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Chemosensory-Mediated Deposit Feeding in the Spionid Polychaete Dipolydora Quadrilobata.

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CHEMOSENSORY-MEDIATED DEPOSIT FEEDING IN THE SPIONID POLYCHAETE *DIPOLYDORA QUADRILOBATA*

By

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Thesis Advisor: Dr. Sara M. Lindsay

An Abstract of the Thesis Presented
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Deposit feeding organisms live and feed in marine soft-sediment habitats. This sediment makes up a majority of the material ingested by deposit feeders and contains a variety of edible material that may constitute their principal nutrient source. However, the specific components that are assimilated by these organisms, and the strategies they employ to efficiently collect those components, remain unclear.

Sensory interactions between an organism and its surrounding environment typically play an important role in helping the organism detect and locate potential food. Accordingly, chemical sensing by deposit feeders is most likely involved in feeding, yet few specifics about this role and its ecological implications are known. This study, a multi-disciplined investigation of chemoreception, focuses on putative chemosensory structures located on the palps of the deposit-feeding spionid polychaete *Dipolydora quadrilobata*. Using behavioral studies, neurophysiological methods, and molecular

biological techniques, this study examines the sensory capabilities of this deposit feeder and their potential role as a mediator of selective feeding.

A series of behavioral assays tested for feeding responses to a selected number of potential cues that might be used to indicate food availability or quality. Two sets of glass beads, one with and one without covalently bound compounds such as single amino acids, mixtures of amino acids, and single simple sugars, were separately presented to an organism. The differences in observed responses were used to identify these compounds as stimulatory, inhibitory, or inactive.

Stimulatory cues identified in the behavioral studies were then used to label, in an activity-dependent manner, putative receptor neurons in the palps. Stimulatory cues were perfused over the palp in the presence of the cationic molecule agmatine. Agmatine can enter into stimulated receptor neurons via activated non-selective cation channels. Those cells containing agmatine are then stained using an anti-agmatine antibody followed by silver intensification. Four putative sensory cell types located in the palps were identified by comparing cell labeling in response to the perfusion of a mixture of amino acids in the presence of agmatine to controls of agmatine in the absence of stimuli. Two of these cells types appear to be mechanosensory in function, and two appear to be chemosensory in function.

Finally, molecular biological techniques were employed in attempts at isolating gene sequences that code for chemoreceptor proteins. Using RNA isolated from two tissues, *D. quadrilobata* palps and tails, single-stranded complementary DNA was constructed and amplified via the polymerase chain reaction. Gene expression patterns in

the two tissues were compared (*i.e.* differential display) in order to isolate genes differentially expressed in the palps with the goal of finding receptor gene sequences.

These studies indicate that chemoreception is an important influence in particle selection by this organism, and similarly suggest that this influence is at least partially mediated via chemoreceptor structures of the palp.

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CHAPTER 1

THE SENSORY INTERACTIONS OF AN ORGANISM WITH ITS ENVIRONMENT

INTRODUCTION

The survival of an organism depends on continual detection of and reaction to changes within its immediate environment. These interactions are predominantly sensory in character and are vital to a wide variety of processes including orientation, defense, predation, persuasion, and homeostasis. Sensory interactions occur primarily as transfers of information from the environment to the organism. The information arising from the environment (which may also include other organisms) is transmitted in several forms: temperature, light, sound, pressure and chemical cues. This information is obtained by the organism's sensory receptors, decoded (often transformed into another form), and used to initiate a proper response.

The importance of these sensory interactions to the full spectrum of an organism's behavior has led researchers to focus on tracing this flow of information as a means of determining the sensory and information-processing abilities of various organisms. A detailed understanding of these abilities, however, requires knowledge not only of the external mechanisms underlying the capture of information (*i.e.* sensory ecology), but also the internal mechanisms (*i.e.* sensory physiology), as well as the development and adaptation of those mechanisms (*i.e.* molecular biology/evolution).

Sensory ecologists tend to focus directly on the transfer of information from the environment to an organism, with the goal of identifying the strategies used to locate resources, the information used, and how that information is obtained (Dusenbery, 1992). This discipline is grounded firmly in the physics of the movement of both the information and the organism. Studies have focused on examining how stimuli move through the environment and how they are detected by different organisms, with the aim of

identifying important stimuli and the information that they transmit. Studies of the physical properties of stimuli in marine habitats and their interaction with the environment have been critical in determining the types of stimuli that are available to varied groups of organisms (Atema, 1988; Carr, 1988; Decho et al., 1998). For example, Decho et al. (1998) recently measured bacterial uptake rates of polypeptides and their individual components, finding that polypeptides are more slowly assimilated. This suggests that this persistence may be a rationale for the selection of polypeptides over single amino acids as chemical cues by many organisms. Other studies, using a more organism-centered approach, have attempted to determine classes and threshold levels of stimuli that can be detected and how searching behavior toward stimuli sources may be directed (Lenhoff & Lindstedt, 1974; Mackie & Grant, 1974; Berg and Purcell, 1977; Croll, 1983; Carr and Derby, 1986a; Derby and Atema, 1988, Browne et al., 1998).

Sensory physiology, on the other hand, focuses primarily on the cellular processes that are responsible for decoding information once it is obtained. The different forms of information are processed by corresponding receptors that measure the signals and typically convert them into an electrical form that is transmitted to the central nervous system (CNS) where an appropriate response is fashioned. The transmission of the signal to the CNS is dependent upon a series of biochemical events that drive the polarization and depolarization of a succession of neuronal cells. Researchers have exploited this sequence of events in attempts to determine signal identities and the modifications they elicit. For example, electrophysiological methods are often used to identify stimuli components and characteristics that evoke responses in receptor cells (*e.g.* odorant mixtures in spiny lobster, reviewed by Derby, 2000). More recent work using calcium-

sensitive fluorescent dyes has begun to discriminate the sensitivities of individual neurons (Fetcho & O'Malley, 1995; Fetcho et al., 1998), as well as allow simultaneous measurements of whole populations of neurons, which are significantly more difficult to accomplish using standard electrophysiological techniques.

Most recently, research regarding these sensory interactions has expanded to include the field of genetics. Researchers have begun to address these questions using molecular biological techniques to examine how chemoreceptor systems may be coded in an organism's genome. This not only provides information about the evolutionary adaptation of the receptor systems of an organism and how they may compare to others, but can also help in determining the organization and specificity of these systems.

Progress in the fields of sensory ecology, sensory physiology, and molecular biology has reached the point where it is now feasible (and arguably necessary) to integrate these three disciplines to approach a more complete picture of the interactions of an organism with its environment. Such an integration of disciplines (albeit in a limited and preliminary manner) in the study of a spionid polychaete, *Dipolydora quadrilobata*, is the focus of this study, with the goal of obtaining a comprehensive view of how this deposit-feeding organism detects and obtains nutrient resources.

Sensory Ecology of Deposit Feeders

Deposit feeders are a group of benthic marine organisms identified as frequently ingesting sedimented material of low bulk food value (Jumars et al., 1984; Lopez & Levington, 1987). This group of organisms is fairly diverse, including polychaetes, bivalves, gastropods, holothuroids and some crustaceans. Although the ingestion of

benthic sediments is the primary characteristic that connects deposit feeders to one another, the actual biological and chemical components of the sediment material that are assimilated by these organisms have not been characterized. This question has been approached using a variety of methods (reviewed by Jumars, 1993), including a significant focus on identifying potential sensory interactions that may direct feeding (Robertson et al., 1981; Rittschof & Buswell, 1989; Weissburg & Zimmer-Faust, 1991; Ferner & Jumars, 1999).

Research on the sensory capabilities of deposit feeders has been dominated by behavioral studies attempting to determine the specific stimuli to which organisms respond and how the response is carried out. These studies show that feeding rates can be either stimulated or depressed by particle size (Fenchel et al., 1975; Whitlatch, 1974; Whitlatch, 1980; Jumars et al., 1982; Taghon, 1982; Self & Jumars, 1988; Taghon, 1988), and by specific chemical cues, either adsorbed to particles or in dissolved form (Robertson et al., 1981; Self & Jumars, 1988; Valiella et al., 1979; Forbes & Lopez, 1986; Miller & Jumars, 1986; Karrh & Miller, 1994; Ferner & Jumars, 1999; Kihslinger and Woodin, 1999).

The mechanisms behind the detection and recognition of these qualities, however, remain unclear. Sensory organs have been identified in many deposit feeders, yet little is known about their roles in detecting and locating specific resources. Among polychaetes, for example, several sensory structures have been identified (Dorsett & Hyde, 1969; Boilly-Marer, 1968; Jouin et al., 1985; Storch & Schlotzer-Schrehardt, 1988), but with the exception of a pheromone receptor in the nereid *Platynereis dumerilii* (Boilly-Marer,

1968, 1974, 1978, & 1980; Ram et al., 1999), demonstration of the functions of these structures is largely lacking.

The genetic basis for these sensory systems is even less well known. Multiple chemoreceptor genes have been identified in several vertebrates (human, rat, catfish, chicken and frog) as well as two invertebrate species, *Drosophila melanogaster* and *Caenorhabditis elegans* (Buck & Axel, 1991; Clyne et al., 1999; Freitag et al., 1993; Nef, 1993; Ngai et al., 1993; Troemel et al., 1995; Voshall et al., 1999). Any homology to deposit feeders is unknown, however, particularly because no marine invertebrate chemoreceptor genes have been identified. The enormous size of the identified gene families and the overall lack of similarity even within the same genome of the organisms examined, however, suggests a high degree of specificity for individual receptors. Therefore, it is unlikely that high levels of similarity between such disparate groups as vertebrates and polychaetes or mollusks are present. However, even short stretches of similarity can be used to screen pools of expressed genes to identify potentially similar function.

Chemosensory-Mediated Deposit Feeding in a Spionid Polychaete

Integrating these three disciplines, I have employed behavioral assays, neurophysiological studies, and molecular techniques in an attempt to characterize the mechanisms underlying chemosensory-mediated feeding in the deposit-feeding spionid polychaete, *Dipolydora quadrilobata*. These studies are important not only to identify potential resources and establish the sensory abilities of this organism, but also to connect these sensory interactions to greater ecological questions regarding deposit feeders.

Sensory interactions are presumed to play important roles not only in feeding, but in recruitment and settlement of many marine invertebrates as well (reviewed by Butman, 1987 and Pawlik, 1992). Most deposit feeders are restricted in their mobility; consequently these organisms spend considerable effort initially, as larvae, selecting a good settlement site. The search is presumably for habitats with high food levels. This selection is most likely mediated by sensory interactions with the benthos, suggesting that receptors for specific food resources that are employed for feeding later in development may also be involved in the settlement process (*e.g.* concomitant expression of genes associated with feeding and digestion systems at larval competence in the red abalone *Haliotis rufescens*, reviewed by Degnan & Morse, 1995). The genetic identification of these receptors is a first step in making this link, and could be an important step in determining the mechanisms behind the population dynamics of these organisms.

CONCLUSIONS

Sensory interactions between a deposit feeder and its surrounding environment presumably play an important role in feeding, specifically in detecting and locating potential food. Examining the behavioral, physiological and genetic aspects that regulate these sensory interactions, therefore, should help in determining not only the specific resources assimilated by these organisms, but also how those resources are detected and preferentially retained.

CHAPTER 2

BEHAVIORAL STUDIES OF CHEMORECEPTION IN *DIPOLYDORA QUADRILOBATA*

INTRODUCTION

The sensory capabilities of organisms in marine habitats are limited by the inherent properties of the habitat. Low light levels and the limit of long distance sound conveyance in water to very low frequencies restrict the propagation of visual and auditory signals. Consequently, marine organisms tend to depend more on chemical signals as vectors of information, particularly at longer distances. Chemical signals are known to mediate many processes including recruitment and metamorphosis (Pawlik, 1992), reproduction (Miller, 1989), escape responses (Mackie, 1970), and feeding (Carr and Derby, 1986a; Ferner & Jumars, 1999). Determining the identity of these chemical signals is critical to understanding the more significant issues involved in the ecological, physiological and evolutionary importance of these processes.

Deposit Feeding

Recent studies of deposit feeding organisms have attempted to determine possible nutrient resources that deposit feeders, such as polychaetes and fiddler crabs, extract from benthic sediments by identifying relevant chemical signals (Robertson et al., 1981; Rittschof and Buswell, 1989; Weissburg and Zimmer-Faust, 1991; Ferner & Jumars, 1999). The ultimate goal of deposit feeding organisms is to concentrate the sparsely distributed edible material available in benthic sediments for digestion. What portion of this edible material deposit feeders actually assimilate remains unclear, but candidates include various combinations of bacteria and their exudates, protozoa, microalgae, non-living particulate detritus and interstitial solutes (Jumars, 1993). Attempts at identifying these resources have focused on examining the organic chemical characteristics of

sediments and the kinetics of deposit feeding. Another possibility is to explore the mechanics of how deposit feeders locate and obtain edible material and what factors may regulate these processes.

To compensate for the poor bulk nutritional value of sediments, deposit feeders have adopted several strategies to concentrate edible particles for digestion. Regulation of ingestion rate and retention time in the gut is the simplest, and therefore most common. High ingestion rates and short residence times allow an organism to process larger volumes of sediment and are common responses to low levels of organic material (Jumars, 1993). The regulation of these rates has a significant influence on the fitness of deposit-feeding organisms, and consequently, a considerable amount of research has focused on what factors may influence that regulation. Studies have shown that deposit feeders have disproportionate gut volumes (Penry & Jumars, 1990), and relatively short residence times (Cammen, 1980).

Another potentially important strategy involves some type of selective feeding. Studies of particle size selection by various deposit feeders indicate a general preference for smaller particles with lower specific gravity, which typically contain more organic matter per unit of volume (Taghon, 1982). Feeding rates have also been shown to increase and then decrease when sediments (or glass beads) are enriched with organic compounds (Taghon & Jumars, 1984; Kihslinger & Woodin, 1999; Taghon & Green, 1990).

It is likely that all deposit feeders selectively feed, but the degree of its importance to the overall feeding strategy should depend on costs of sorting and access to fresh material. For subsurface deposit feeders, selection may be less important (Self &

Jumars, 1988). At the sediment-water interface, however, and particularly for more sessile organisms, selective feeding most likely plays an important role. Advective transport of sediments by current and wave action consistently delivers new particles to an organism's feeding zone at this boundary, effectively swamping ingestion rates of interface feeders and supplying an abundant selection of particles to allow for choice (Miller & Sternberg, 1988).

Selectivity is dependent upon sensory capabilities as regulators of the selection process. These sensory capabilities may include visual and tactile discrimination of different resources, but chemoreception presumably plays the most important role because of the longer range of transmission of chemical signals in marine habitats.

Study Organism

One group of interface feeders, the spionid polychaetes, has been the focus of a number of studies on the selective aspects of deposit feeding, particularly chemosensory influences (Taghon, 1982; Taghon & Jumars, 1984; Taghon & Green, 1990; Kihslinger & Woodin, 1999; Ferner & Jumars, 1999). Spionids are generally classified as "interface" deposit feeders, signifying that they feed at the interface of the sediment surface and the water column. These organisms build tubes, using sediment grains and mucus, within the benthos that emerge from the surface of the sediment, rising several millimeters into the overlying water column. Spionids feed by extending two anterior appendages (*i.e.,* palps) out of these tubes to probe the surface of the sediment and the water column in search of food particles. Polychaete feeding strategies are varied, but many polychaetes employ a fairly continuous bulk ingestion strategy, limiting extensive

particle selection to the digestive and absorptive stages of feeding. Spionids, on the other hand, appear to sort and selectively retain particles prior to ingestion along the ciliated oral groove of the palps and at the pharynx (Dauer et al., 1981; Levin, 1981; Shimeta & Koehl, 1997). Probing of the sediment surface and the water column with the palps may involve passive selection via a mechanical method, due to the strength of adhesion of mucus secreted by the palps used to "grab" particles (Self & Jumars, 1978; Taghon, 1982; Shimeta & Koehl, 1997), or active selection via chemosensory detection.

This potential chemosensory role is supported by recent histological and ultrastructural evidence of putative chemosensory structures located on the palps and the anterior region of the prostomium of several spionid species (Dauer, 1984 and 1997; Qian & Chia, 1997; S. M. Lindsay, unpublished observations). These ciliated structures are regularly dispersed across both areas, and are structurally similar to chemoreceptor cells of sensory buds identified in caudal papillae of another polychaete, *Arenicola marina* (Jouin et al., 1985). Ultrastructurally, these structures contain characteristics typical of invertebrate chemoreceptor cells: short ciliary rootlets and many apical mitochondria.

Ferner and Jumars (1999) recently identified phagostimulatory and phagodepressant cues by exposing several spionid species to short pulses of dissolved stimuli and observing immediate changes in behavior. However, the dissolved nature of these stimuli permits the cues to potentially interact with chemoreceptors of the prostomium and the nuchal organ in addition to any located on the palps. In this study we limited the initial interactions of potential stimuli solely with the palps by covalently binding cues to glass beads and presenting them to one spionid species, *Dipolydora quadrilobata,* as a proxy for sediment of known organic content. Observations of palp

behaviors in response to the addition of the beads were used to determine the ability of this organism to recognize adsorbed cues.

MATERIALS AND METHODS

Collection and Maintenance of Animals

Dipolydora quadrilobata individuals were sieved (0.5 mm) out of cores collected from the mudflats of Lowe's Cove at the University of Maine's Darling Marine Laboratory (Walpole, ME, USA) on several days in June, July, August, and September of 2000. Animals and natural sediments were transported to the University of Maine in Orono and maintained in large culture tanks in an environmental chamber (14 °C:10°C, 12 h L:D cycle). Individual worms that showed no signs of gametogenesis, loss of segments, or other bodily damage and measured 10-20 mm in length were introduced into sediment-filled centrifuge tubes (50 mL, VWR), one worm per tube. Worms that established sediment tubes within 24 hours were used in the experiments.

Experimental Apparatus and Set-up

A single centrifuge tube containing an established *D. quadrilobata* individual was placed in a modified 1-liter plastic beaker filled with filtered seawater. The beaker was modified to allow a continuous flow of seawater across the top of the centrifuge tube (Figure 2.1). A steady stream of filtered seawater from a gravity enteral feeding bag entered into the chamber via a spigot inserted through the beaker wall. A thin plane of plastic was placed around the centrifuge tube, flush with its top rim, and the flow was

directed across this plane to an outlet on the opposite side of the beaker (flow rate = 1 cm sec^{-1}). Laminar flow across the plane was confirmed with dye experiments.

Experiments were carried out in a room cooled to ambient seawater temperature (10°C). Animals were illuminated with cool (fiber-optic) light and their behavior recorded via a video camera attached to a dissecting microscope and mounted on a swing arm above the beaker. Using a small glass pipette, glass beads were deposited in a circle measuring 1.5 to 2 cm in diameter and approximately 2 mm thick, surrounding the sediment tube of each *D. quadrilobata* individual. Palp behavior was recorded 15 minutes prior to and 15 minutes following the addition of the beads.

