**: 105-114.

Nistratova, S. N., I. N. Mitskevich, M. E. Mirabet-Regalado and N. Josef (1993). "Inhibitory effect of the purple fluid produced by a mollusc *Aplysia punctata* on growth of gram-negative marine bacteria." *Microbiology* **61**(5): 630-632.

Nolen, T. G., P. M. Johnson, C. E. Kicklighter and T. Capo (1995). "Ink secretion by the marine snail *Aplysia californica* enhances its ability to escape from a natural predator." *Journal of Comparative Physiology A* **176**: 239-254.

Nolen, T. G. and P. M. Johnson (2001). "Defensive inking in *Aplysia* spp: multiple episodes of ink secretion and the adaptive use of a limited chemical resource." *Journal of Experimental Biology* **204**(7): 1257-1268.

Nystrom, P., O. Svensson, B. Lardner, C. Bronmark and W. Graneli (2001). "The influence of multiple introduced predators on a littoral pond community." *Ecology* **82**(4): 1023-1039.

Painter, S., B. Clough, R. Garden, J. Sweedler and G. Nagle (1998). "Characterization of *Aplysia* attractin, the first water-borne peptide pheromone in invertebrates." *Biological Bulletin* **194**(2): 120-131.

Paul, V. J. and S. C. Pennings (1991). "Diet-derived chemical defenses in the sea hare *Stylocheilus longicauda* (Quoy et Gaimard 1824)." *Journal of Experimental Marine Biology and Ecology* **151**: 227-243.

Paul, V. J. (1992). *Ecological roles of marine natural products*. Ithaca, Comstock Publishing Associates.

Pawlik, J. R., B. Chanas, R. J. Toonen and W. H. Fenical (1995). "Defenses of Caribbean sponges against predatory reef fish. I. Chemical deterrency." *Marine Ecology Progress Series* **127**: 183-194.

Peckarsky, B. L. and A. R. McIntosh (1998). "Fitness and community consequences of avoiding multiple predators." *Oecologia* **113**(4): 565-576.

Pennings, S. C. (1990a). "Multiple factors promoting narrow host rage in the sea hare, *Aplysia californica*." *Oecologia* **82**: 192-200.

Pennings, S. C. (1990b). "Predator-prey interactions in opisthobranch gastropods: effects of prey body size and habitat complexity." *Marine Ecology Progress Series* **62**: 95-101.

Pennings, S. C. (1990c). "Size-related shifts in herbivory: specialization in the sea hare *Aplysia californica* Cooper." *Journal of Experimental Marine Biology and Ecology* **142**: 43-61.

Pennings, S. C. and V. J. Paul (1993). "Sequestration of dietary secondary metabolites by three species of sea hares: location specificity and dynamics." *Marine Biology* **117**: 535-546.

Pennings, S. C. (1994). "Interspecific variation in chemical defenses in the sea hares (Opisthobranchia: Anaspidea)." *Journal of Experimental Marine Biology and Ecology* **180**: 203-219.

Pennings, S. C., V. J. Paul, D. C. Dunbar, M. T. Hamann, W. A. Lumbang, B. Novack and R. S. Jacobs (1999). "Unpalatable compounds in the marine gastropod *Dolabella auricularia*: distribution and effect of diet." *Journal of Chemical Ecology* **25**(4): 735-755.

Pennings, S. C., S. Nastisch and V. J. Paul (2001). "Vulnerability of sea hares to fish predators: importance of diet and fish species." *Coral Reefs* **20**(3): 320-324.

Petzelt, C., G. Joswig, H. Stammer and D. Werner (2002). "Cytotoxic cyplasin of the sea hare, *Aplysia punctata*, cDNA cloning, and expression of bioactive recombinants in insect cells." *Neoplasia* **4**: 49-59.

Prince, J. S., T. G. Nolen and L. Coelho (1998). "Defensive ink pigment processing and secretion in *Aplysia californica*: concentration and storage of phycoerythrobilin in the ink gland." *Journal of Experimental Biology* **201**: 1595-1613.

Prince, J. S. and P. M. Johnson (in prep). "Comparative ultrastructural analysis of the purple, white, and mixed ink glands of three species of seahare (Gastropoda: Opisthobranchia)."

Quiñoa, E., L. Castedo and R. Riguera (1989). "The halogenated monoterpenes of *Aplysia punctata*. A comparative study." *Comparative Biochemistry and Physiology* **92B**(1): 99-101.

