NEURAL AND BEHAVIORAL RESPONSES TO COMPLEX ODOR STIMULI USING CRAYFISH AS A MODEL SYSTEM

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A Dissertation

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The physical environment in which animals live has a profound impact on the sensory information they receive and how they respond behaviorally to that information. This is particularly true for chemical cues that are transported through moving fluids. Natural habitats provide a complex environment that structures and alters chemical cues such as food and predator, as well as mating pheromones as they are transported through changing habitats. For example, a natural river bed consists of a mixture of different substrates over which water flows. These different substrates exhibit different levels of roughness elements that alter the turbulence structure of the flow over these areas. Because the distribution of chemical cues or odor plumes is highly influenced by turbulence structure of a moving fluid, you would expect changes in the available chemical information as an odor plume is transported over different substrates (i.e. habitats) within a river. Changes in the structure and availability of relevant chemical information can overwhelmingly affect behavioral strategies used to orient toward or avoid a particular odor cue. These behavioral strategies in turn are mediated by the underlying neural processing of external environmental stimuli. In crayfish, orientation behavior has been thought to be particularly influenced by the spatial and temporal fluctuations of turbulent odor plumes, especially by changes in the intermittency, duration and concentration of individual odor filaments. The focus of this compendium of work is to provide a comprehensive investigation into the influence that physical odor plume dynamics in natural habitats have on neural processing and orientation behavior. The overall objective of this collection of investigations is
to understand how the physical environment structures complex information and how the nervous system has evolved to process this information to ultimately mediate behaviors.
DEDICATION

I would like to dedicate this dissertation to my parents, John and Yvonne Wolf. You have always been there to support and encourage me in everything I have endeavored to accomplish. My accomplishments throughout my life are a tribute to your courage and strength. I love you both very much and am so proud to be your daughter. Thank you for being the parents that you are and for teaching me what really matters in life. This is for you.
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CHAPTER I

GENERAL INTRODUCTION

Chemical signal use

Chemical signals play an important role in conveying specific information about the environment to organisms in both the terrestrial and aquatic world. This information is used by organisms to locate food, mates, habitat, and to avoid predation (Ameyaw-Akumfi and Hazlett 1975; Hazlett 1985; Chivers and Smith 1993; Mathis and Smith 1993; Mafra-Neto and Carde 1995a; Williamson 1995; Tamburri et al. 1996; Zhou and Rebach 1999; Zulandt Schneider et al. 2000). For example, male moths use pheromone plumes to locate female moths in order to mate (Mafra-Neto and Carde 1995b). Bees use odor information to discriminate between different floral odors (Wright and Smith 2004) and to recognize nestmates from non-nestmates (Breed 1998; Downs and Ratnieks 1999). Pigeons use olfactory cues in order to home from unfamiliar areas (Budzynski et al. 1998; Wallraff 2004). In aquatic ecosystems, mayflies alter life history strategies when they experience predator odor in the area around them (Peckarsky 1996). As seen in these examples, chemical signals are widely used as a means of gathering information about the surrounding environment and organisms utilize chemical signals through a variety of ways.

Strategies utilized by aquatic organisms to orient toward a chemical signal are wide ranging. Blue crabs use an odor-gated rheotaxis to locate food by using the flow of water as spatial information and the concentration differences within and along the edges of the plume as temporal information to guide their movements (Weissburg and Zimmer-Faust 1993; Zimmer-Faust et al. 1995). Lobsters seem to rely more on a chemotactic strategy, using both the temporal and spatial components of an odor plume to orient (McLeese 1973; Moore et al. 1991b; Atema
Crayfish appear to use a chemotactic strategy that is guided by spatial information within the odor plume (Moore and Grills 1999; Kraus-Epley and Moore 2002). How these animals respond to chemical signals is influenced by many factors, both external (e.g. flow, substrate, conflicting odor information) and internal, (e.g. processing of odor information from the sensory organs to how the brain encodes this information). Investigating the different factors that influence the distribution and perception of chemical signals can give insight into what guides certain behaviors.