Stimuli Preparation and Quantification

Chemical cues were bound covalently to glass microbeads (45-63 μm in diameter, 2.5 specific gravity from MO-SCI Corporation Rolla, Missouri, USA) and used as a proxy for sediment of known organic content in the behavioral assays. Amino acids were covalently attached to the beads via a peptide bond to the free amino group of a linker molecule, aminopropyltriethoxysilane (APTS) (Sigma Chemical Co.). APTS links the amino acids to the beads by binding directly to the silanol groups on the surface of the glass beads (Yoshioka et al., 1991). Simple sugars were also bound via an APTS linker molecule, although by reductive amination with cyanoborohydride anion in aqueous solution at pH 7 (Gray, 1974; Roy et al., 1983).

Cues were bound to the glass beads by first creating alkylamine beads using APTS and then incubating these beads in concentrated solutions of the different cues (Brotherton et al., 1976; Clements, 1984; Taghon & Jumars, 1984). In the first step, the

beads were rinsed in distilled water, aspirated to a damp cake and air-dried. 50 g of the dry, clean beads were weighed into a flask and incubated in 12.5 mL of distilled water and 25 μL of APTS at room temperature for 1 hour. The beads were then washed in several changes of distilled water, and again aspirated to a damp cake and air-dried.

The alkylamine beads were soaked in 100 mL of distilled water for 15 minutes and incubated in either 200 mL of an amino acid solution (1mM in 0.1 M sodium phosphate buffer) at 4°C for 1 hour (swirling the flask periodically), or 50 mL of a sugar solution (10 mM in 50 mL 0.2 M borate buffer + 125 mg sodium cyanoborohydride) at 37°C for 3-4 days (again swirling periodically). Beads were subsequently washed in three changes of distilled water, soaked in 1 M NaCl for 20 minutes, and then frozen with a small volume of 0.45-μm filtered seawater.

The concentration of the amino acids bound to the glass microbeads was determined using a spectrophotometric assay of the reaction of amino acids with ninhydrin. Ninhydrin reacts with free amino groups to give off a characteristic color determined by the concentration of the amino groups present (Rosen, 1957; Moore, 1968; McGrath, 1972). The concentration can be determined by comparing the absorbance of the solution at λ=570 to a standard curve of solutions of known amino acid concentration.

Brotherton et al. (1976) found that only 3 percent of the total number of free amine groups from the APTS molecules bound to glass beads is bound by protein. Consequently, these free amine sites on the beads would also be included in a direct measurement of the concentration of any amino acids bound to the beads. As a result, we chose instead to determine the concentration of the amino acids that remained in the incubation solution and subsequent washes, assuming that subtracting this value from the

initial concentration of the incubation solution yields the concentration actually bound to the beads. The incubation solution and the three wash solutions were combined and mixed, and three 1-mL aliquots were extracted. These aliquots were mixed gently with 1 mL of the ninhydrin reagent (Sigma) and 2 mL of distilled water in a small test tube. The solutions were then incubated in a boiling water bath for 10 minutes, allowed to cool to room temperature, and diluted with 5 mL of 95% ethanol. Three aliquots of the solutions were placed in a spectrophotometer and the absorbance at λ=570 were read vs. a solution of distilled water and ninhydrin reagent as a blank.

A standard curve of the absorbance at λ=570 of five solutions of known amino acid concentration (0 mM to 1 mM) was simultaneously prepared to which the samples were compared (Figure 2.2). The calculated concentrations of the three samples were averaged to obtain a measurement of the total concentration of amino acids in the mixture of solutions. Assuming that everything not in the incubation solution and washes was bound to the beads, we subtracted the calculated value from the concentration of the original incubation solution to obtain an estimate of the concentration of the amino acids bound to the beads.

The concentrations of the sugars bound to the beads were determined with a spectrophotometric assay of the reaction of the sugars with phenol and concentrated sulfuric acid. This reaction also yields a color change that can be quantified spectrophotometrically by comparing absorbance at λ=490 to that of a calibration curve of known sugar concentrations (Dubois et al., 1956). Because there were no other groups bound to the beads that would complicate this assay, we were able to obtain a direct measurement of the concentration of sugars actually bound to the beads. Three 1 mL

aliquots of the thoroughly mixed reaction solution of the beads and sugar solution were washed six times with 0.2 M NaOH and once with 4% phenol by centrifugation in a clinical centrifuge. The beads were drained and mixed with 2.0 mL of 4% phenol and 5.0 mL of concentrated sulfuric acid, added rapidly via pipette. The mixture was allowed to stand for 10 minutes and then stirred rapidly for 1 minute on a vortex mixer. The absorbance at λ=490 was read vs. a solution of distilled water, phenol, and sulfuric acid as a blank.

A standard curve of absorbance at λ=490 of five solutions of known sugar concentration (0 mM to 1mM) was simultaneously prepared to which the samples were compared (Figure 2.2). The calculated concentrations of the three samples were averaged to obtain a measurement of the total concentration of the sugars bound to the beads.

Behavioral Assay

Each worm was exposed to two trials: one with glass beads bound with a chemical cue and one with beads bound only with the APTS linker molecule. The two trials were performed on separate days and each worm was exposed to just one cue. A total of seven trials were conducted; three with single amino acids (proline, alanine, or threonine) one with a mixture of amino acids (proline + alanine + threonine + valine + taurine + glycine) and three with simple sugars (glucose, maltose, or galactose). Responses to the cues were quantified by scoring the videotaped records of the palp behaviors for the time spent in a suite of behaviors prior to and following the addition of the beads. The classification of palp behaviors was modified from Ferner and Jumars (1999). Behaviors were separated into three classes: inactive, active but non-feeding, and

actively feeding. The active classes were separated further into types of searching behavior and rates of feeding (Table 2.1). Feeding was defined as active collection of particles (either natural sediments or glass beads) with the palps and transport along the food grove to the mouth. Actual ingestion occurred inside the tube and out of view of the camera, but particles or beads that entered the tube typically reappeared solely in the form of fecal rods, indicating that ingestion had occurred. Feeding rates were inferred by the percent coverage of the palps with particles and the speed of transport of those particles (Ferner & Jumars, 1999).

To identify the cues as stimulatory, inhibitory, or inactive, we compared the behavior of each worm in response to the addition of beads bound with a cue to their behavior in response to the addition of unbound beads (paired Students' t-tests). Observations of behavior prior to the addition of beads were used to document background behavior patterns and verify that active levels were similar for all trials.

RESULTS

Stimuli Quantification

The concentrations of the single amino acids bound to the beads ranged from ca. 175 μg/gram of beads to 180 μg/gram of beads. The concentration of the amino acid mixture bound was ca. 580 μg/gram of beads, and the concentration of the sugars bound ranged from ca. 30 μg/gram of beads to 50 μg/gram of beads (Table 2.2). Each worm was exposed to approximately 1 gram of beads per trial and therefore the total cue concentrations presented were in the range of 0.3-0.4 mM for the sugars, and 2-5 mM for the single amino acids and amino acid mixture.

Feeding Behavior

Palp behavior was observed for 15 minutes prior to bead addition and in all trials *D. quadrilobata* individuals were active for some portion of that time (Figure 2.3). The total time that worms were active before and after the addition of beads was not significantly different across all treatments, indicating that bead addition did not adversely affect general activity levels (Figure 2.3). Worms spent a majority of their active time probing the sediment surface and feeding (Figure 2.4). Comparisons of feeding time prior to bead addition found no significant differences between treatments and controls for all trials (Figure 2.5), indicating that any difference in behaviors observed in response to the addition of beads were not an artifact of differences in baseline feeding behavior. Compared to natural sediments, however, bead addition, depressed the total time worms spent feeding in all treatments (Figure 2.5).

Nonetheless, we saw significant feeding responses by *D. quadrilobata* to several chemical cues bound covalently to glass beads (Figure 2.5). The ratio of the time worms spent feeding to their total active time was significantly higher in response to beads bound with alanine, the amino acid mixture, glucose and maltose than in response to control beads. Responses to beads bound with proline, threonine, and galactose were not significantly different than in response to control beads.

Responses to Bound vs. Dissolved Cues

We were concerned that the cues may not have remained bound to the beads and instead became waterborne during deposition through the water column. Therefore, we performed the behavioral assays under slow flowing seawater in an attempt to flush the

chamber of any potential waterborne cues. As a measure of the effectiveness of the cue binding procedure and the effort to flush the chamber of dissolved cues, we compared the time period between the addition of the beads and the first extension of a palp out of the tube in response to the treatments and controls.

As beads were deposited around *P. quadrilobata* tubes, worms typically withdrew the palps into the safety of their tubes, and remained withdrawn for anywhere from a few seconds to a few minutes. This withdrawal behavior is most likely in response to the vibrations produced in the water column by the bead deposition. Emergence of the palps out of the tube is primarily dependent upon the cessation of the vibrations, but may also be influenced by the presence of other signals. The presence of a stimulus representing a food source may motivate a worm to reappear faster than in the absence of such a stimulus. Thus, if the cues were becoming waterborne (and assuming they were stimulatory), we expected to see shorter reappearance times in response to the addition of treated beads compared to the addition of control beads. This was not apparent for any of the treatments (Figure 2.6), indicating that either the cues were remaining bound to the beads or that the flow was effective in flushing the chamber of dissolved cues.

After emergence from the tube, worms actively probed the sediment surface for a varying amount of time prior to picking up particles and transporting them along the palp to the mouth. The length of this time period (*i.e.,* from emergence to initial feeding) could represent the ability of *D. quadrilobata* individuals to immediately recognize the cue (or lack thereof) bound to the beads using the putative sensory structures on the palps. Accordingly, we compared this time period (*i.e.,* the time to initial ingestion) in response to the addition of the treated beads *versus* control beads. Only in response to beads bound

with the amino acid mixture was this time period significantly shorter than in response to the addition of the control beads (Figure 2.7, t-test p=0.0003). However, the other identified stimulatory cues (alanine, glucose and maltose) show a similar trend even though the results are not statistically significant (Figure 2.7, p=0.45, p=0.44 & p=0.23, respectively).

DISCUSSION

The results demonstrate the ability of one spionid polychaete species, *Dipolydora quadrilobata,* to detect and respond to several bead-bound cues. We were primarily interested in determining the ability of individuals to recognize adsorbed cues and not necessarily in determining all the cues that might regulate feeding or the ecological significance of those cues. The cues used in the assay were chosen based on prior results showing stimulatory interactions with several marine organisms (Carr, 1988; Ferner & Jumars, 1999), reasoning that stimulatory effects would be easier to detect when compared to a control of little to no feeding response. We selected amino acids and simple sugars as potential feeding cues because low molecular-weight compounds are particularly common feeding cues (Carr, 1988). These cues were not chosen to represent any ecologically relevant food source nor were any attempts made to determine threshold levels or dose-response curves for any of the cues. Nonetheless, the phagostimulatory effect of the simple sugars may indicate plant material as a potential food source for this polychaete (Self et al., 1995). The prevalence of almost all amino acids in most organisms makes it difficult to speculate on possible sources of the monomeric cue

alanine, and similarly suggests that it is unlikely that such a ubiquitous signal would be used as a cue for food.

The response to the amino acid mixture however, is more intriguing. It is likely that oligomers or mixtures of monomers would be more informative signals due to better specificity to a food source. Although all organisms contain the full complement of amino acids, a mixture of particular amino acids or an oligomer may be more distinctive to an individual organism. In addition, free amino acids are also more rapidly taken up by bacteria than short peptides, suggesting that peptides are more likely to persist in marine environments and thus would serve as better stimuli (Decho et al., 1998)

Mixtures of cues can also act to stimulate a wider diversity of membrane receptors, and it has been shown in some invertebrates that mixtures can result in a summation electrophysiological response in which the signal is of greater intensity in response to the mixture than in response to any of the individual components, though the salience of each component is not always lost (Carr and Derby, 1986b; Livermore et al., 1997). This may explain the difference in response time (*i.e.*, initial ingestion) to the amino acid mixture compared to the other cues even though the concentration of each cue presented was relatively equal. The summation nature of a mixture may furnish an organism the capacity to increase its sensitivity and detect stimuli concentrations that are at subthreshold levels for the individual components (Carr and Derby, 1986a).

For deposit feeding spionids, ingestion rate is most likely regulated by a variety of factors, including the continual assessment of the quality of particles during their passage from the palps through the pharynx and gut (Dauer et al., 1981; Levin, 1981; Taghon, 1982; Self & Jumars, 1978; Shimeta & Koehl, 1997). As a result, the ingestion rate can

be elevated or depressed by a variety of interactions. In this study, the background feeding times prior to bead addition were significantly higher than in response to the addition of beads (either treated or controls). The decrease seen after bead addition may be a result of a negative feedback from interactions later in bead handling. Although the beads were coated with a potentially stimulatory compound, they have no inherent food value. Consequently, the beads should be recognized as valueless particles at least in the gut if not earlier, and this may result in depression of ingestion rate. *D. quadrilobata* defecates approximately once every fifteen to twenty minutes when feeding at a moderate rate (T. Riordan, pers. obs). This retention time falls within our observation period, and therefore it is likely that these organisms are receiving feedback from the gut regarding little to no nutrient adsorption from the beads. This would most likely result in a depression of the ingestion rate, resulting in the decrease in total feeding time seen.

Mechanical selectivity has also been shown to be an important regulator of spionid feeding rates (Jumars et al., 1982; Self & Jumars, 1988). Adhesive mucus secreted and distributed along the palps of spionids acts to increase particle retention after contact. Particle retention appears to be size and density dependent, with particles of lower weight per unit of surface area more likely to be collected (Jumars et al., 1982). Smaller and lighter particles tend to have more organic matter per unit of volume (Taghon, 1982); therefore this fairly simple passive selection mechanism can significantly increase ingestion of food-rich particles. This passive selection may explain the ingestion of a limited amount of the control beads that have no food value or cues attached, and suggests a two-pronged selective feeding approach. Spionids may use chemosensory cues to focus particle collection in food-rich patches and utilize

mechanical selection of particles with lower specific gravity as a default strategy. This type of partial active preference should ensure that the organism ingests particles most likely to have some food value even when cues are too dilute for chemosensory detection to be efficient.

As Jumars (1993) states in reference to other studies of particle preference, without documentation of behavioral changes, such as ciliary reversals, that lead to preferential retention or rejection of beads coated with potential cues when compared to clean beads, studies that show preference can be interpreted as simply showing a mechanical consequence of the greater 'stickiness' of the coated beads (*e.g.*, Taghon, 1982). We were not able to observe such behavioral changes in this study, however using beads coated with the linker molecule APTS as controls may circumvent this problem. Assuming that 'stickiness' is a function of the size of the compounds coating the particles, and given that only approximately 3% of the APTS binding sites are typically bound with a cue (Brotherton et al., 1976) and that the APTS compound is bigger or of comparable size to the amino acids and the sugars used in this assay, we would not expect the treated beads to be significantly more sticky than those of the control. Consequently we can fairly conclude that the increased feeding responses seen are a response to the cues bound to the beads.

CONCLUSIONS

These experiments were conducted as a first survey of the ability of spionid polychaetes to recognize specific sediment-bound chemical cues. Recent histological and ultrastructural findings show that the palps of some spionids bear ciliated structures that

appear to be sensory in function. This suggests that the palps play not only a mechanical role in deposit feeding, collecting particles and transporting them to the mouth, but a sensory role as well. Prior research has shown that spionid feeding rates are influenced by dissolved chemical cues, as well as sediment enrichments, but prior to this study no attempt at localizing the stimuli to specific sensory structures had been made. Even here, we have not shown conclusively that the stimulatory effects seen in response to the bead-bound cues are a direct result of interactions of the cues with the putative receptors located on the palps. Separating that interaction from those that occur later in bead handling (*i.e.* at the pharynx, or in the gut) is difficult in such a behavioral study, but studies of neurophysiological interactions (*e.g.*, activity-dependent labeling experiments such as those of Michel et al., 1999) may help clarify the distinction.