Rajaganapathi, J. and K. Kathiresan (2002). "Heparinase in purple fluid of the sea hare, *Bursatella leachii*." *Current Science* **82**(3): 264-266.

Rankin, C. H. (1987). "Development of learning and memory of *Aplysia*. 1. Functional assembly of gill and siphon withdrawal." *Journal of Neuroscience* **7**(1): 120-132.

Rittschof, D. (1990). "Peptide-mediated behaviors in marine organisms." *Journal of Chemical Ecology* **16**(1): 261-272.

Rogers, C. N., R. de Nys, T. S. Charlton and P. D. Steinberg (2000a). "Dynamics of algal secondary metabolites in two species of sea hare." *Journal of Chemical Ecology* **26**(3): 721-744.

Rogers, C. N., R. de Nys and P. D. Steinberg (2000b). "Predation on juvenile *Aplysia parvula* and other small anaspidean, ascoglossan, and nudibranch gastropods by pycnogonids." *Veliger* **43**(4): 330-337.

Ross, T. L., J. S. Prince and T. G. Nolen (1995). "Acetylcholine induces ink secretion from isolated ink vesicles in *Aplysia*." *Society for Neuroscience Abstracts* **21**: 178.

Russell, E. (1966). "An investigation of the palatability of some marine invertebrates to four species of fish." *Pacific Science* **20**: 452-460.

Ryan, M. J. and A. S. Rand (1990). "The sensory basis of sexual selection for complex calls in the Tungara frog, *Physalaemus pustulosus* (sexual selection for sensory exploitation)." *Evolution* **44**(2): 305-314.

Sadowski, J. A., A. J. Moore and E. D. Brodie, III. (1999). "The evolution of empty nuptial gifts in a dance fly, *Empis snoddyi* (Diptera:Empididae): bigger isn't always better." *Behavioral Ecology and Sociobiology* **45**: 161-166.

Sakamoto, Y., T. Nakajima, S. Misawa, H. Ishikawa, Y. Itoh, T. Nakashima, T. Okanoue, K. Kashima and T. Tsuji (1998). "Acute liver damage with characteristic apoptotic hepatocytes by ingestion of *Aplysia kurodai*, a sea hare." *Internal Medicine* **37**(11): 927-929.

Sarver, D. J. (1978).The ecology and energetics of *Aplysia juliana* (Quoy and Qaimard, 1832). Ph.D., University of Hawaii.

Schmitz, O. J. and L. Sokol-Hessner (2002). "Linearity in the aggregate effects of multiple predators in a food web." *Ecology Letters* **5**(2): 168-172.

Schnitzler, I., G. Pohnert, M. E. Hay and W. Boland (2001). "Chemical defense of brown algae (*Dictyopteris* spp.) against the herbivorous amphipod *Ampithoe longimana*." *Oecologia* **126**: 515-521.

Schuhmacher, H. (1973). "Notes on occurrence, feeding, and swimming behavior of *Notarchus inicus* and *Melibe bucephala* at Elat, Red Sea (Mollusca: Opisthobranchia)." *Israeli Journal of Zoology* **22**: 13-25.

Sih, A., G. Enlund and D. Wooster (1998). "Emergent impacts of multiple predators on prey." *Trends in Ecology and Evolution* **13**(9): 350-355.

Stopfer, M., X. Chen and T. J. Carew (1993). "Evoked ink release in *Aplysia* produces inhibition of the siphon withdrawal reflex in neighboring conspecifics." *Behavioral and Neural Biology* **60**(3): 196-204.

Stowe, M. K. (1988). Chemical mimicry. In: *Chemical mediation of coevolution*. K. C. Spencer, Ed. New York, Academic Press: 513-580.

Stowe, M. K., T. C. J. Turlings, J. H. Loughrin, W. J. Lewis and J. H. Tumlinson (1995). "The chemistry of eavesdropping, alarm, and deceit." *Proceedings of the National Academy of Science USA* **92**: 23-28.

Sullivan, B. and L. Webb (1983). "Siphonodictidine, a metabolite of the burrowing sponge *Siphonodictyon* sp. that inhibits coral growth." *Science* **221**: 1175-1176.

Sung, D.-Y., W. W. Walthall and C. D. Derby (1996). "Identification and partial characterization of putative taurine receptor proteins from the olfactory organ of the spiny lobster." *Comparative Biochemistry and Physiology B* **115**(1): 19-26.