**Physics of odor plumes**

The fine-scale structure (i.e. the spatial and temporal aspects) of chemical signals provides information for olfactory-mediated behaviors (Moore et al. 1989; Mafra-Neto and Carde 1995a; Finelli et al. 1999; Moore et al. 2000). Changes in the temporal and spatial distribution of odor signals influence how organisms perceive and respond to chemical signals in their environment. Ultimately, the distribution of chemical signals is influenced by many variables present in moving fluids.

There are three major factors likely to influence the spatial and temporal distribution of chemical signals: 1) the rate of release of a chemical signal (Zimmer et al. 1999), 2) the hydrodynamic characteristics of the environment (Westerberg 1989), and 3) the spatial location of odor sources in the habitat (Westerberg 1989; Keller et al. 2001). For example, increases in turbulence as well as changes in pulse rate of the chemical signal alter different aspects of odor plume structure such as concentration within individual filaments and intermittency between filaments (Westerberg 1989; Moore et al. 1994; Zimmer et al. 1999; Finelli et al. 2000; Moore et al. 2000). This increase in turbulence can be attributed to many factors within the environment such as changing substrate in flowing aquatic systems to vegetation cover in terrestrial systems.
These habitats alter the fluid flow and ultimately how the signal is transmitted (Davidson et al. 1995; Weissburg 2000). Changes in fine-scale structure, either temporal or spatial, can introduce an aspect of complexity that animals in flowing systems must adapt to in order to carry out essential behaviors.

_Crayfish orientation behavior_

Crayfish live in complex environments and must adapt to these surroundings in order to survive. In a typical stream system there can be a number of different habitats, such as rocky riffles, sandy pools, gravely straight flow, as well as dead fall along the shoreline that add complexity to this environment. In addition to the numerous habitats within a stream system, more complexity can be added to a system when multiple chemical signals from various sources mix together. These complex odor signals convey information concerning the type of signal it is (predator, mate, or food) and its location in relation to the animal.

Crayfish must distinguish between complex odor signals and respond appropriately to locate mates, find food, and to avoid predators (Ameyaw-Akumfi and Hazlett 1975; Keller et al. 2001; Tomba et al. 2001). Here, we define complexity as an increase in temporal or spatial fluctuations within an odor plume. This complexity challenges the ability of organisms to extract the necessary information to locate the source of an odor signal (Keller et al. 2001; Tomba et al. 2001).

Changes in odor plume structure have a strong effect on the efficiency of organisms to orient toward an odor source (Weissburg and Zimmer-Faust 1993; Moore and Grills 1999). Orientation behavior in crayfish becomes more efficient with increased complexity of the odor signal (Moore and Grills 1999; Keller et al. 2001; Tomba et al. 2001). Changes in the physical habitat (i.e., substrate of the stream) alter the complexity of odor quality, and the spatial
distribution of odor sources can alter orientation behavior of crayfish (Moore et al. 2000, Keller et al. 2001). What is unclear from these studies is whether changes in orientation strategies are dependent on spatial or temporal fluctuations of the chemical signal. How crayfish respond to changes in temporal and spatial characteristics of chemical signals is also dependant on how the brain encodes the temporal and spatial aspects odorant information.

*Neural processing of information*

In order to understand behavioral patterns seen in natural systems fully, we must know how the nervous system filters the temporal and spatial information of turbulent odor plumes. Odor signals are detected in the environment when odorant molecules contact receptors on the antennules and other chemoreceptors on the body. The information contained in these odorant molecules (i.e. whether it is food, mate or predator, and its location) is extracted through neural processes in the brain resulting in the behaviors we observe. What we do not know is how information carried in the form of odor signals is processed in regards to natural signal structure.

One question facing sensory ecologists is how olfactory information is coded in the nervous system and how that is correlated with behavioral strategies. To understand these sensory/behavioral strategies more fully, investigation of the neural processing of odor signal dynamics is essential. One way of elucidating this process is by recording the neurological function within the olfactory lobe while the animal is stimulated with odor signals that resemble natural signal structure seen in various habitats.