Table 2.1. Behavior Classification (adapted from Ferner & Jumars, 1999)

Inactive:

1. Palps withdrawn
2. Palps slightly extended
3. Palps extended but stationary (on sediment or in water column)

Active: Non-feeding (no palp coverage)

4. Searching
5. Probing
6. Tube building

Active: Feeding (palps covered in beads to some degree)

7. Searching/probing/feeding
8. Probing/Feeding
   - -Low coverage (<25% of palp area) & slow transport
   - -Medium coverage (25-75% of palp area) & slow transport
   - -High coverage (>75% of palp area) & slow transport
   - -Low coverage (<25% of palp area) & fast transport
   - -Medium coverage (25-75% of palp area) & fast transport
   - -High coverage (>75% of palp area) & fast transport

Others:

9. Palp retraction
10. Twisting and knotting of palps
11. Fecal pellet removal
12. Bead removal

Table 2.2. Concentrations of cues bound to beads and total concentration presented in each trial

Cue	Concentration Bound to Beads	Concentration Presented Per Trial
Amino Acid Mixture	580 μg/g beads (total)	2.3 mM (each)
Proline	178 μg/g beads	3.9 mM
Alanine	178 μg/g beads	5.0 mM
Threonine	176 μg/g beads	3.7 mM
Glucose	31 μg/g beads	0.4 mM
Maltose	46 μg/g beads	0.3 mM
Galactose	30 μg/g beads	0.4 mM

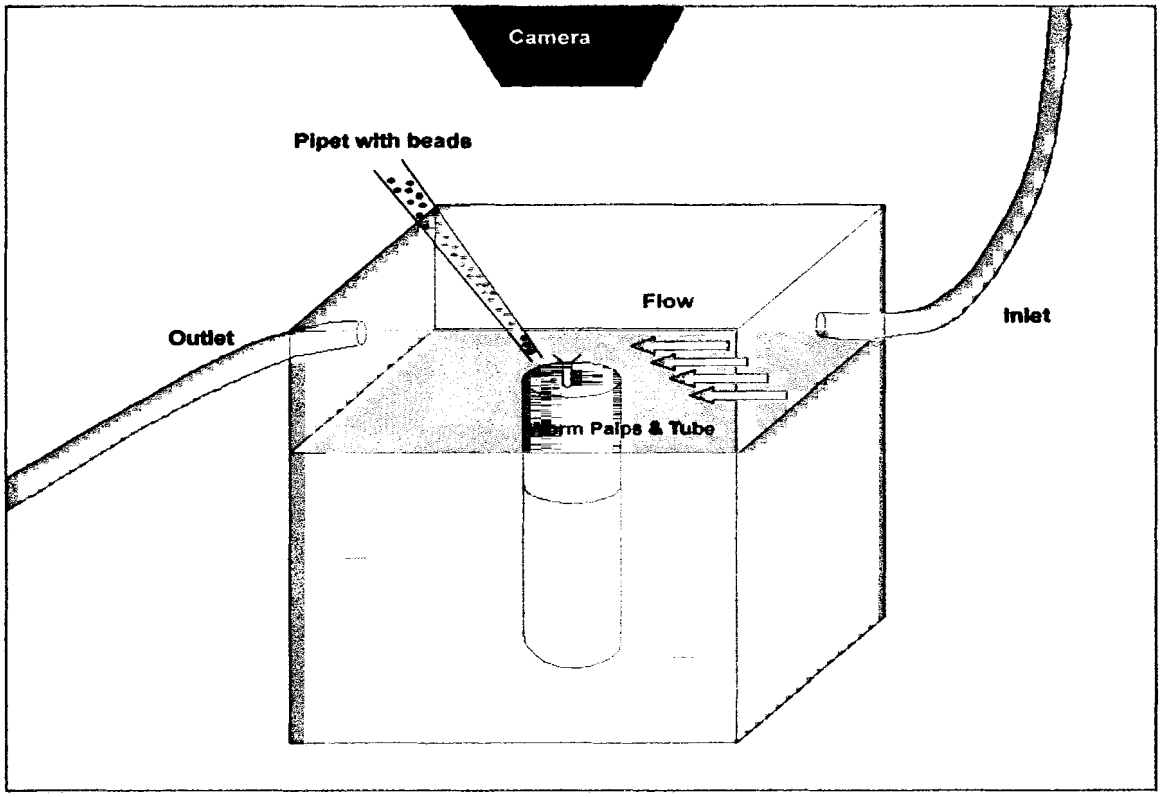
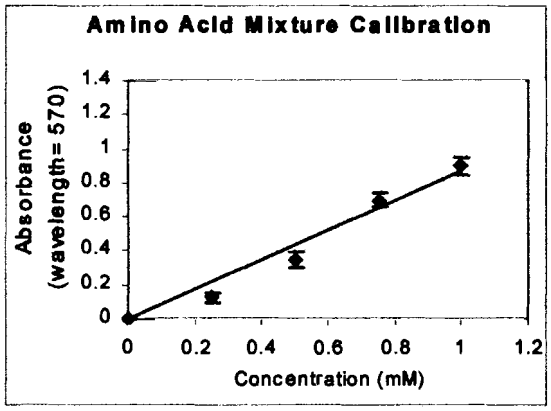
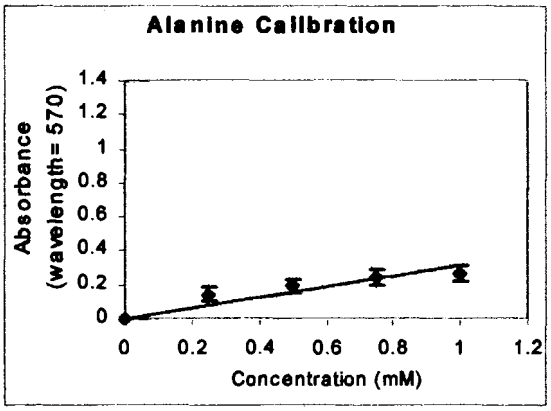
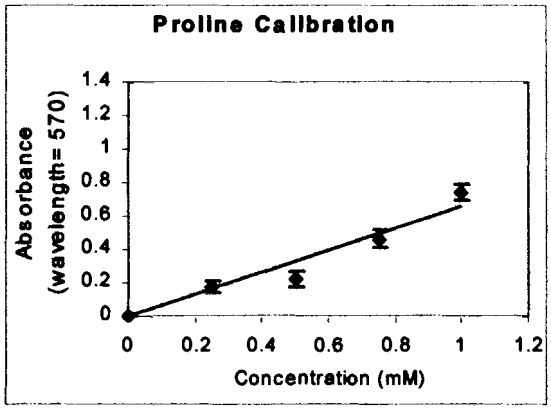
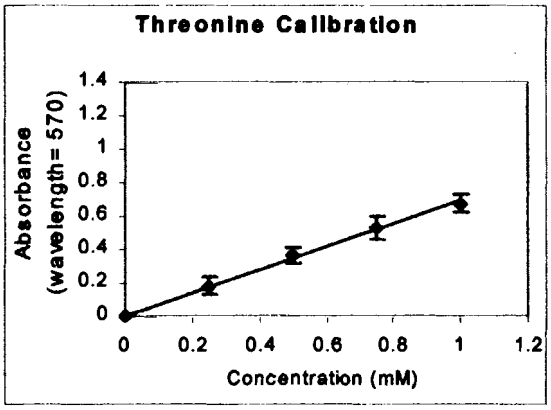


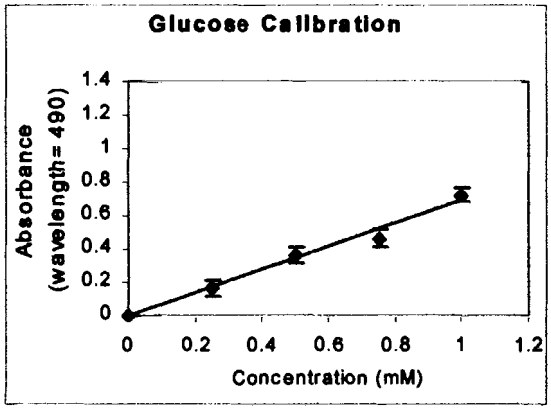
Figure 2.1. Side view of apparatus used in behavior trials (see text for description).

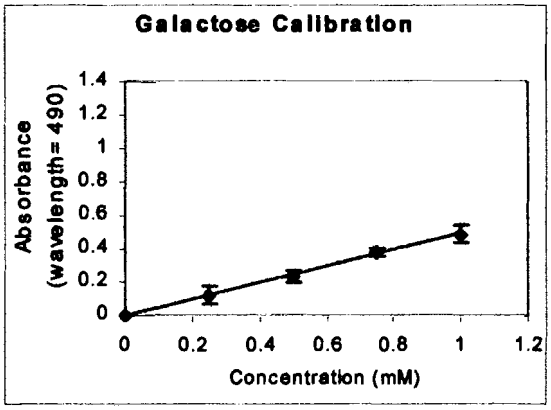












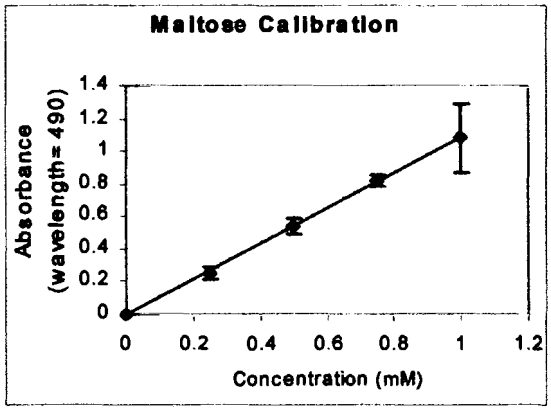


Figure 2.2. Calibration curves for ninhydrin assay of amino acid concentrations and phenol assay of sugar concentrations. Error bars are +/- 1 standard error.

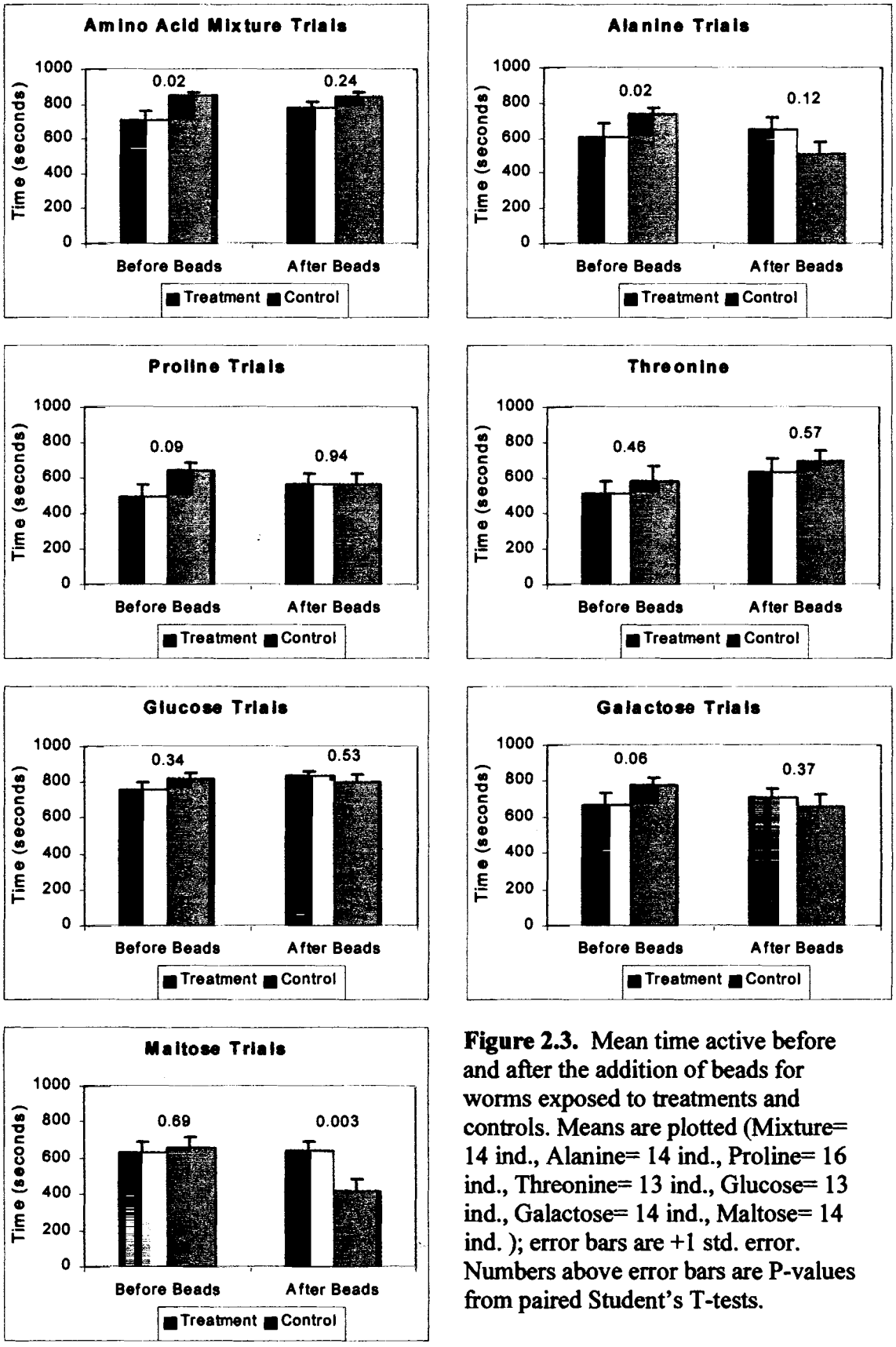


Figure 2.3. Mean time active before and after the addition of beads for worms exposed to treatments and controls. Means are plotted (Mixture= 14 ind., Alanine= 14 ind., Proline= 16 ind., Threonine= 13 ind., Glucose= 13 ind., Galactose= 14 ind., Maltose= 14 ind.); error bars are +1 std. error. Numbers above error bars are P-values from paired Student's T-tests.

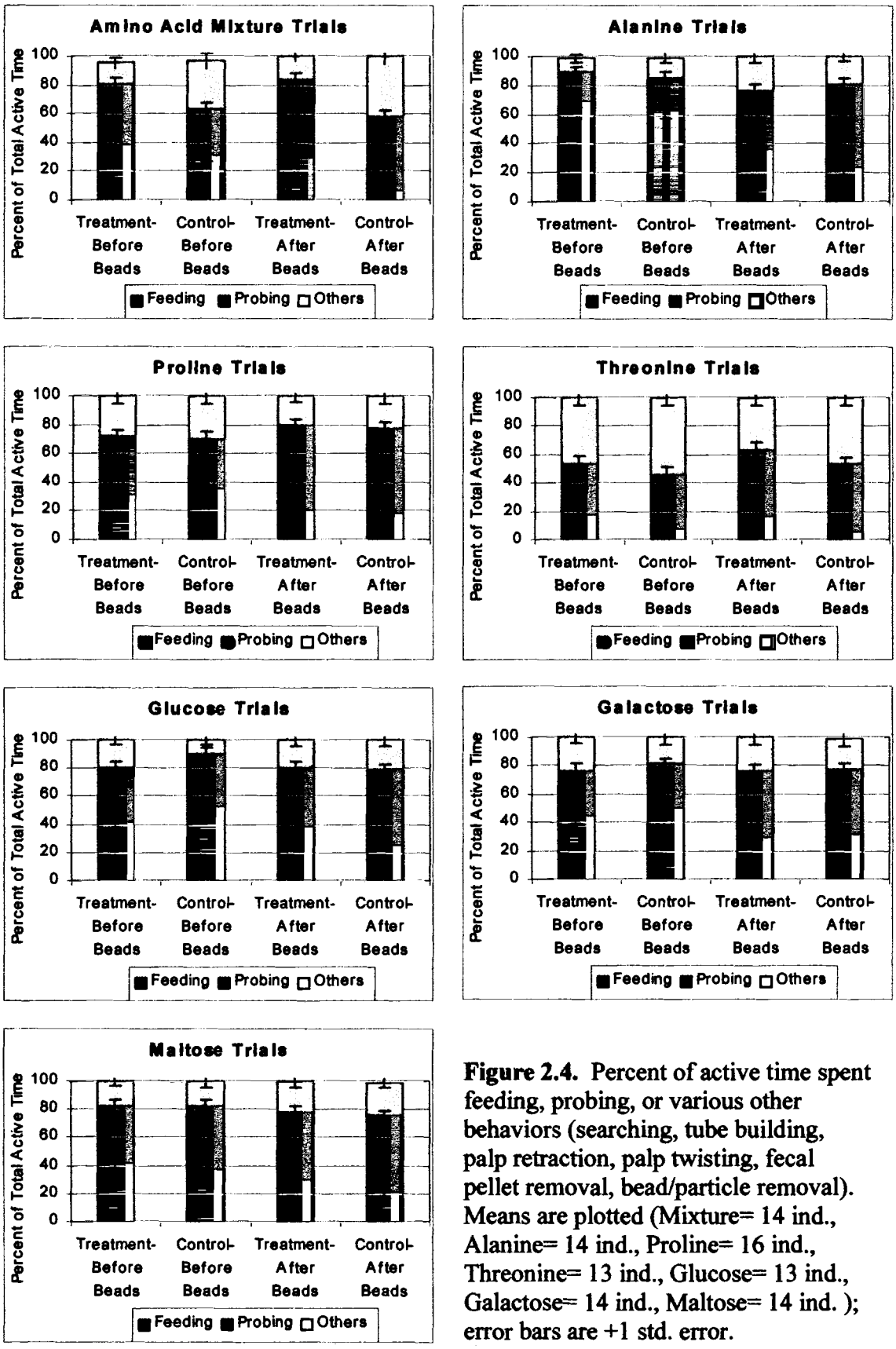


Figure 2.4. Percent of active time spent feeding, probing, or various other behaviors (searching, tube building, palp retraction, palp twisting, fecal pellet removal, bead/particle removal). Means are plotted (Mixture= 14 ind., Alanine= 14 ind., Proline= 16 ind., Threonine= 13 ind., Glucose= 13 ind., Galactose= 14 ind., Maltose= 14 ind.); error bars are +1 std. error.

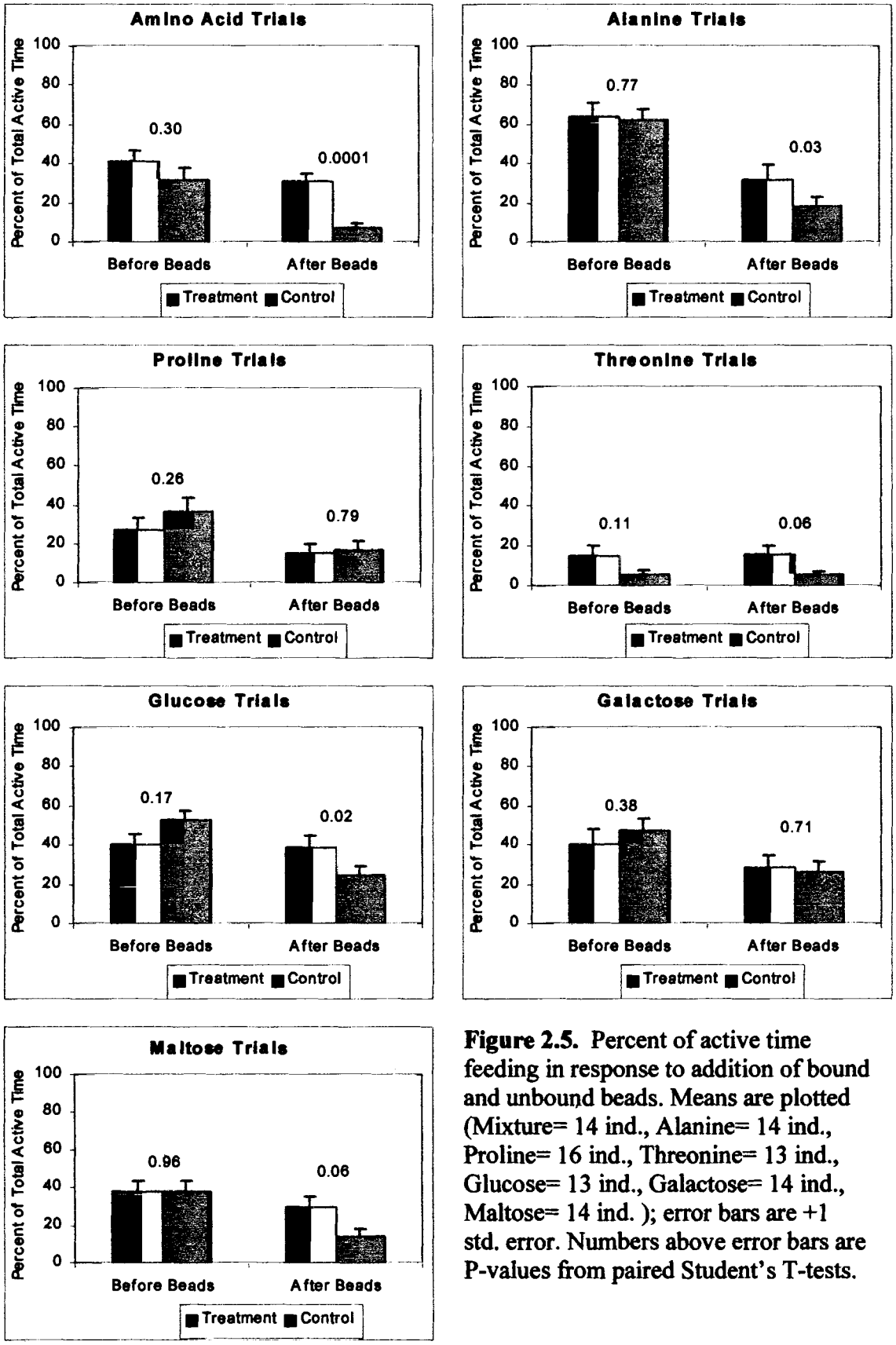


Figure 2.5. Percent of active time feeding in response to addition of bound and unbound beads. Means are plotted (Mixture= 14 ind., Alanine= 14 ind., Proline= 16 ind., Threonine= 13 ind., Glucose= 13 ind., Galactose= 14 ind., Maltose= 14 ind.); error bars are +1 std. error. Numbers above error bars are P-values from paired Student's T-tests.