Susswein, A. J., S. Gev, Y. Achituv and S. Markovich (1984). "Behavioral patterns of *Aplysia fasciata* along the Mediterranean coast of Israel." *Behavioral and Neural Biology* **41**: 7-22.

Tachibana, K., M. Sakaitanai and K. Nakanishi (1984). "Pavoninins: shark-repelling ichthyotoxins from the defense secretion of the Pacific sole." *Science* **226**: 703-707.

Tachibana, K. (1988). Chemical defenses in fishes. In: *Bioorganic marine chemistry*. P. J. Scheuer, Ed. Berlin, Springer-Verlag: 117-138.

Thompson, S. A., K. Tachibana, K. Nakanishi and I. Kubota (1986). "Melittin-like peptides from the shark-repelling defense secretion of the sole *Pardachirus pavoninus*." *Science* **233**: 341-343.

Thompson, T. E. (1960a). "Defensive adaptations in opisthobranchs." *Journal of the Marine Biological Association U.K.* **39**: 123-134.

Thompson, T. E. (1960b). "Defensive acid-secretion in marine gastropods." *Journal of the Marine Biological Association U.K.* **39**: 115-22.

Thompson, T. E. (1976). *Biology of opisthobranch molluscs*. London, The Ray Society.

Thompson, T. E. and D. R. Seaward (1989). "Ecology and taxonomic status of the aplysiomorph *Akera bullata* in the British Isles." *Journal of Molluscan Studies* **55**: 489-496.

Tinbergen, N. (1951). *The study of instinct*. Oxford, Clarendon Press.

Tobach, E., P. Gold and A. Ziegler (1965). "Preliminary observations of the inking behavior of *Aplysia* (*Varria*)." *Veliger* **8**: 16-18.

Tobach, E., A. Zaferes and L. Migenis-Lopez (1989). "*Aplysia* ink and opaline: exploration of their relation to predation." *Bulletin of Marine Science* **45**(3): 664-670.

Tomaschko, K. H. (1994). "Ecdysteroids from *Pycnogonum litorale* (Arthropoda, Pantopoda) act as chemical defense against *Carcinus maenas* (Crustacea, Decapoda)." *Journal of Chemical Ecology* **20**(7): 1445-1453.

Trapido-Rosenthal, H. G. (1995). The use of the spiny lobster as a tool for the study of chemosensory biochemistry. In: *Experimental cell biology of taste and olfaction: current techniques and protocols*. A. I. Spielman and J. G. Brand, Eds. Boca Raton, CRC Press: 169-179.

Tritt, S. H. and J. H. Byrne (1980). "Motor controls of opaline secretion in *Aplysia californica*." *Journal of Neurophysiology* **43**(3): 581-594.

Tritt, S. H. and J. H. Byrne (1982). "Neurotransmitters producing and modulating opaline gland contraction in *Aplysia californica*." *Journal of Neurophysiology* **48**(6): 1347-1361.

Troxler, R. R., G. D. Offner and T. R. Capo (1981). "Structural studies on aplysioviolin." *Biological Bulletin* **161**: 339.

Van Buskirk, J. (2001). "Specific induced responses to different predator species in anuran larvae." *Journal of Evolutionary Biology* **14**(3): 482-489.

Walters, E. T. and M. T. Erickson (1986). "Directional control and the functional organization of defensive responses in *Aplysia*." *Journal of Comparative Physiology A* **159**: 339-351.

Walters, E. T., P. A. Illich and C. Hickie (1993). "Inking and siphon response plasticity in *Aplysia*: anti-predator and alarm signal functions." *Society for Neuroscience Abstracts* **19**: 578.

Wee, S. L. and K. H. Tan (2001). "Allomonal and hepatotoxic effects following methyl eugenol consumption in *Bactrocera papayae* male against *Gekko monarchus*." *Journal of Chemical Ecology* **27**(5): 953-964.

Willan, R. and J. Morton (1984). *Marine Molluscs. Part 2. Opisthobranchia.* Leigh, N.Z., Leigh Marine Laboratory, University of Auckland.

Willan, R. C. (1979).The ecology of two New Zealand opisthobranch molluscs. Ph.D. Dept. of Biology, University of Auckland.

Winkler, L. R. (1957).The biology of California sea hares of the genus *Aplysia*. Ph.D., University of Southern California.