There is a large body of work that has investigated the coding to odor quality in the olfactory systems of organisms ranging from insects to mammals. These studies have shown that the olfactory system can encode odor quality and discriminate between similar odor types based on the pattern of cell firing within the olfactory centers. When sections of the olfactory bulb are
ablated the ability to discriminate odor types is seriously diminished (Stopfer et al. 1997). These studies have focused on the identification of odorants but not on the processing of aspects that encompass the fine-scale structure of odor plumes, namely the fluctuations of concentration, intermittency and duration of individual odor filaments. Because the fluctuations of these fine-scale dynamics play a major role in eliciting behaviors it is important to know how the brain is processing this information.

The spatial or temporal distribution of an odor signal is critical for orientation behavior. Investigating how signals distribute through natural environments, such as a flowing stream system, will facilitate in understanding of how crayfish are adapted to respond to complex signals. By characterizing the signal structure of odor signals moving through various stream environments (i.e. riffle, cobble, sand, pools, and deep flowing habitats) we can bring this information into the laboratory and consequently mimic the natural environment to observe responses to complex signals within the brain and in the whole animal. Therefore, the purpose of this dissertation is to tie together the signal structure from the environment these animals live in with the behaviors we observe and to link central olfactory processing of odorant information from the environment to the various behavioral strategies that are available to an organism found in diverse habitats.
CHAPTER II

CHEMOSENSORY SIGNALS IN STREAM HABITATS: IMPLICATIONS FOR ECOLOGICAL INTERACTIONS\(^1\)

INTRODUCTION

Ecological interactions in aquatic environments are mediated by the information gathered through sensory systems. Inter- and intra-specific interactions rely on the sensory information available and this in turn can impact survivorship and reproduction of organisms that inhabit aquatic ecosystems. Organisms have a variety of adaptations based on sensory systems that allow them to avoid predators (Peckarsky 1996), locate mates (Endler 1987), forage for food (Moller 2002), and locate suitable habitats (Tamburri et al. 1996). Weakly electric fish integrate multiple sensory cues (visual, mechanical, electrical) to locate prey, avoid predators and attract mates (Moller 2002; Curtis and Stoddard 2003). Habitat conditions can enhance or detract from the information being transmitted and animals are able to integrate multiple cues to respond appropriately (Moller 2002). The common theme of these investigations is that the environment in which an organism resides influences how that organism adapts, evolves, and uses sensory signals that are relevant for survival. Therefore, to know how stream organisms have adapted to respond appropriately to sensory signals, it is imperative to know how the environment influences the distribution of those signals.

To begin to understand how sensory signals modulate the behavior of organisms, we need to understand how sensory information is distributed in space and time in natural habitats. The physics of natural habitats influence or constrain how stimulus information is transmitted through environments. Scattering, reflection, and refraction of light are three physical processes that are influenced by the physical structure of aquatic and terrestrial habitats. This in turn, influences how organisms use visual stimuli in structurally different habitats (Endler 1987). Auditory information, such as echolocation and bird song, is altered through interactions with physically different habitat types as well (Richards and Wiley 1980; Waser and Brown 1984; Patriquin et al. 2003; Schnitzer et al. 2003). Chemical signals are influenced by the environment through which they travel. In the aquatic realm, changes in the fine-scale distribution of chemical signals occur through interactions with various substrate types and flow regimes (Moore and Grills 1999; Finelli 2000; Finelli et al. 2000; Weissburg 2000). Variations in flow regime and substrate type alter the turbulent energy within local environments, and it is this energy that shapes the temporal and spatial distribution of chemical stimuli (Moore and Crimaldi 2004). The sensory information available within a habitat is determined by the interaction between chemosensory signals and fluid dynamics of the environment.