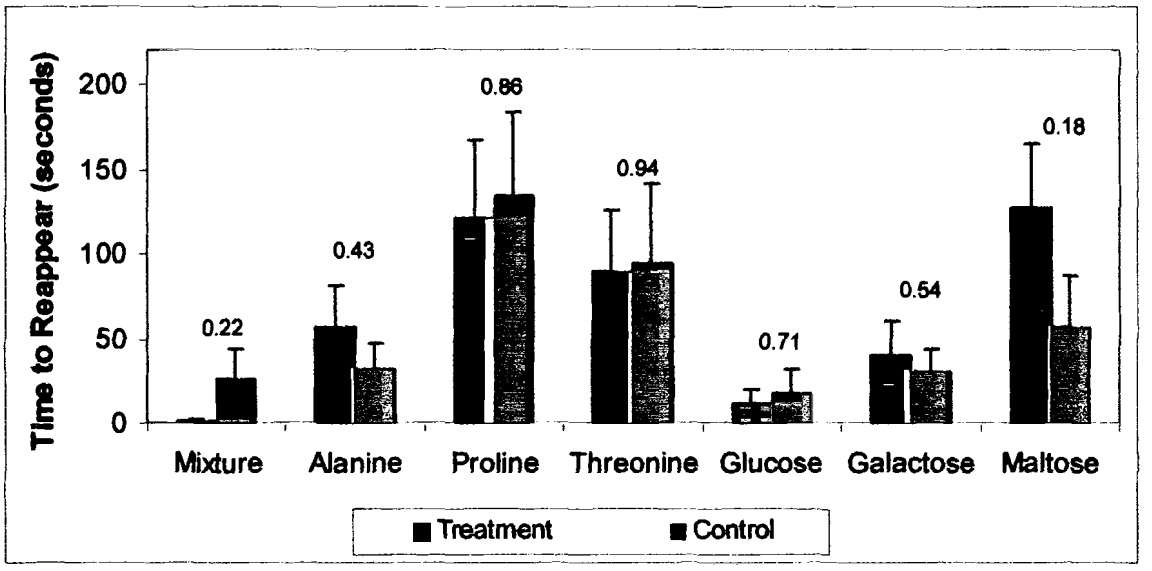


Figure 2.6. Time to reappear after addition of beads. Means are plotted (Mixture= 14 ind., Alanine= 14 ind., Proline= 16 ind., Threonine= 13 ind., Glucose= 13 ind., Galactose= 14 ind., Maltose= 14 ind.); error bars are +1 std. error. Numbers above error bars are P-values from paired Student's T-tests.

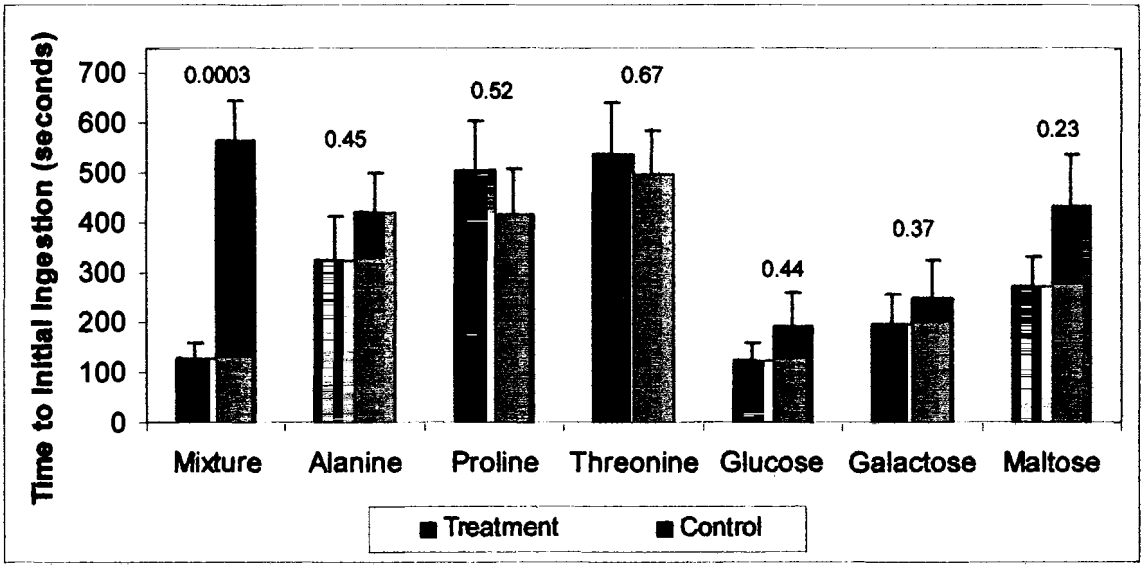


Figure 2.7. Time between addition and initial ingestion of beads. Means are plotted (Mixture= 14 ind., Alanine= 14 ind., Proline= 16 ind., Threonine= 13 ind., Glucose= 13 ind., Galactose= 14 ind., Maltose= 14 ind.); error bars are +1 std. error. Numbers above error bars are P-values from paired Student's T-tests.

CHAPTER 3

NEUROPHYSIOLOGICAL STUDIES OF CHEMORECEPTION IN *DIPOLYDORA QUADRILOBATA*

INTRODUCTION

Marine organisms are exposed to a vast array of chemical stimuli. These chemicals commonly appear as components of plant and animal metabolites, excreta, secretions, and autolytic products from dead and dying organisms, and are typically polar amino acids, peptides, and nucleotides (Carr, 1988). These chemicals often function as important stimuli, transmitting information about the surrounding environment to an organism and (when present in appropriate intensities, combinations or patterns) eliciting specific behavioral responses.

The behavioral responses to chemical cues are mediated by chemosensory receptors located on the external surface of an organism's body. These cells are responsible for detecting chemical stimuli and decoding the information they transmit. In its raw form, this information typically consists of the identity, quality, and quantity of a given stimulus. Such information is transmitted to the central nervous system for processing and the formulation of a response. The information gleaned from the decoding of complex signals can supply the organism with knowledge of the location and approximate distance of the source, as well as its implications (*i.e.* danger, food source, potential mate, etc.).

Responses to Chemical Cues by Spionid Polychaetes

Studies have shown that chemical cues have a direct influence on spionid polychaete feeding rates (Ferner & Jumars, 1999; and see Chapter 2). However, the mechanisms underlying that influence are less well known. The prevalence and organization of chemoreceptors within spionids has not been fully explored, with the

exception of the nuchal organs (Schlotzer-Schrehardt, 1986 & 1987). Nuchal organs are found on most polychaetes; typically as paired epidermal structures located on the dorsal side of the peristomium or prostomium, and are presumed to be chemosensory in function. In addition, sensory cells have been identified on several other polychaete species, including epidermal papillae of the deposit-feeding lugworm *Arenicola marina* (Jouin et al., 1985), compound sensory organs on the prostomial cirri and palps of *Nereis diversicolor* (Dorsett & Hyde, 1969), and the parapodial cirri of nereid polychaetes (Boilly-Marer, 1972). Ciliated papillae have been identified on the palps of several spionids and have been postulated to have a sensory function (Hempel, 1957; Dauer, 1987, 1991 & 1994; Qian & Chia 1997), but no direct evidence has been found thus far.

The function of the palps (*i.e.* locating and collecting food resources from the deposited material that makes up the benthos or from the water column) however, suggests that they may serve in some sensory capacity. This capacity would most likely involve the detection of dissolved or adsorbed cues indicating food availability, quality, or location. If these are legitimate palp functions, the surface of the palp should be equipped with chemoreceptor structures.

The palps are innervated, with connections to the anterior region of the brain (Bullock & Horridge, 1965; Schlotzer-Schrehardt, 1987). In addition, recent histological and ultrastructural evidence suggests that putative sensory structures are present along the lateral and abfrontal surface of the palps of several spionid polychaete species (Dauer, 1984 and 1997; S. M. Lindsay, unpublished observations). Further examination of functions and sensitivity of such structures should yield important information about the

sensory role the palps may play in spionid deposit feeding, as well as the overall chemoreceptive abilities of spionids.

Chemoreceptive Transduction

A great diversity of chemosensory organs exists among marine organisms, from crustacean aesthetasc hairs to molluscan osphradium and polychaete nuchal organs (Laverack, 1968). Despite this diversity, such structures share common features. The transduction of externally detected signals to the brain follows a similar pathway. In all cases this pathway starts with the activation of a chemoreceptor neuron that leads to the central nervous system.

Chemoreceptor neurons are generally bipolar neurons whose dendrites carry the molecular elements necessary for signal transduction and whose axons extend into and synapse with the central nervous system. The apical ends of the dendrites typically branch off into cilia or microvilliar extensions that are in direct contact with the environment, though they may be covered in a mucus secretion. These extensions increase the surface area of the cell and allow for greater access to potential stimuli. Invertebrates appear to use primary bipolar receptor neurons for both olfactory and gustatory functions, in contrast to vertebrates who have distinct taste buds for gustation (Finger & Simon, 2000). As a result, the transduction mechanisms for both olfaction and gustation are likely to be similar.

Distinguishing between smell and taste in marine organisms is difficult, but this similarity between the two processes physiologically in invertebrates may make the distinction less important. The olfactory and gustatory transduction pathways are both

multi-step processes that start with the binding of a ligand with a receptor on the membrane of the dendrites of a sensory cell (Dionne & Dubin, 1994). Recent evidence suggests that the receptor-bound ligand activates a membrane-bound GTP-binding protein that, in turn, stimulates the production of an intracellular second messenger, typically either adenosine 3'5'-cyclic monophosphate (cAMP) or inositol-1,4,5-trisphospate (IP_3) (reviewed by Lancet and Ben-Arie, 1993; Nef, 1993). These second messengers target membrane-bound ion channels, producing the initial depolarizing current that propagates an action potential. cAMP typically targets nonspecific cation channels causing an influx of calcium ions and a depolarization of the cell (Baumann et al., 1994, Hatt & Ache, 1994, Coburn & Bargmann, 1996). The depolarization caused by the influx of calcium ions induces further channel activation and a concomitant depolarization of the cell, ultimately pushing the cell potential past a threshold that results in the propagation of an action potential. The IP_3 pathway is less understood, but in some invertebrates it also appears to function in a stimulatory pathway, suggesting that it may work in parallel or as an alternative to the cAMP pathway (Fadool & Ache, 1992; Boekhoff et al., 1994; Hatt & Ache, 1994).

Recent Studies of Chemoreception

Studies of chemoreceptive abilities have focused primarily on behavioral demonstrations of cue recognition; however, increasing knowledge of the physiological mechanisms behind cue recognition has provided other avenues for research. Connecting a behavioral response to a specific receptor cell, or even groups of cells, requires finer-scale investigation. Similarly, the spatial determination of chemoreceptive capacity is

dependent upon the visualization of individual neuronal activity. This has been attempted with various methods, notably utilizing electrophysiological recordings (Erickson & Caprio, 1984; Mackay-Sim & Kesteven, 1994; Scott et al., 1997) and voltage and calcium sensitive dyes (Cinelli & Kauer 1992; Kent & Mozell 1992; Fetcho & O'Malley, 1995; Fetcho et al., 1998).

More recently, a high-resolution method of functionally labeling olfactory neurons in both vertebrates and invertebrates has been developed (Michel et al., 1999). This method exploits the ability of cationic guanidinium analogs to enter into stimulated neurons and metabolically active cells (Dwyer et al., 1980; Picco & Menini, 1993). These analogs gain access to active neurons through nonspecific cation channels activated and opened by the binding of a ligand with its receptor protein. Sequestration of the analogs in these cells allows for the activity-dependent labeling of individual receptor neurons.

One guanidinium analog, 1-amino-4-guanidobutane (= agmatine), has been shown to enter into receptor neurons through these open cation channels (Yoshikami, 1981). Agmatine has been coupled with known stimulatory cues in solution and perfused over olfactory organs, causing the stimulation of odorant receptors and the entrapment of agmatine in the corresponding cells (Michel et al., 1999; Steullet et al. 2000). Cells that have accumulated agmatine can be identified using an anti-agmatine IgG antibody followed by silver intensification labeling (Marc, 1995, 1999a & b), allowing for the identification of individual neurons activated by a specific cue.

I have adapted this method in studies with the spionid polychaete *Dipolydora quadrilobata*, in an attempt to show odor-stimulated activity of putative chemoreceptors located on the surface of the palps. Using phagostimulatory cues identified in behavioral

assays (see Chapter 2), I show that this technique is applicable to this organism and that the presumed sensory structures located on the palps of *D. quadrilobata* are activated by the same chemical cues that elicit the behavioral responses.

MATERIALS AND METHODS

Collection and Maintenance of Animals

Dipolydora quadrilobata individuals were sieved (0.5 mm) out of cores collected from the mudflats of Lowe's Cove at the University of Maine's Darling Marine Laboratory (Walpole, ME, USA) on several days in September and October of 2000, and March, April, and May of 2001. Animals and natural sediments were transported to the University of Maine in Orono and maintained in large culture tanks in an environmental chamber (14 °C:10°C, 12 h L:D cycle). Individual worms that showed no signs of gametogenesis, loss of segments, or other bodily damage and measured 10-20 mm in length used in the experiments.

Activity-Dependent Agmatine Labeling Procedure

Individual *D. quadrilobata* were immersed in artificial seawater (ASW: see Solutions and Chemicals section) inside a small cover-slip perfusion chamber (Warner Instruments, Model # RC 21B). Odorant stimuli were added to the ASW perfusion fluid in 5 second pulses every 60 seconds for 60 minutes. The ASW and the odorant stimuli solutions were held in 60-mL syringes connected to the perfusion chamber via rubber tubing and a manifold. Fluid flow from the syringes was via gravity feed and flow rates (0.5 cm sec^{-1}) were controlled by stopcocks; flows were turned on and off by

electronically activated pinch valves. Stimuli included 20 mM AGB in ASW (control) and 20 mM AGB plus a mixture of amino acids (proline + alanine + threonine + valine + taurine + glycine: 1mM each) in ASW (treatment). Following the 60-minute stimulation period, ASW was perfused over the worms for 5 minutes to remove residual AGB. Worms were then immersed in fresh ASW and relaxed by placing them in a freezer (-20°C) for 10 minutes prior to fixing. Whole worms were placed in fixative (see Solutions and Chemicals section) overnight to several days.

Tissue Processing, Immunolabeling, and Visualization

Fixed worms were rinsed in a phosphate buffer (PB) and dehydrated through a graded series of absolute ethanol and acetone. The dehydrated tissue was embedded in Epon 812 resin, cured and sectioned using a microtome and glass knife. Semi-thick sections (2 μm) were placed in 7 millimeter wells of a teflon-coated spot slide (Erie Scientific), deplasticized in a 1:5 v/v solution of mature sodium ethoxide in anhydrous ethanol, and subsequently washed in three changes of anhydrous ethanol. The slides were dipped briefly in deionized water, air-dried and then incubated overnight in a 1:100 dilution of a polyclonal anti-AGB IgG antibody (Signature Immunologics, Salt Lake City, Utah). The anti-AGB antibody was raised in rabbits against a glutaraldehyde-conjugated AGB-albumin complex.

The slides were then rinsed in PB, washed in 1% goat serum in phosphate buffer plus 0.05% thimerosal (1% GSPBT) for 10 minutes, and incubated in a 1:50 dilution of a 1 nm gold conjugated anti-rabbit IgG for 60 minutes. The slides were rinsed quickly again in PB, washed in fresh PB for one hour, dipped in deionized water, and air-dried.

Visualization of labeled cells was accomplished using silver intensification (Marc, 1999a & b). Briefly, the sections were exposed to a silver nitrate solution (see Solutions and Chemicals section) for 4-6 minutes in a dark location and the reaction was stopped with a brief dip in 5% acetic acid. Finally, the slides were washed in deionized water for 10 minutes, air-dried, and mounted in Permount (Fisher) for visualization on a light microscope.

Image Digitization and Analysis

Images of the sections were captured digitally using an Olympus light microscope (Olympus BX 60) with a bright field video camera (Javelin, Model JE12HMV) attached to a framegrabber board (Scion LG 3) in a computer. The images were analyzed using the Scion ImagePC software (Scion Corporation, Frederick, MD).

Cells labeled with agmatine were identified by quantifying the pixel intensity inside a cell of interest in the digitized images and comparing it to the pixel intensity of an unlabeled region adjacent to the cell (*e.g.* Michel et al., 1999). Specifically, the mean and the standard deviation of the pixel intensity inside the adjacent unlabeled region were used to calculate a 95% confidence interval for background staining. The upper limit of this confidence interval was applied as a cut-off for discriminating agmatine-labeled cells. The mean and standard deviation of the pixel intensity inside cells of interest were also used to calculate a 95% confidence interval, and cells were counted as labeled if the calculated lower limit of this interval was higher than the upper limit of the background staining interval.

Labeled cells were grouped by type according to location within the sections. Cells located behind a straight line drawn across the sections and tangent to the back of the food groove were called abfrontal cells. Cells located between the food groove and the frontal cilia were called lateral cells. Cells immediately adjacent to the frontal cilia were called latero-frontal cells. And cells located within the frontal cilia were called frontal cells (Figure 3.1). The ratios of the number of each cell type to the total number of sections analyzed for treatment *versus* control trials were compared using a two-sample Students' t-test. To obtain a rough estimate of the numbers of each cell type present in a given length of palp, groups of serial sections were also stained with a toluidene blue stain. Toluidene blue stains acidic cell parts (*i.e.* nucleus) and allows for the identification of the number of each cell type present per section.