Winkler, L. R. (1959). "Intraspecific variation in the purple secretion of the California sea hare, *Aplysia californica* Cooper." *Pacific Science* **13**: 357-61.

Winkler, L. R. and B. E. Tilton (1962). "Predation on the California sea hare, *Aplysia californica* Cooper, by the solitary great green sea anemone, *Anthopleura xanthogrammica* (Brandt), and the effect of sea hare toxin and acetylcholine on anemone muscle." *Pacific Science* **16**: 286-290.

Winkler, L. R. (1969). "Distribution of organic bromine compounds in *Aplysia californica* Cooper, 1863." *Veliger* **2(3)**: 268-71.

Wooton, R. J. (1990). *Ecology of Teleost Fishes*. London, Chapman and Hall.

Wright, W. G. and T. J. Carew (1995). "A single identified interneuron gates tail-shock induced Inhibition in the siphon withdrawal reflex of *Aplysia*." *Journal of Neuroscience* **15**(1 Part 2): 790-797.

Yamazaki, M., J. Kisugi, M. Ikenami, H. Kamiya and D. i. Mizuno (1984). "Cytolytic factor in eggs of the sea hare *Aplysia kurodai*." *Gann* **75**: 269-274.

Yamazaki, M., J. Kisugi, K. Kimura, H. Kamiya and D. i. Mizuno (1985). "Purification of antineoplastic factor from eggs of a sea hare." *FEBS* **185**(2): 295-298.

Yamazaki, M., K. Kimura, J. Kisugi and K. Hisao (1986). "Purification of a cytolytic factor from purple fluid of a sea hare." *FEBS* **198**: 25-28.

Yamazaki, M., K. Kimura, J. Kisugi, K. Muramoto and H. Kamiya (1989a). "Isolation and characterization of a novel cytolytic factor in the purple fluid of the sea hare, *Aplysia kurodai*." *Cancer Research* **49**: 3834-3838.

Yamazaki, M., S. Tansho, J. Kisugi, K. Muramoto and H. Kamiya (1989b). "Purification and characterization of a cytolytic protein from the purple fluid of the sea hare, *Dolabella auricularia*." *Chemical and Pharmaceutical Bulletin* **37**(8): 2179-2182.

Yamazaki, M., H. Ohye, J. Kisugi and H. Kamiya (1990). "Bacteriostatic and cytolytic activity of purple fluid from the sea hare." *Developmental and Comparative Immunology* **14**: 379-383.

Yamazaki, M. (1993). "Antitumor and antimicrobial glycoproteins from sea hares." *Comparative Biochemistry and Physiology* **105C**(2): 141-146.

Zhu, J., R. C. Unelius, K.-C. Park, S. A. Ochieng, J. J. Obrycki and T. C. Baker (2001). "Identification of (Z)-4-tridecene from defensive secretion of green lacewing, *Chrysoperla carnea.*" *Journal of Chemical Ecology* **26**(10): 2421-2434.

Zimmer-Faust, R. K. (1987). "Crustacean chemical perception: towards a theory on optimal chemoreception." *Biological Bulletin* **172**: 10-29.

CURRICULUM VITAE

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PLACE OF BIRTH	Terre Haute, Indiana, 1971

EDUCATION

2000-	Georgia State University, Department of Biology: Visiting Ph.D. candidate in the lab of Dr. Charles Derby
2000	Ph.D. Candidacy at the University of Washington, Department of Zoology
1997-	Entered University of Washington, Department of Zoology Ph.D. Program
1989-94	University of Miami, B.S., Marine Science/Biology, minor: Chemistry
1989-94	University of Miami, B.A., Religion, minor: Psychology

EMPLOYMENT

1994-1997	Japan Exchange and Teaching (JET) Program, Assistant Language Teacher, Mie Prefecture Board of Education, Japan
1993-94	University of Miami, Department of Biology, Computer Consultant

PROFESSIONAL EXPERIENCE

Research Activities:

1997-	Effects of seahare chemical defenses on multiple predator species
1991-94	Neural basis of chemical defense and conspecific signaling in *Aplysia* spp.