Solutions and Chemicals

The composition of artificial seawater (ASW) was, in mM: 423 NaCl, 9 KCl, 13 $CaCl_2$, 23 $MgCl_2$, 26 $MgSO_4$ (Cavanaugh, 1975) pH adjusted to 7.2. Agmatine sulfate was purchased from Sigma Chemicals. Phosphate Buffer (PB) was 1.76 g $NaH_2PO_4 \bullet H_2O$ + 7.67 g Na_2HPO_4 in 1 L of deionized water. The fixative was prepared by mixing 5 mL of 4% paraformaldehye, 2 mL of 25% glutaraldehyde, 13 mL of 0.2 M PB and 2 g of sucrose. The silver nitrate solution was prepared by mixing 5 mL of solution A + 1 mL of solution B + 1 mL of solution C (A= 114 mg citric acid + 342 mg sodium citrate in 6 mL of deionized water; B= 0.5 g hydroquinone in 15 mL of deionized water; C= 1% aqueous silver nitrate).

RESULTS

This preliminary study of the chemosensory capabilities of *Dipolydora quadrilobata* establishes the labeling of chemically stimulated cells using the cationic molecule agmatine technique as a viable method. Time limits prohibited a more comprehensive survey of potential stimuli and a better characterization of receptor specificities. However, the single cue assayed, a mixture of amino acids, was known to elicit fairly strong behavioral responses (see chapter 2). Consequently, the physiological responses observed appear to be behaviorally relevant.

Four different types of putative sensory cells were labeled by the perfusion of AGB+ the amino acid mixture over the palps of *D. quadrilobata*: frontal, latero-frontal, lateral, and abfrontal cells (Figures 3.1,3.2, 3.3, 3.4, 3.5 & Table 3.1). These four cell types all have cellular processes extending through the epidermis to the surface of the palp (Figure 3.2), many with visible ciliary projections extending from the surface of the palp, indicative of a sensory function. Frontal and latero-frontal cells were generally found in groups of several cells in close proximity to one another, and often most of these cells were labeled. The lateral and abfrontal cells, on the other hand, were always found in isolation.

Stimulation with a mixture of amino acids (proline + alanine + threonine + valine + taurine + glycine) in the presence of AGB resulted in a significantly higher ratio of the number of labeled abfrontal and lateral cells to the number of sections analyzed when compared to a control of no odor + AGB (Figure 3.6; t-Test $p=0.03$). The ratio of the number of labeled frontal and latero-frontal cells to the total number of sections viewed

was not significantly different between the treatment and the control trials (Figure 3.6; t-Test $p=0.17$ & $p=0.29$)

Counts of the number of cells of each type labeled by the toluidine blue stain give a rough estimate of the number of each type per distance of the palp (Figures 3.7 & 3.8, & Table 3.2). These numbers allow for a very rough estimate of the percentages of each cell type that are stimulated by the cue.

DISCUSSION

This study presents the first physiological evidence (albeit preliminary) of detection of chemical stimuli by putative sensory receptors on the palps of spionid polychaetes. Although a definitive identification of the function of these putative sensory cells cannot be fully established thus far, the association of the activity of these cells to stimulation with a behaviorally relevant cue strongly suggests a chemosensory function.

It has been previously established that spionid palps function in a passive mechanical selective role via mucus adhesion strength (Frankboner, 1978; Jumars et al., 1982; Taghon, 1982; Cameron & Frankboner, 1984; Dauer, 1985). Qian & Chia (1997) have speculated about a possible sensory role the palps may play in selective feeding, and putative sensory structures have been identified on the palps (Dauer, 1984 and 1997; S. M. Lindsay, unpublished observations), but prior to this study no direct evidence linking sensory cells to selective behavior has been found. These results are a significant step towards confirming this speculation by showing that these putative sensory cells are activated by a cue that elicits a selective feeding response.

Identification of these labeled cells as sensory in function is based not only on the mechanics of the labeling process (which requires the activation of membrane-bound ion channels to allow agmatine into the cell and is indicative of a sensory cell), but also on a combination of structural features, including their location in the epithelial cell layer, distal processes that extend through the epithelium reaching the surface of the palp, and apical cilia that protrude from that surface. This location, the cell structure, and the presence of cilia in sensory organs of invertebrates are well established (Ache, 1982; Ache & Derby, 1985; reviewed by Laverack, 1988). Axonal processes were not observed from any of the labeled cells, however it is possible that they were directed out of the plane of the sections.

Staining sections with toluidene blue provides some indication of the total number of these cell types present in a given length of palp. These numbers are by no means precise, only cells that had visible cellular processes extending to the palp surface were counted, which conceivably excluded many cells whose distal ends projected vertically out of the sections. As a result, these counts are most likely fairly conservative. However, these counts do show that not all of the cells of each type were labeled in either the control or treatment trials, and allow a very rough estimate of the percentages of the total numbers of cell type labeled by a single cue. Our conservative estimates suggest that approximately 20% of the total number of lateral and abfrontal cells, 9% of the total number latero-frontal cells, and 2% of the total number of frontal cells were labeled.

Mechanosensory vs. Chemosensory Receptors

It is important to note, however, that this method of labeling active sensory cells does not discriminate between types of cells. Thus, it is possible that the labeled cells include a variety of different sensory cell types. Probable candidates include mechanoreceptors and chemoreceptors.

Although the transduction pathway for mechanoreceptors is not yet fully determined, the initial events appear to have some similarity to those for chemoreceptor transduction. The initial stimulation of a mechanoreceptor (*i.e.* stretching or bending of the cell membrane or a protruding cilia) in at least one invertebrate (the crayfish) opens a stretch-activated ion channel that appears to be permeable to divalent cations (Edwards et al., 1981). Chemoreceptor transduction proceeds similarly, with the binding of a ligand to the receptor in the membrane causing the activation and opening of ion channels. These cation-permeable channels should both be permeable to agmatine, and therefore both mechanoreceptors and chemoreceptors could conceivably be labeled using this technique.

As a result, the labeled cells in the palps could be functioning in a variety of ways. A mechanosensory function is likely for at least some of these cell types, while others may function as chemosensory cells, most likely as olfactory receptors or gustatory receptors.

Mechanoreception

It is very likely that spionid palps are equipped with mechanosensory cells. Many spionids (including *D. quadrilobata*; T. Riordan, pers. obs.) are known to switch feeding modes, from deposit feeding to suspension feeding, in the presence of higher flow rates

(Dauer et al., 1981). This switch is most likely mediated by mechanosensory detection of flow rates. In addition, when suspension feeding, spionids collect suspended material through direct impaction of the particles on the palps. This material is corralled into the food groove and directed towards the mouth using cilia lining the edge of the groove (*i.e.* latero-frontal cilia) as well as those inside the groove (*i.e.* frontal cilia) (Dauer 1984, 1985 & 1987). In one spionid species, the latero-frontal cilia beat only when contacted by a suspended particle (Dauer, 1985), similarly suggesting a mechanosensory influence.

Consequently, the frontal and latero-frontal cells labeled by agmatine in this study may function as mechanosensory receptors with many of these same roles. This might explain the lack of a significant difference between the treatment and control trials in the numbers of labeled frontal and latero-frontal cells. These cells could be activated by the flow across the palps through the perfusion chamber, which may activate membrane-bound ion channels and allow agmatine to enter through the activated cation channels. This activation should occur similarly in both trials and thus label similar numbers of mechanoreceptor cells.

Chemoreception: Olfaction vs. Gustation

The significant difference between the treatment and control trials in the numbers of lateral and abfrontal cells, on the other hand, suggests that these cells are chemosensory in function. In prior behavioral assays, the cue used in this study (a mixture of six amino acids) elicited a significant feeding response when bound to glass beads and presented to *D. quadrilobata* (see Chapter 2). This suggests that the significantly higher rate of activation of the lateral and abfrontal cells in the treatment

trials is a result of the chemosensory detection of the cue, which could possibly indicate presence of a food source.

The differentiation of these sensory interactions as gustatory or olfactory, however, is a more difficult distinction. Although comparatively little is known about the sensory systems of invertebrates, preliminary studies suggest that gustatory and olfactory sensory neurons show a considerable amount of structural similarity (Dionne & Dubin, 1994). However, their primary functional roles are significantly different. Gustatory receptors (*i.e.*"taste") are utilized for contact chemoreception detecting stimuli at close range. Olfactory receptors (*i.e.* "smell"), on the other hand, are responsible for longer distance stimuli detection. Laverack (1988) suggests that in aquatic organisms, organs that have a dual mechanical and chemical function in which contact is essential should be considered the equivalent of taste, while those with more discrete chemoreceptor populations in which simultaneous contact is not essential should be considered the equivalent of smell. The apparent ability of the sensory structures on the palps of *D. quadrilobata* to recognize both dissolved (*i.e.* lacking contact) as well as adsorbed (*i.e.* contact necessary) cues does not help to clarify this question.

Taste cells are generally considered to be cells situated in the epithelial layer of body parts involved in the manipulation and ingestion of food, including (in various animals) the lips, oral cavity, tongue, pharynx, and cephalic appendages. The lateral and abfrontal cells are present in the epithelial layer of the palps, which are involved in such functions; therefore it is conceivable that these putative sensory cells may function as taste receptors identifying food resources for immediate collection and ingestion.

A function as olfactory receptors for these cells is not intuitively obvious, particularly as mediators of feeding interactions. Spionids are essentially sessile organisms. As a result, searching for the source of a stimulus is not realistic, unless it is within reach of the palps. However, spionids live in a dynamic environment, where currents and wave action frequently supply fresh deposited material around a worm's tube (Miller & Sternberg, 1988). Spionids could use the palps as an early detection system during and immediately after one of these resuspension events to probe for the presence of potential food and determine its proximate location. In addition, the detection of other stimuli, such as the presence of predators, would be a likely and necessary function. Alternatively, the palp may be equipped with both gustatory and olfactory receptor cells. This is likely, particularly considering the duality in feeding mode (*i.e.* both deposit and suspension feeding) of most spionids.

Solitary Chemosensory Cells: A Structural Analog?

Structurally, the lateral and abfrontal cells may also represent an analog to a more general chemosensory system in many vertebrate fish, the solitary chemosensory cells (SCCs). SCCs are thought to be involved in predator detection (reviewed by Kotrschal 1995), though in some species they appear to mediate feeding (Silver & Finger, 1984). Unlike olfactory and gustatory receptors that are typically grouped together in an olfactory epithelium or taste bud and concentrated in particular areas, SCCs are generally found embedded between unspecialized epidermal cells and are evenly distributed over the entire body surface (Kotrschal, 1991 & 1992; Whitear, 1992). Structurally SCCs appear to be precursors to the taste buds (Whitear, 1971 & 1992; Kotrschal, 1991), but

functionally their ability to detect low-threshold levels of stimuli (Kotrschal, 1991 & 1995) aligns them more with long-distance receptors (olfactory receptors).

The lateral and abfrontal cells are similar to these SCCs in their distribution pattern (at least along the palps) and their relative isolation from one another as well as other sensory cells. Functionally, the similarities are not quite as clear, although the abilities of spionids to detect predators using sensory mechanisms is unknown and the potential participation of SCCs in feeding has not been explored in a majority of fish.

CONCLUSIONS

This preliminary study provides the first documented physiological evidence of the involvement of spionid palps in the detection of chemical stimuli. More studies are needed to determine receptor specificities and distributions, as well as a better characterization of potential stimuli; however, these results offer a promising technique to accomplish these goals.

Determining the functional roles of these cells as smell or taste receptors will be more challenging. Such a distinction can only be made after consensus is reached on the distinction, if any, between smell and taste in aquatic habitats.

Table 3.1. Numbers of cells labeled with agmatine by type. Numbers in parentheses indicate total length of palp represented by sections (under Total Section Analyzed column) and estimates of the numbers of each cells labeled in a 100 μm length of palp.

Worm	Total Sections Analyzed	Frontal Cells	Latero-frontal Cells	Lateral/Abfrontal Cells
13	152 (304 um)	4 (1.32)	2 (0.66)	11 (3.62)
15	169 (338 um)	1 (0.30)	11 (3.25)	3 (0.89)
16	183 (366 um)	0 (0)	7 (1.91)	5 (1.37)
21	252 (504 um)	5 (0.99)	38 (7.54)	20 (3.97)
22	60 (120 um)	0 (0)	5 (4.17)	7 (5.83)
23	86 (172 um)	4 (2.33)	7 (4.07)	5 (2.91)
26	264 (528 um)	0 (0)	5 (0.95)	4 (0.76)
31	196 (392 um)	2 (0.51)	1 (0.26)	6 (1.53)
33	143 (286 um)	0 (0)	2 (0.70)	5 (1.75)
35	84 (168 um)	3 (1.78)	8 (4.76)	8 (4.76)
38	131 (262 um)	1 (0.38)	2 (0.76)	7 (2.67)
Treatment Average	156.36 (312.72 um)	1.82 (0.58)	8 (2.56)	7.36 (2.35)
Worm	Total Sections Analyzed	Frontal Cells	Latero-frontal Cells	Lateral/Abfrontal Cells
17	124 (248 um)	5 (2.02)	24 (9.68)	6 (2.42)
18	69 (138 um)	4 (2.90)	12 (8.70)	4 (2.90)
19	351 (702 um)	1 (0.14)	19 (2.71)	5 (0.71)
20	226 (452 um)	1 (0.22)	9 (1.99)	8 (1.77)
24	61 (122 um)	4 (3.28)	1 (0.82)	1 (0.82)
29	23 (46 um)	0 (0)	0 (0)	0 (0)
39	250 (500 um)	0 (0)	2 (0.40)	4 (0.80)
Control Average	157.71 (315.43 um)	2.14 (0.68)	9.17 (3.03)	4 (1.27)

Table 3.2. Numbers of cells by type stained with toluidene blue. Numbers in parentheses indicate total length of palp represented by sections (under Total Section Analyzed column) and estimates of the numbers of each cells present in a 100 μm length of palp.

Worm	Total Sections Analyzed	Frontal Cells	Latero-frontal Cells	Lateral/Abfrontal Cells
1	45 (90 um)	26 (28.89)	34 (37.78)	15 (16.67)
2	63 (126 um)	41 (32.54)	35 (27.78)	10 (7.94)
3	63 (126 um)	22 (17.46)	24 (19.05)	9 (7.14)
4	72 (144 um)	44 (30.56)	44 (30.56)	21 (14.58)
Average	60.75 (121.5 um)	33.25 (27.37)	34.25 (28.19)	13.75 (11.32)

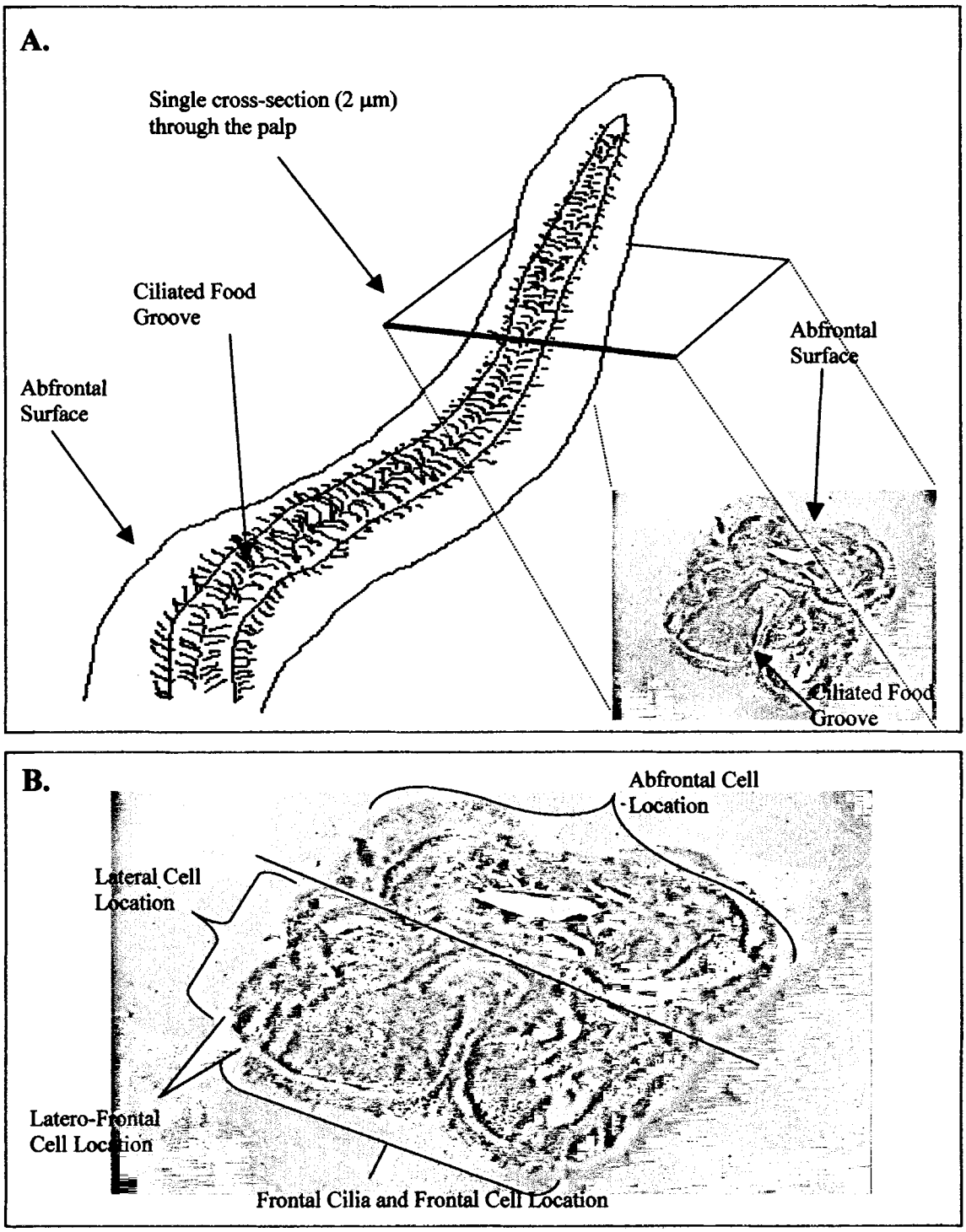


Figure 3.1. A. Palp morphology and section placement. **B.** Cell type location within sections. Cells along the food groove and within the frontal cilia were designated frontal cells. Cells immediately adjacent to the frontal cilia were designated latero-frontal cells. Cells behind a line drawn across the back end of the food groove were designated abfrontal cells. Cells in front of that line and lateral to the frontal surface were designated lateral cells.

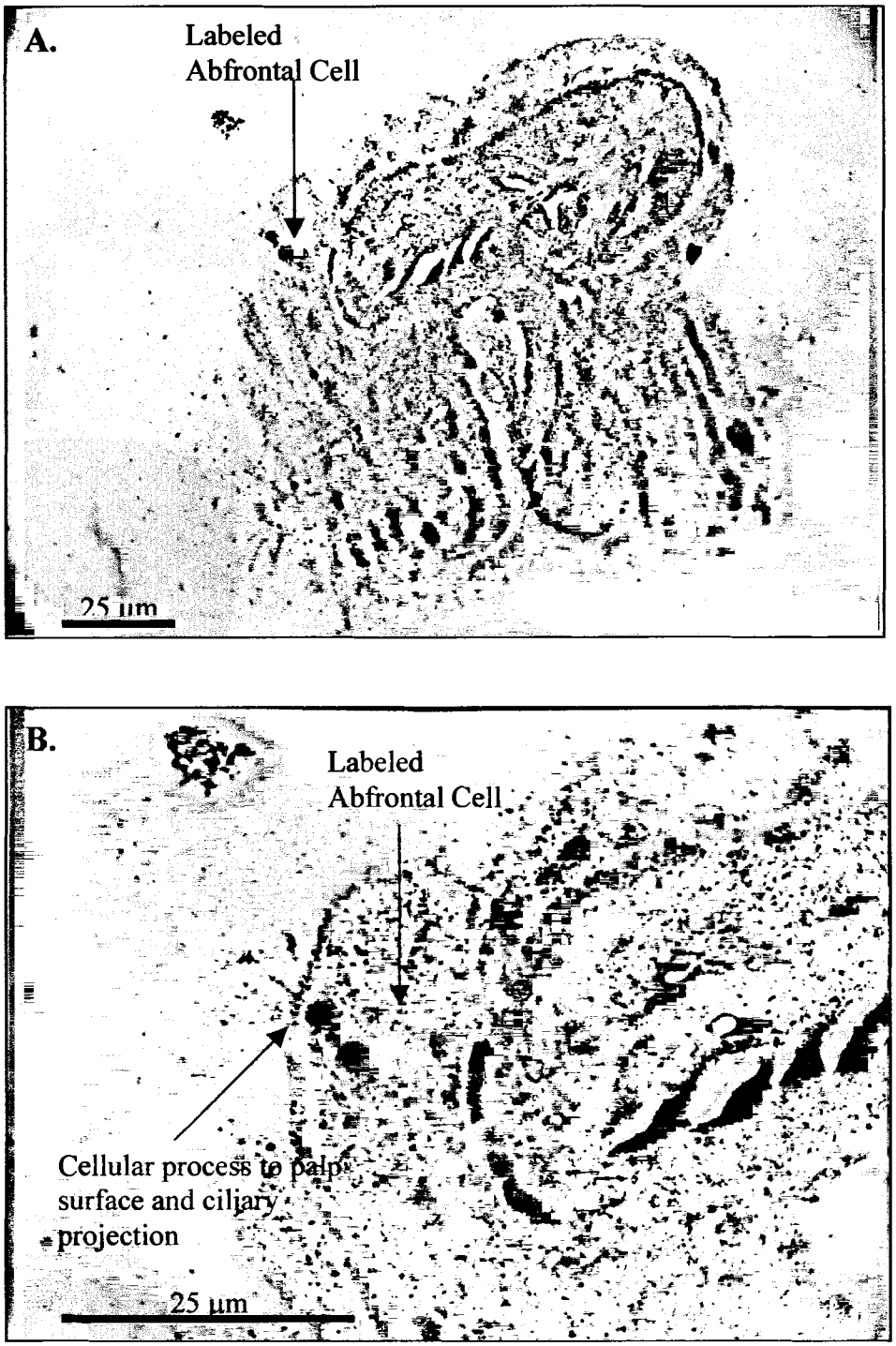


Figure 3.2. Semi-thick section from palp of *D. quadrilobata* with an abfrontal cell labeled by agmatine + amino acid mixture (treatment). **A.** Whole section 160X. **B.** Abfrontal area 400X.

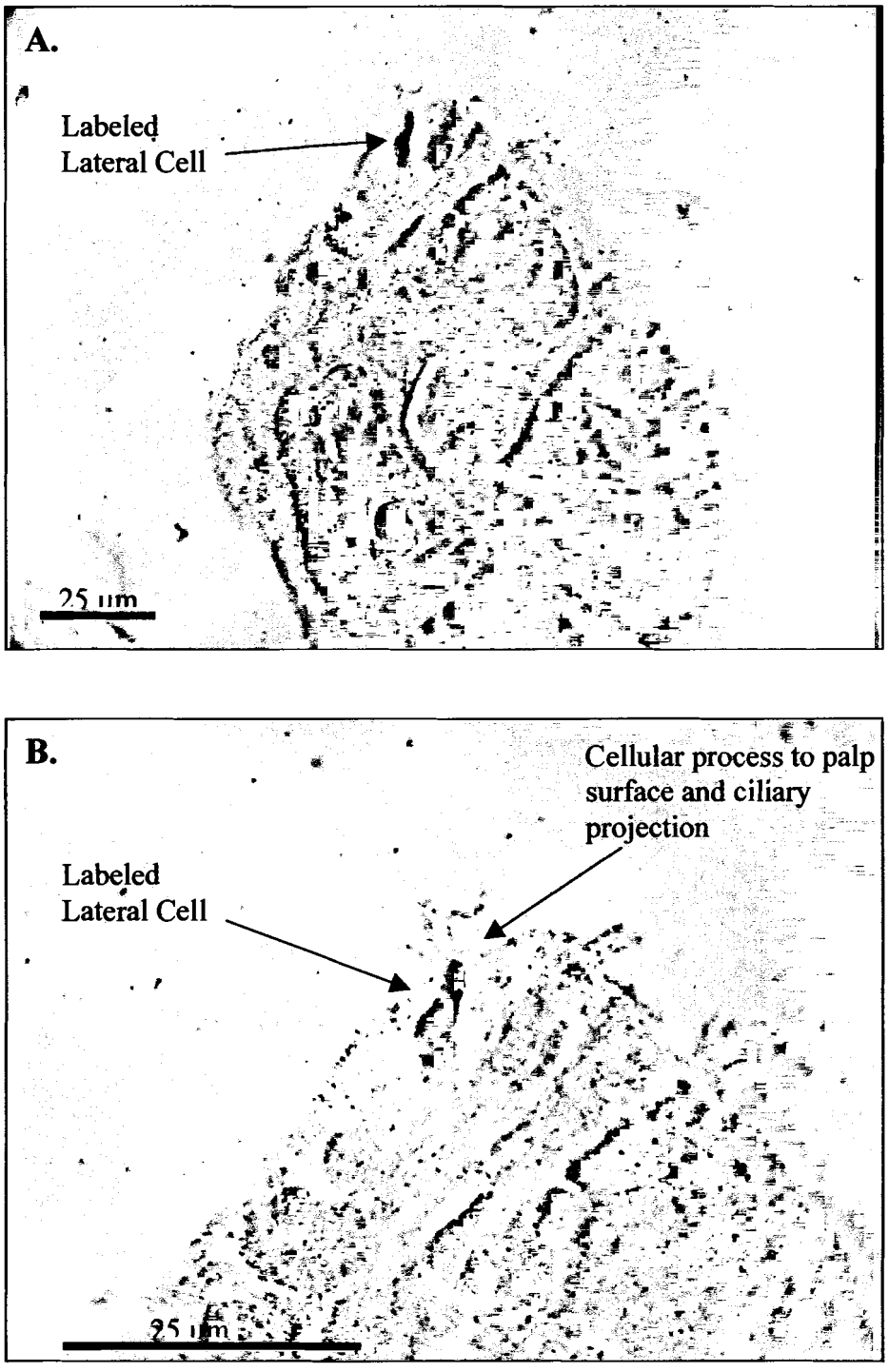


Figure 3.3 Semi-thick section from palp of *D. quadrilobata* with a lateral cell labeled by agmatine + amino acid mixture (treatment). **A.** Whole section 160X. **B.** Lateral area 400X.

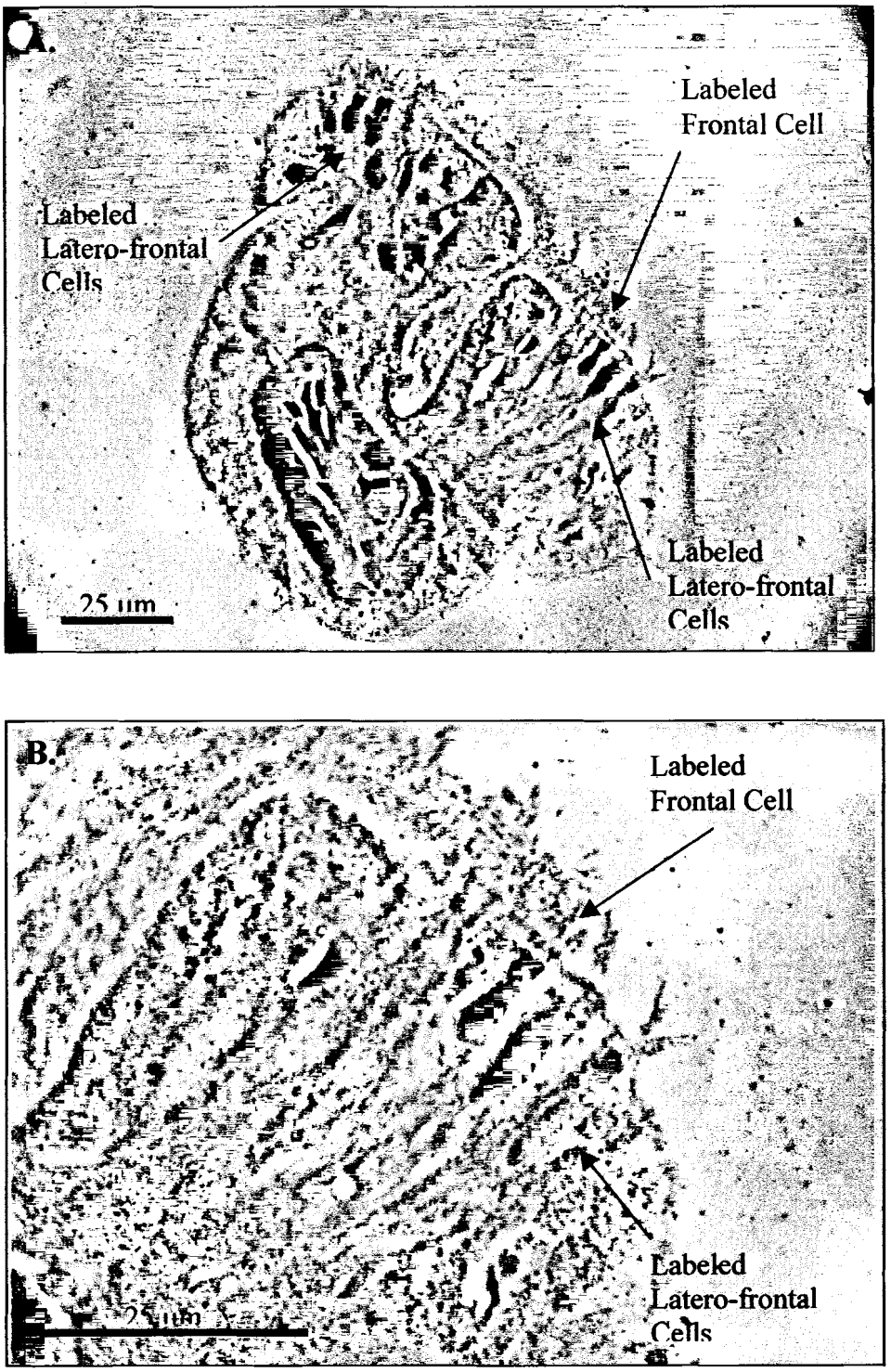
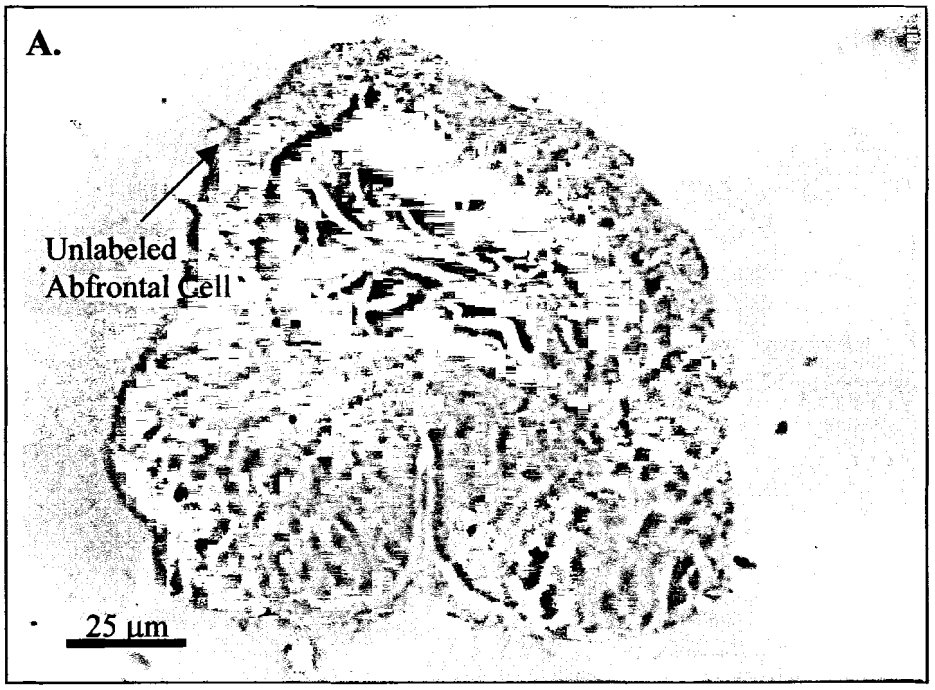


Figure 3.4. Semi-thick section from palp of *D. quadrilobata* with a frontal and latero-frontal cells labeled by agmatine + amino acid mixture (treatment). **A.** Whole section 160X. **B.** Frontal area 400X.



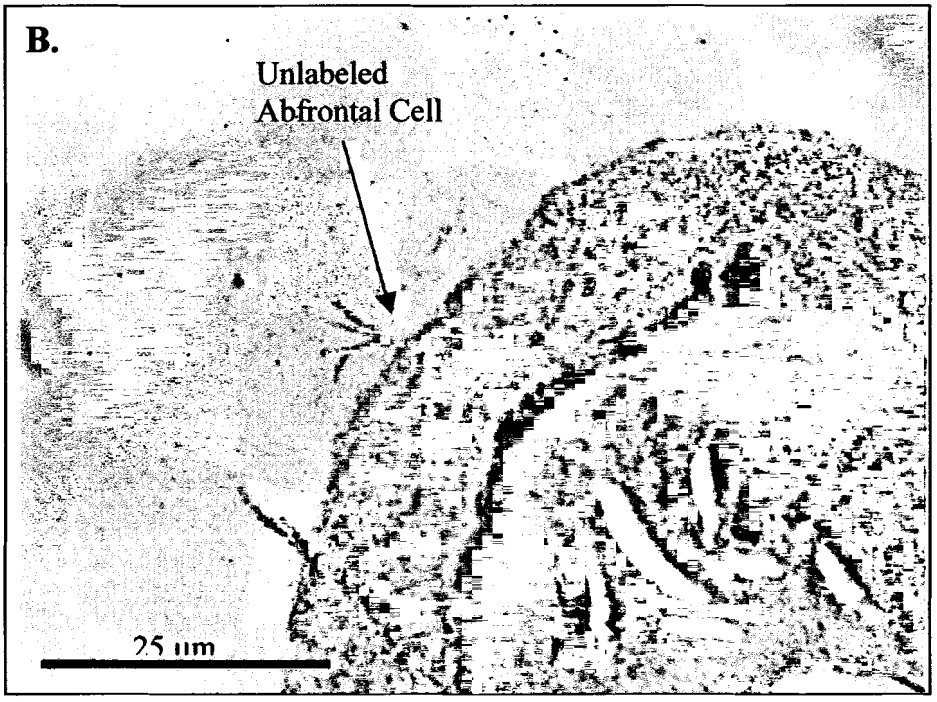


Figure 3.5. Semi-thick section from palp of *D. quadrilobata* with an abfrontal cell unlabeled by agmatine + artificial sea water (control). **A.** Whole section 160X. **B.** Abfrontal area 400X.

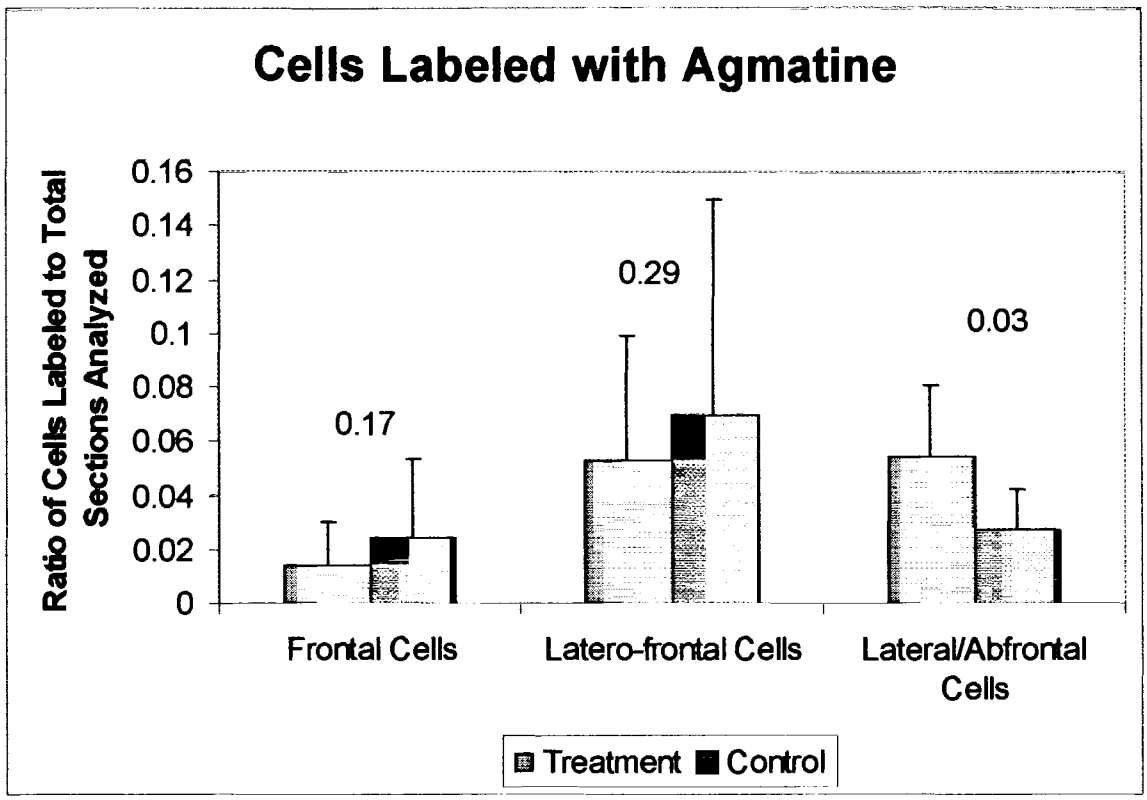


Figure 3.6. Ratio of the number of cells of each type labeled to the total number of sections analyzed. Means are plotted (Treatment= 11 individuals, Control= 7 individuals). Error bars are + 1 standard deviation. Numbers above error bars are p-values from Student's t-Tests.