Honors and Awards:

2002	Center for Behavioral Neuroscience Postdoctoral Fellowship
1997	NSF Graduate Research Fellowship in Neuroscience
1997	Recruitment Award, University of Washington, Department of Zoology
1994	Outstanding Graduating Senior in Marine Science, University of Miami
1994	Outstanding Graduating Senior in Religion, University of Miami
1994	Outstanding Honors Senior Thesis Award in Biology, University of Miami
1994	Departmental Honors in Biology, University of Miami
1994	Selected by the Japanese government to participate in the Japan Exchange and Teaching (JET) Program
1993	President of the Marine Science Honor Society
1989-94	Received a 5-year half-tuition scholarship from the University of Miami

PUBLICATIONS

Nolen, T.G. and **Johnson, P.M.** 2001. Defensive inking in *Aplysia* spp.: multiple episodes of ink secretion and the adaptive use of a limited chemical resource. *J. Exp. Biol.* 204: 1257-1268.

Johnson, P.M. and Willows, A.O.D. 1999. Defense in sea hares (Gastropoda: Opisthobranchia: Anaspidea): multiple layers of protection from egg to adult. *Mar. Frsh. Behav. Physiol.* 32: 147-180.

Nolen, T.G., **Johnson, P.M.**, Kicklighter, C.E., Capo, T. 1995. Ink secretion by the marine snail *Aplysia californica* enhances its ability to escape from a natural predator. *J. Comp. Phsyiol. A.* 176:239-254

OTHER PUBLICATIONS

Published Abstracts from International Meetings (*Presented at the meeting)

***Johnson, P.M.**, Paul, V.J., Cruz-Rivera, E., Derby, C.D. 2002. Chemical Attractants as Chemical Defenses? *Chem. Senses* 27

Johnson, P.M., Yang, H., Paul, V.J., Tai, P.C., Derby, C.D. 2001. Sea hare defensive secretions function by repelling some predators and attracting others. *Amer. Zool.* 41: 1488.

Nolen, T.G., Robinson, S., **Johnson, P.M.** and Kicklighter, C.E. 1994. Non-noxious stimulation elicits graded inking in *Aplysia. Soc. Neurosci. Abstr.* 20: 68.

***Johnson, P.M.** Evoy, W.H., Nolen, T.G. 1993. Distributed mechanical stimulation of the skin triggers ink release in *Aplysia. Soc. Neurosci. Abstr.* 19: 167.

Kicklighter, C., **Johnson, P.M.** Robinson, S., Augustines, M. Nolen, T.G. 1993. Ink release by *Aplysia* enhances its ability to escape from a natural predator. *Soc. Neurosci. Abstr.* 19: 167.

Kicklighter, C.E., ***Johnson, P.M.** and Nolen, T.G. 1992. Chemically mediated defensive inking in *Aplysia californica.* Soc. Neurosci. Abstr. 18: 346.

***Johnson, P.M.** and Nolen, T.G. 1991. Conspecific ink is aversive to juvenile *Aplysia californica. Amer. Zool.* 31(5): 112A

Papers in Preparation

Johnson, P.M., Cruz-River, E., Paul, V.J. (in prep). Species differences, but not defensive secretions, important in sea hare palatability to reef fish.

Prince, J.S. and Johnson, P.M. (in prep). Comparative ultrastructural analysis of the purple, white, and mixed ink glands of three species of seahare (Gastropoda: Opisthobranchia)

INVITED & SYMPOSIUM PRESENTATIONS

"Mechanisms of Chemical Defense: One Prey Species vs. Multiple Predators" at Georgia Tech, Dept. of Biology, April, 2002.

"Seahare Defensive Secretions Function Differently Against Three Generalist Predators", Opisthobranch Symposium at the Western Society of Malacologists 34th Annual Meeting, June, 2001.

"Chemical Defense in a Deep-Sea Polychaete" at the University of Guam Marine Laboratory, April, 2001.

TEACHING ACTIVITIES

University of Washington

2000	Teaching Assistant: Introductory biology for majors
1999	Teaching Assistant: Senior level animal physiology
1998	Teaching Assistant: Graduate level marine invertebrate zoology at the Friday Harbor Marine Laboratory

Japan, Mie Prefecture

1994-97	Assistant Language Teacher, JET Program. English instructor for high school students, English teachers, and other Japanese high school faculty.

Cornell University

1994	Teaching Assistant: Field marine science at the Shoals Marine Laboratory

University of Miami

1992	Teaching Assistant: Transmission electron microscopy

UNIVERSITY SERVICE

1998-2000	Department of Zoology senator for the University of Washington Graduate and Professional Student Senate
1998-99	Member of the President's Student Forum: a student advisory board (including 6 graduate students) to University of Washington president, Richard McCormick