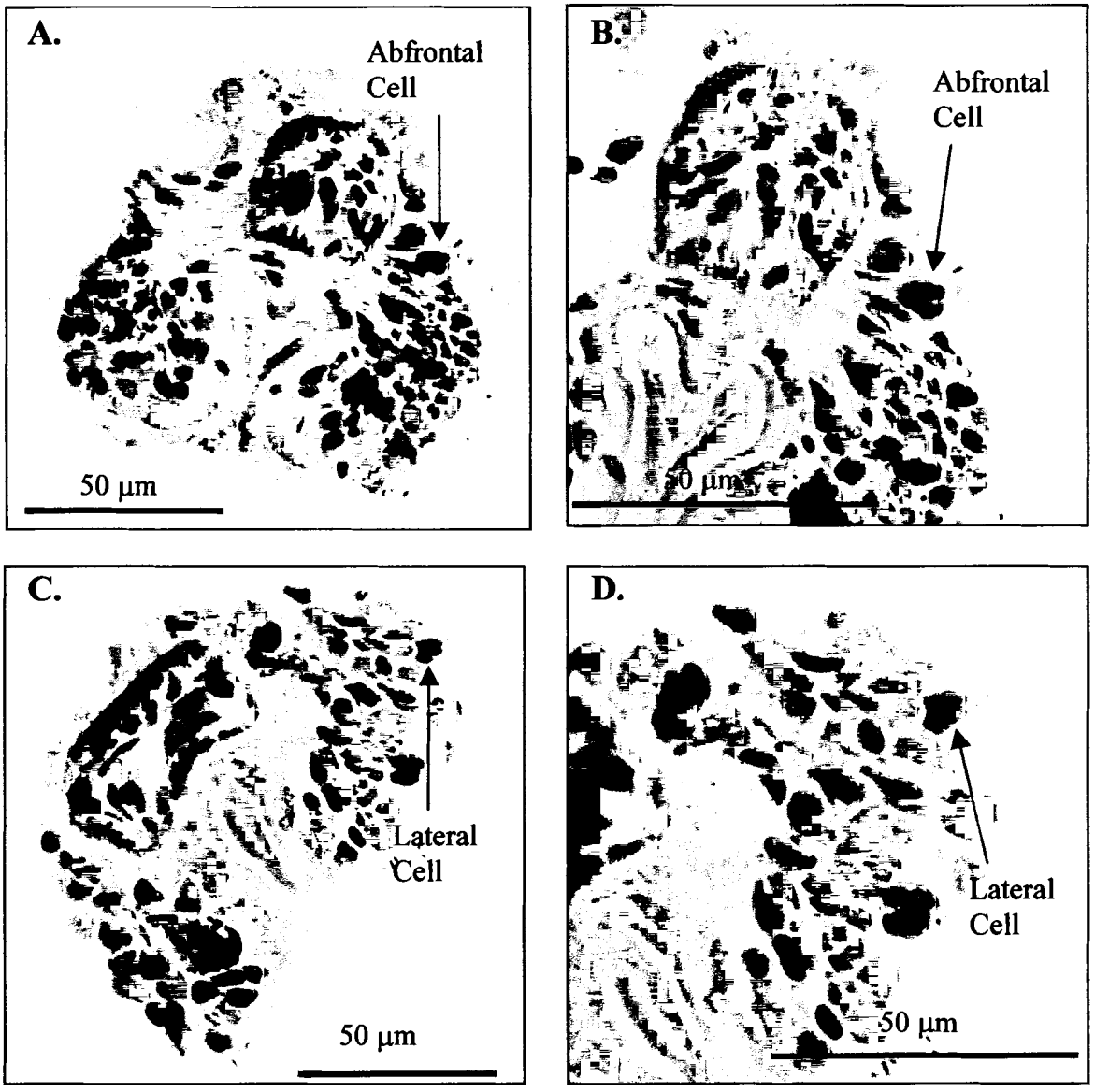


Figure 3.7. Semi-thick sections from palp of *D. quadrilobata* with an abfrontal and lateral cells stained with toluidene blue. **A.** Stained abfrontal cell 150X **B.** Stained abfrontal cell 250X **C.** Stained lateral cell 150X **D.** Stained lateral cell 250X.

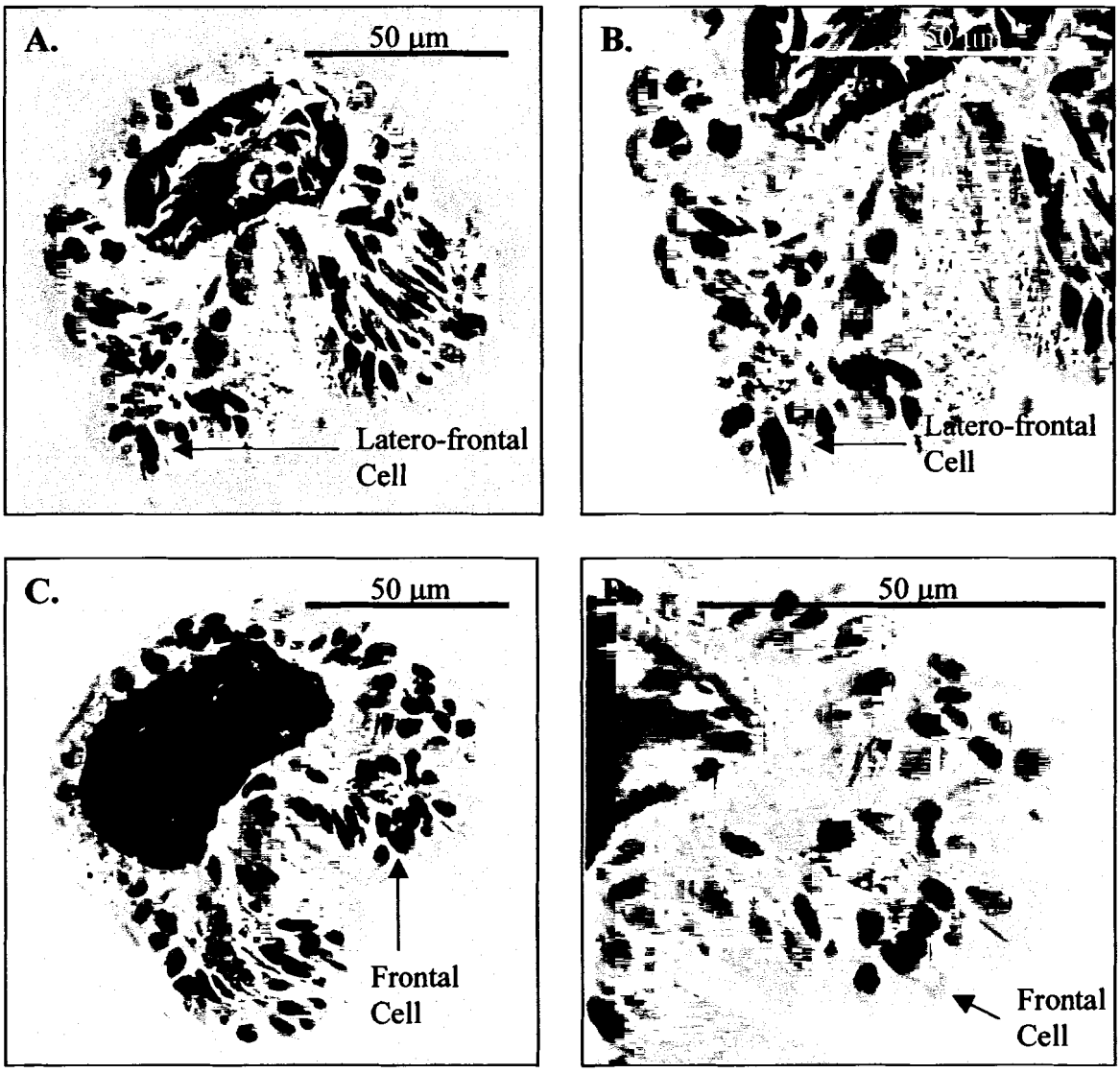


Figure 3.8. Semi-thick sections from palp of *D. quadrilobata* with frontal and latero-frontal cells stained with toluidene blue. **A.** Stained latero-frontal cell 150X **B.** Stained latero-frontal cell 250X **C.** Stained frontal cell 150X **D.** Stained frontal cell 250X.

CHAPTER 4

GENETIC STUDIES OF CHEMORECEPTION IN *DIPOLYDORA QUADRILOBATA*

INTRODUCTION

Chemical cues invoke physiological, and subsequently behavioral, responses by binding to receptor proteins in the membranes of chemosensory neurons. Recent evidence has linked olfactory receptor proteins to a second-messenger-dependent pathway belonging to the seven-helix family of G-protein-coupled receptors (Lancet & Ben-Arie, 1993; Nef, 1993; Dionne & Dubin, 1994). The search for these proteins has focused on identifying gene sequences that encode potential receptor proteins using molecular biological techniques. This approach is based on the rationale that seven-transmembrane domains most likely contain regions of conserved sequence across species (Buck & Axel, 1991). Multiple olfactory receptor genes have been identified in several vertebrates (human, rat, catfish, chicken and frog) as well as two invertebrate species (*Drosophila melanogaster* and *C. elegans*) (Buck & Axel, 1991; Freitag et al., 1993; Nef, 1993; Ngai et al. 1993; Troemel et al., 1995; Clyne et al., 1999; Voshall et al., 1999). Overall, sequence similarity between organisms is low, particularly between vertebrates and invertebrates, where there is almost no similarity. Among vertebrates, however, there are some short conserved regions, primarily in the seven-transmembrane domains and sites of interaction with G-protein intermediates.

Multiple families of receptor genes have been identified in all species examined so far. Estimates of the size of these gene families establish them as the largest in each species' genome. Although in some species (*e.g.* mammals) receptor gene families include psuedogenes, the maintenance of such large gene families is likely due to the fact that each receptor interacts with specific ligands (Selbie et al., 1992; Ben-Arie et al., 1994; Glusman et al., 1996; Buettner et al., 1998; Rouquier et al., 1998). Unlike

photoreception, in which three photoreceptors can absorb light across the entire visible spectrum, chemoreceptors appear to be much more specialized, capable of recognizing only a small number of chemical ligands (Voshall et al., 1999). As a result, organisms require a much larger repertoire of chemoreceptors in order to recognize the vast number of chemicals they encounter.

Among vertebrates, the receptor gene repertories of aquatic organisms are typically significantly smaller than those of terrestrial organisms. Such a pattern is consistent with the observation that there are generally fewer cues available in aquatic habitats than in terrestrial habitats. Classes of aquatic cues are constrained by the requirement of being soluble in water, limiting these cues to amino acids, quaternary ammonium bases, nucleotides and nucleosides, and organic acids (Carr, 1988). Terrestrial cues, on the other hand, are typically volatile, and are therefore hydrophobic compounds of low molecular weight including alcohols, aldehydes, esters, organic acids, and aromatic hydrocarbons (Dusenbery, 1992). Even so, it is likely that the specificity of the receptors still supports a fairly large repertoire of genes for marine invertebrates, similar to estimates for catfish and zebrafish (~100 genes).

Chemical stimuli are known to mediate a variety of marine invertebrate processes, including recruitment and metamorphosis (Pawlik 1992), reproduction (Miller 1989), escape responses (Mackie 1970), and feeding (Carr and Derby 1986a, Ferner & Jumars 1999). Numerous sensory organs have been identified throughout the marine invertebrate taxa that recognize these stimuli (reviewed in Laverack, 1988). In deposit feeding polychaetes, for example, sensory cells have been identified on several species, including the epidermal papillae of the deposit-feeding lugworm *Arenicola marina* (Jouin et al.,

1985), the compound sensory organs on the prostomial cirri and palps of *Nereis diversicolor* (Dorsett & Hyde, 1969), and the parapodial cirri of nereid polychaetes (Boilly-Marer, 1972). Little is known, however, about the genes that are responsible for these functions in any marine invertebrate.

Research specifically concerning the proteins responsible for recognizing chemical cues in marine invertebrate taxa has been limited mainly to studies of the spiny lobster, *Panularis argus*. The sensory organs that are responsible for olfaction in the spiny lobster are located on the antennules (Halberg et al., 1992). Biochemical studies have shown that saturable and reversible binding of radiolabled chemical cues occurs with high affinity to protein fractions from the dendritic membrane of these antennules (Olson et al., 1992). The binding properties of the stimuli to these protein fractions agree with electrophysiological studies of excitation of receptor neurons, suggesting that the proteins are involved in olfactory transduction. Further characterization of these proteins and their genomic basis, however, has not been achieved.

Our attempts at identifying and characterizing chemoreceptor proteins of the spionid polychaete *Dipolydora quadrilobata* have focused on finding potential genes that code for the receptor proteins. We have assumed that these genes are homologous to olfactory receptor genes characterized in other organisms and that some degree of sequence similarity is shared between vertebrates and invertebrates. Preliminary behavioral and neurophysiological studies suggest that *D. quadrilobata* have chemoreceptor cells located along their feeding palps that are involved in detecting and locating food resources (see Chapters 2 & 3). Our efforts at isolating chemoreceptor protein sequences from these cells have concentrated on using reverse transcriptase

polymerase chain reaction (RT-PCR) based methodologies to detect the expression of potential genes of interest. These methods can be used to greatly amplify specific genes of interest from RNA pools, even if the genes are expressed at very low levels.

MATERIALS AND METHODS

Collection and Maintenance of Animals

Dipolydora quadrilobata individuals were sieved (0.5 mm) out of cores collected from the mudflats of Lowe's Cove at the University of Maine's Darling Marine Laboratory (Walpole, ME, USA) on several days in June, July, August, and September of 2000. Animals and natural sediments were transported to the University of Maine in Orono and maintained in large culture tanks in an environmental chamber (14 °C:10°C, 12 h L:D cycle). Palps and tails were dissected from living worms, relaxed by exposure to isotonic magnesium chloride, and frozen in RNAlater (Amersham), which preserves RNA in tissues for later use.

RNA Isolation and cDNA Construction

I attempted to use degenerate to isolate potential nucleotide sequences for *D. quadrilobata* receptor proteins. This technique depends upon the isolation of high quality cellular RNA to ensure that sufficient copies of full-length receptor protein mRNA are available as template for subsequent first-strand cDNA construction and PCR reactions to amplify potential receptor genes. Total RNA was isolated from approximately 60 *D. quadrilobata* palps and 5 tails (~30 mg) from several organisms using an RNeasy Kit (Qiagen). RNA isolation followed the manufacturer's protocol including the use of a

Qiashredder column to aid in tissue disruption and a DNAse digestion step to remove any contaminating genomic DNA. The concentration and purity of the total RNA isolated was determined using a spectrophotometer.

First strand cDNA was synthesized by adding 1µl RNAsin (40units/µl), 2µl of 1mg/ml RaceAD1 primer (see Table 4.1), 1 µg of total RNA, and water up to 24µl. The reaction was kept on ice until all components were assembled, then incubated at 70°C for 10 min and cooled to 42°C at which time 8µl of 5x reverse transcriptase buffer, 4µl 0.1M DTT, and 2 µl of 10mM dNTPs were added. Following incubation at 42°C for 2 minutes, 1µl of Superscript II (Gibco; 200 units/µl) was added and the reaction was allowed to proceed at 42°C for 90 min, after which it was heated to 70°C for 15 minutes to inactivate the reaction. Excess primer, dNTPs and enzyme were removed from the reaction using a PCR spin column (Qiagen) and the first strand cDNA was eluted into 40 µl of TE buffer and stored at - 20°C.

Degenerate PCR

Sequence alignments from several vertebrate receptor protein sequences (catfish, chicken, rat, and frog) were used to identify conserved amino acid residues within the receptor protein sequences. Similar to experiments on vertebrate olfactory receptors (Ngai et al., 1993; Raming et al., 1993; Byrd et al., 1996), two degenerate primers were designed based on the conserved amino acid sequences at the 3' end of the third and seventh transmembrane domains for use in degenerate PCR (Table 4.1).

For each reaction, 1µl of first strand cDNA, 2.5µl 10x PCR Buffer, 0.75µl 50mM $MgCl_2$, 0.5µl 10mM dNTPs, 0.5µl of a forward degenerate primer (WOR1; 250µM) and

an adapter primer targeting the 3' end of RACEAD1 (UNAD1; 10μM), 0.2μl *Taq* polymerase (Gibco; 5 units/μl), and 19.5μl ddH_2O were combined, heated initially to 94°C for 2 min and then incubated for 30 cycles of 94°C for 30 sec, 40-55°C for 90 sec, and 72°C for 150 sec. A 5 minute extension step at 72°C was added to finish the reaction.

The products were separated through non-denaturing agarose gel electrophoresis and visualized using an ethidium bromide stain. Bands that appeared only in lanes representing palp tissues were excised from the gel and used in a second round of PCR. These reactions employed a second primer (WOR2), located downstream of WOR1 within the sequence of interest, and thus, reamplification with WOR2 should confirm that fragments isolated with WOR1 contain appropriate sequences.

For each reaction 1μl of the PCR product, 2.5μl 10x PCR Buffer, 0.75μl 50mM $MgCl_2$, 0.5μl 10mM dNTPs, 0.5μl of a forward degenerate primer (WOR2; 250μM) and an adapter primer targeting the 3' end of RACEAD1 (UNAD1; 10μM), 0.2μl *Taq* polymerase (Gibco; 5 units/μl), and 19.5μl ddH_2O were combined, heated initially to 94°C for 2 min and then incubated for 30 cycles of 94°C for 30 sec, 50-55°C for 90 sec, and 72°C for 150 sec. A 5 minute extension step at 72°C was added to finish the reaction. These products were separated through gel electrophoresis, gel purified, and sequenced via direct sequencing with primer WOR1 on an ABI 377 automated sequencer following standard protocols.

Differential Display

Differential display (DDRT-PCR) combines these techniques (RNA isolation, RT, and PCR) to isolate and amplify expressed gene sequences in two different tissue types for comparisons of gene expression and levels of expression (Figure 4.1). As a result, genes of interest can be identified and isolated by comparing gene expression in a tissue where the genes are expected to be expressed (*i.e.* the palps) to gene expression in a tissue where the genes should not be expressed (*i.e.* the tails). DDRT-PCR typically employs nine different downstream oligo-dT primers with two additional bases (combinations of A, G, and C) added to the 3' end of the primer and 24 different upstream primers to screen the isolated RNA pool from each tissue type. Therefore, up to 216 combinations of these primers can be used to detect and amplify expressed genes for comparison between tissue types.

A more directed approach can be used, however, if a portion of the sequence of the gene of interest is known. This region of known sequence can be used to design upstream primers of more specificity, and can greatly reduce the detection and amplification of many of the differentially expressed genes from the two tissues that are involved in functions not of primary interest. Therefore, the two degenerate primers given above, were uses in a targeted attempt at DDRT-PCR.

RESULTS

RNA isolation was highly successful. Recoveries were as high as 0.2 μg/μL and the purity was near 100%. We were able to amplify some DNA fragments from the first strand cDNAs using the WOR1 primer, that were differentially expressed in the palp

tissue when compared to tail tissue (Figure 4.2). Reamplification of these fragments with WOR2, however, did not amplify any appropriate products (Figure 4.3). A variety of PCR protocols, involving different annealing temperatures, buffers, and primer combinations, were used in attempts to more specifically amplify sequences. In addition, PCR products were also separated through denaturing polyacrylamide gel electrophoresis and visualized with a silver stain in attempts at more precise separation of bands. However, these modifications failed to yield differentially expressed products.

DISCUSSION/FUTURE DIRECTIONS

RT-PCR and DDRT-PCR with degenerate primers are ways of identifying and isolating sequences for proteins which have not been previously identified in an organism. In this case, we were looking for transmembrane olfactory receptor proteins expressed in the palps of *D. quadrilobata*. The use of degenerate primers takes the amino acid and nucleotide sequence variations of genes from different organisms into account to include all possible sequence variants when attempting to amplify genes of interest from among a pool of expressed genes in tissues and species of interest. This variability can be problematic however, primarily because the variation of the degenerate primers necessitates running the reactions at lower stringencies which decreases the specificity PCR reactions. This is likely to be a severe problem associated with the methods described here, as the levels of similarity among olfactory receptors necessitates that primers have a high degree of degeneracy.

DDRT-PCR is a powerful method for identifying genes that have tissue-specific expression patterns. However, the technique is prone to amplifying numerous

differentially expressed genes that have no involvement in the function of interest and that take considerable time to analyze and eliminate or confirm as putative receptors. In an attempt to avoid a large number of false positives, we used a pair of targeted primers in the place of the nonspecific but comprehensive primer system normally used in DDRT-PCR. Based on sequence similarities that exist among known vertebrate chemoreceptor gene sequences, the pair of targeted primers was expected to be specific to the transmembrane regions and sites of interaction with G-proteins (particularly near the 3rd transmembrane domain) of chemoreceptor gene sequences in *D. quadrilobata.*

Isolation and amplification of DNA sequences using WOR1 did not produce appropriate sequences upon reamplification with WOR2 however, suggesting that this expected similarity does not occur. This apparent lack of similarity can be a result of a variety of factors: 1) the receptor proteins may be homologous to vertebrate receptors (*i.e.* they are G-protein coupled receptors) but the similarity of these regions are too low for use in isolating sequences, 2) these regions are of high similarity, but the intervening regions between conserved sequence are too varied for proper alignment to known sequences in gene databases, 3) the receptor proteins are not homologous to G-protein coupled receptors found in vertebrates, or 4) DDRT-PCR may only amplify fragments of sequences prohibiting comparison of full sequence data to gene databases. In addition, the limits of degenerate PCR are even more pronounced in DDRT-PCR because reactions are run at high stringency.

Receptor sequences identified in *Drosophila* and *C. elegans* do not share high levels of sequence similarity, and even between gene families within the same organism similarity is low. This variability between two invertebrate species indicates that primer

sequences based on limited sequence similarity to vertebrates are unlikely to be sufficiently similar to receptor sequences of *D. quadrilobata*. Consequently, further attempts at designing primers based on other conserved regions in receptor genes, which are even shorter and more variable, are similarly unlikely to be useful in isolating receptor sequences. Alternatively, I suggest that there are two approaches that may yield future success in detecting and isolating receptor genes: 1) a more comprehensive use of the DDRT-PCR methodology or 2) a back-door approach that starts by isolating receptor proteins themselves.

A more comprehensive attempt at DDRT-PCR would likely prove more successful. Using the full 216 combinations of the random primers available should greatly improve the probability of isolating any sequences of interest by amplifying virtually all of the expressed genes in a tissue. However, this increased probability of amplifying a sequence of interest also results in an increased probability of amplification of numerous other genes expressed in the tissue. Considerable time and effort (both of which were limited for this study) are necessary to fully screen the expressed genes in these tissue types in hopes of finding putative receptor sequences.

Another, potentially more specific technique could involve working backward from the receptor protein itself. Biochemical studies of binding specificity of olfactory and gustatory receptor proteins have been made in several organisms (Krueger & Cagan, 1976; Cagan & Zeiger, 1978; Rhein & Cagan, 1980; Brown & Hara, 1981; Fresenko et al., 1983; Rehnberg & Schreck, 1986; Kalinoski et al., 1987; Bruch & Rulli, 1988; Olson et al., 1992). These studies suggest that techniques employing known stimuli that can preferentially bind to the chemoreceptor proteins could be used to isolate these proteins

from tissues for further analysis. Our behavioral assays (Chapter 2) have already identified potential stimuli. Techniques for isolating membrane fractions from tissues are well established (*e.g.*, Lesko et al., 1973; Fleischer et al., 1983; Jacobson et al., 1992), and could be used to obtain tissue enriched in dendritic membrane from receptor cells. Similar to column chromatographic techniques, a column could be derivatized with a known stimulatory compound (such as an amino acid, or even a short peptide) and used to isolate receptor proteins specific to the stimulus from the tissues of interest.

Once potential proteins are isolated, techniques are available for analyzing short sections of amino acid sequence at the end of a protein or cleavage site using C- or N-terminal degradation (Bergman et al., 2001). Identification of short sections of sequence could then be used to design more species-specific primers for use in further attempts at gene sequencing through degenerate RT-PCR.

CONCLUSIONS

Although this study did not produce any potential receptor sequences, the methods explored show that such an attempt is feasible. The isolation of RNA from *D. quadrilobata* tissues and the creation and amplification of first strand cDNA from that RNA, are viable techniques for identifying the genes that code for these receptor proteins. Of the two alternate methods proposed to successfully accomplish the goals of this study, a more comprehensive attempt at DDRT-PCR is most likely to succeed, given the validation of the technique with other organisms. Identifying gene sequences by first sequencing small parts of the protein has also been fairly well applied in identifying the sequences of different proteins from a variety of organisms, but it requires the accurate

isolation of a considerable amount of the protein of interest. However, the technique has the potential to be much less time consuming than DDRT-PCR and, given that it is based on proteins that have known functional significance, considerably more specific.

Table 4.1. Sequences of UNAD1 and degenerate primers based on conserved regions at the third and seventh transmembrane domain across four vertebrates (sequences from EMBL database): catfish (*Ictalurus punctatus*, IR3; acc. no. H45774), rat (*Rattus norvegicus*, IR3; acc. no. P23269), frog (*Xenopus laevis*, acc. no. Y08353) and chicken (*Gallus gallus*, acc. no. Z79586). *A* = Adenosine, *C* = Cytosine, *G* = Guanine, *T* = Thymine. D=*A*+*G*+*T*, H=*A*+*C*+*T*, M=*A*+*C*, N=*A*+*C*+*G*+*T*, S=*C*+*G*, W=*A*+*T*, Y=*C*+*T*

AMINO ACID SEQUENCE AT END OF 3RD TRANSMEMBRANE DOMAIN

Catfish (IP3)	Alanine	Tyrosine	Aspartic Acid	Arginine
Rat (I3)	Alanine	Leucine	Aspartic Acid	Arginine
Frog	Alanine	Phenylalanine	Aspartic Acid	Arginine
Chicken	Serine	Tyrosine	Aspartic Acid	Arginine
Nucleotide Sequences	GCT AGC T A G	TTT CAC A G	GAT C	AGA C G
Degenerate Primer (WOR1)	DSN	YWN	GAY	MG

AMINO ACID SEQUENCE AT END OF 7TH TRANSMEMBRANE DOMAIN

Catfish (IP3)	Proline	Isoleucine	Isoleucine	Tyrosine
Rat (I3)	Proline	Phenylalanine	Isoleucine	Tyrosine
Chicken	Proline	Phenylalanine	Isoleucine	Tyrosine
Nucleotide Sequences	CCT C A G	ATT T C C A G	ATT C A	TAT C
Degenerate Primer (WOR2)	CCN	HTN	ATH	TA

PRIMER SEQUENCES

M13R Tail

WOR1 – $^{5'}$ACAATTTCACACAGGA| DSN YWN GAY MG$^{3'}$

WOR2 – $^{5'}$ACAATTTCACACAGGA| CCN HTN ATH TA$^{3'}$

UNAD1 – $^{5'}$GGC CAC GCG TCG ACT AGT AC $^{3'}$

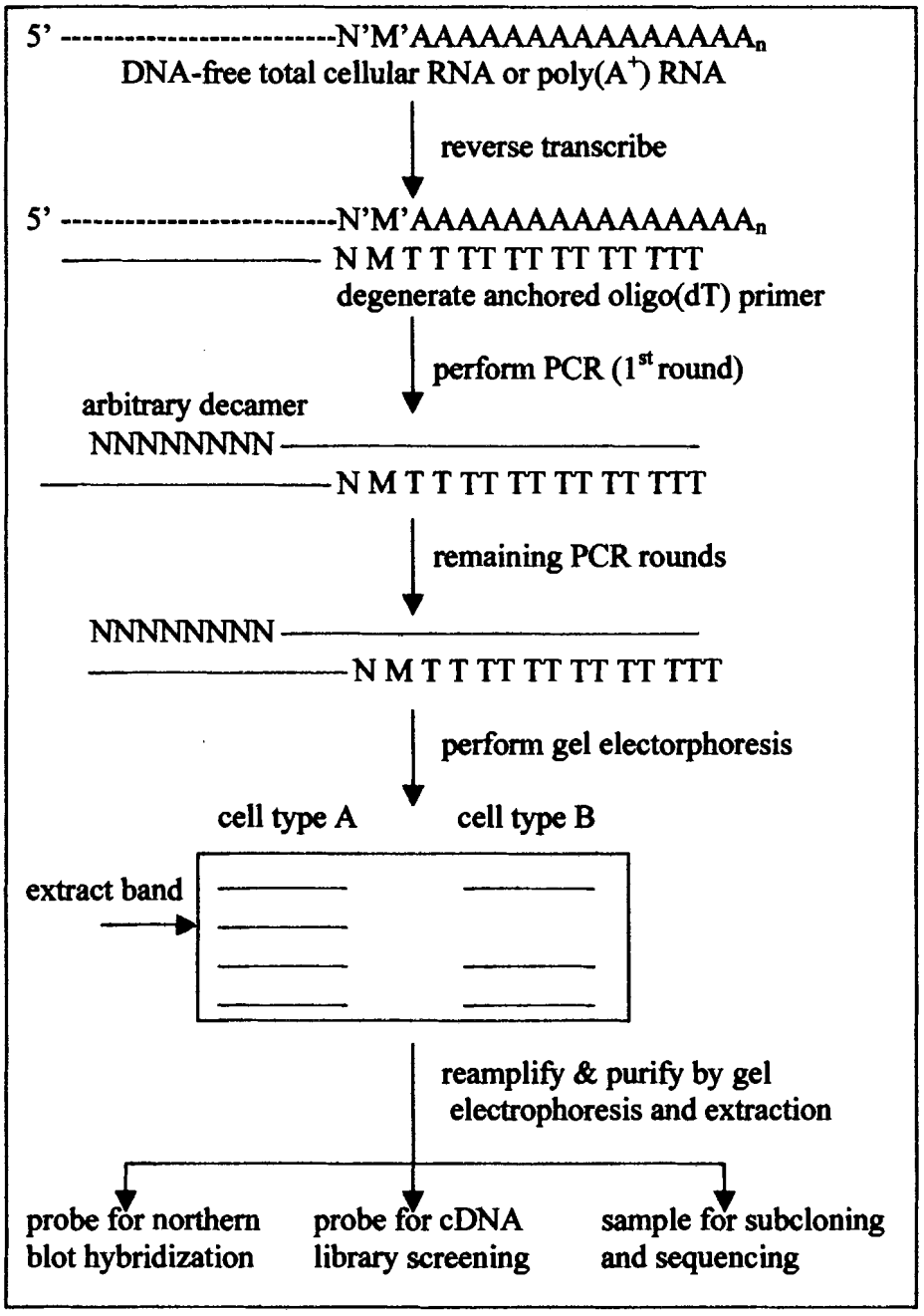


Figure 4.1. Schematic representation of differential display. (Adapted from Liang and Pardee, 1995)

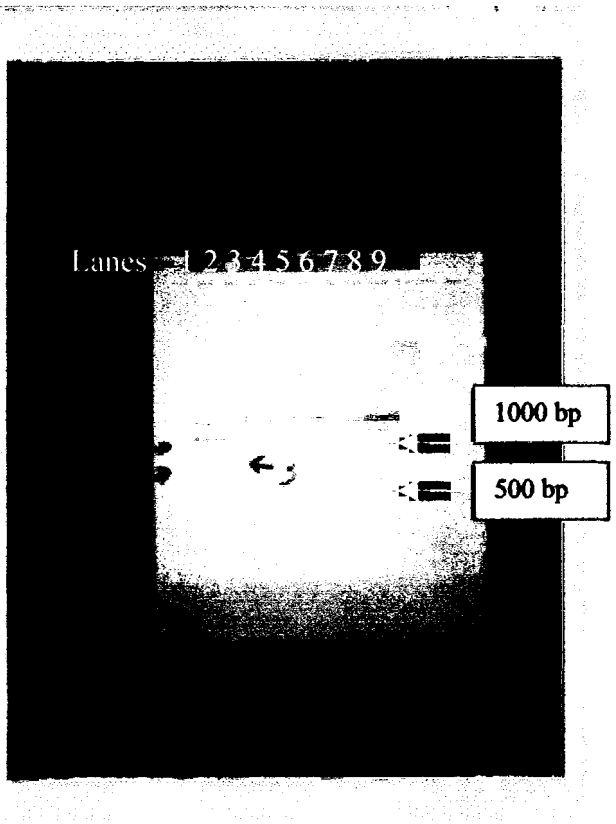


Figure 4.2. Initial amplification of *D. quadrilobata* palp and tail cDNA using WOR1 and UNAD1. Lanes 1, 3, 5, & 7 show amplification of palp cDNAs and lanes 2, 4, 6, &8 show amplification of tail cDNAs. Results show 3 differentially expressed products around 700 to 1000 bp (Lanes 1 & 3), which are reasonably sized products based on known receptor sequences. These three bands (indicated by numbered arrows) were cut out for reamplification with WOR2. The marker in lane 9 is Life Technologies 1Kb Plus Ladder.

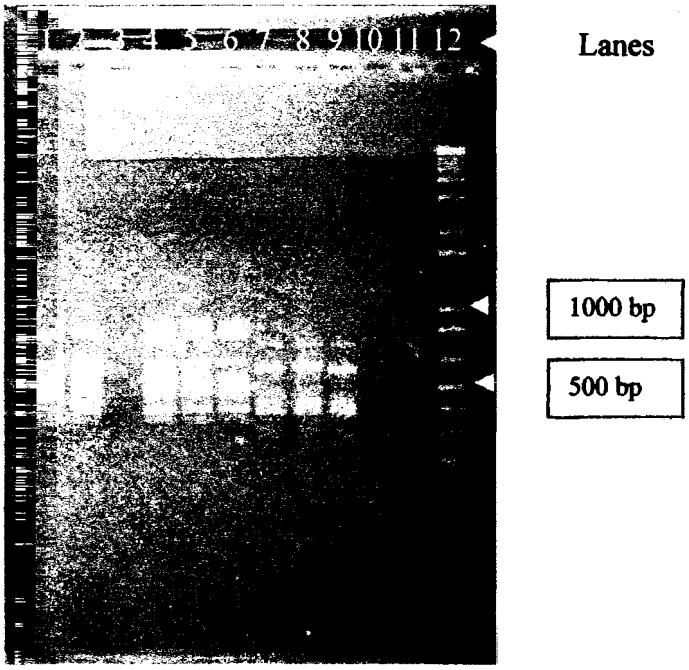


Figure 4.3. Reamplification PCR products using WOR1 & WOR2. Lanes 1, 4, & 7 show reamplification of isolated bands 1, 2, and 3 respectively, with WOR1 at 55°C. Lanes 2, 5, & 8 show reamplification of isolated bands 1, 2, and 3 respectively, with WOR2 at 55°C. Lanes 3, 6, & 9 show reamplification of isolated bands 1, 2, and 3 respectively, with WOR2 at 50°C. Lanes 10 & 11 are control blanks. The marker in lane 12 is Life Technologies 1Kb Plus Ladder.

CHAPTER 5

CONCLUSIONS

This project hypothesized that sensory interactions are an important influence on the selection and collection of nutrient resources by deposit feeders, and that the study of these interactions could help in the identification of these components. The behavioral study of feeding in the spionid polychaete *Dipolydora quadrilobata* (Chapter 2), suggests that selectivity based on sensory detection of potential food sources plays some part in determining the material that is ingested. The neurophysiological study (Chapter 3) begins to reveal how that selectivity is mediated physiologically. These results suggest that further study of sensory-mediated feeding in deposit feeders would be conducive to the determination of the biological and chemical components of benthic sediments that are assimilated by these organisms.

This project also points to promising new directions for the study of sensory interactions in deposit-feeding organisms. The obvious importance of sensory capabilities to a variety of processes in these organisms (*e.g.* recruitment and metamorphosis, predator detection, etc.) necessitates further study of all aspects of these types of interactions. The integration of behavioral, physiological, and genetic techniques should be very useful towards this end. The dearth of such integrated information, particularly of the genetic basis for sensory receptors in marine invertebrates, suggests that there is much to be discovered.

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Tim Riordan was born in Chicago, Illinois on November 3, 1975. He grew up in the suburban oasis of Glencoe, Illinois and graduated from New Trier High School in 1994. He then headed west to attend the University of Washington, where he developed a great interest in all things marine, working on projects as varied as examining dominance hierarchies in Nurse sharks to documenting the invasion of the salt marsh cord grass, *Spartina anglica*, into Puget Sound. In 1999, Tim graduated with a Bachelor's degree in Zoology from the University of Washington. After a short stint at the Washington State Department of Natural Resources, he headed back east and entered the School of Marine Science's graduate program in marine biology at The University of Maine in the fall of 1999.

After receiving his degree, Tim will once again strike out towards the west, seeking fame and fortune in Portland, Oregon. Tim is a candidate for the Master of Science degree in Marine Biology from the University of Maine in August, 2001.