Chemical signals are transported and distributed through natural habitats by two processes. Molecular diffusion occurs at small spatial scales and low flow velocities, while turbulent advection occurs at large macroscopic scales and high flow velocities. These processes can be differentiated by the use of a non-dimensional ratio called the Peclet number (Pe). Though molecular diffusion (Pe < 1) is an important process at the level of sensory receptors, appendages, and small microscopic organisms, we are more concerned in this study with how information is transported in the turbulent world of macroscopic organisms (Pe > 1). Although
molecular diffusion is an important factor for chemical detection at the edges of chemical filaments, it is the role turbulent advection plays in the distribution of chemical signals that we shall focus on in this study.

The turbulent nature of fluid flow is dependent on various physical characteristics of the environment (Fischer et al. 1979). Fluid flow in natural streams is turbulent due to the velocity of the flow and the interaction of the water with varying substrates and obstructions within the stream bed (Fischer et al. 1979; Tennekes and Lumley 1992). These physical parameters can be organized into non-dimensional ratios such as the Reynolds number \( Re = \frac{l \times U}{\nu} \) and roughness Reynolds number \( Re^* = \frac{u^* \times D}{\nu} \) to give an estimate on the turbulent nature of the flow in question. The focus of this paper is to look at how changes in velocity \( (U) \) and substrate type \( (D) \) affect the turbulent mixing and distribution of chemical signals in a natural stream. As turbulent flow changes, so does the dispersion of the chemicals within that flow (Shteinman and Gutman 1993). Turbulence plays a major role in how chemical signals are dispensed thus affecting the behavior of organisms that live in turbulent environments.

Changes in sensory information conveyed within chemical signals can have a profound impact on the behavior of organisms. For instance, orientation behavior toward a host or mate was found to be influenced by the spatial and temporal components of airborne chemical signals (Bursell 1987; Mafra-Neto and Carde 1995b). In aquatic environments various substrate types and flow regimes alter foraging efficiency of organisms through changes in the temporal and spatial characteristics of the chemical signal (Moore and Grills 1999; Webster and Weissburg 2001; Keller and Weissburg 2004). Changes in flow velocity affect prey capture in blue crabs and whelks (Powers and Kittinger 2002; Weissburg et al. 2003; Ferner and Weissburg 2005). Slow moving whelks are more successful in finding prey when they are in low flow regimes,
whereas faster moving blue crabs are less successful in this type of flow (Powers and Kittinger 2002). It is evident from these studies that characteristics of fluid flow influence orientation behavior, but how the flow environment structures chemical signals in a natural aquatic setting, for the most part, has been neglected (Weissburg and Browman 2005).

In the present study, we investigated how chemical signals move through a natural stream consisting of three physically different substrate types and flow regimes. By taking simultaneous flow and in situ odor plume measurements at the same spatial point, we were able to characterize the information transmitted through the natural stream environment at the perceptual level equivalent to large benthic organisms.

MATERIALS AND METHODS

Study Site Description

Three physically different habitats within the Maple River located in Pellston, Michigan, USA were selected. The three habitats chosen were consistent in representing the diverse environment that crayfish and other benthic invertebrates may encounter as they forage for food, mates, or shelter. We performed simultaneous 3-D velocity and in situ odor plume measurements within a gravel, sand, and transition area (Fig. 1). The river channel was 7.6 m wide and measurements were performed 2.5 m from the bank. The depth of the river at each measuring point ranged from 19.05 cm to 25.4 cm in depth. Bed shape was consistent across sampling sites with little to no debris obstruction.

Hydrodynamic Measurements

Vertical profiles of the velocity within each habitat type were taken in triplicate using an Acoustic Doppler Velocimeter (ADV) (NORTEK AS, Rud, Norway). Measurements were
performed in the same location with 2 min intervals between each replicate measurement. The ADV probe was held stationary for each measurement by attaching the probe to a camera tripod. The tripod was placed in the river at each of the measurement locations, leveled and oriented so that wakes generated by the legs did not enter the ADV’s measuring volume. To obtain a vertical profile of the velocity in each habitat, the probe tip was lowered to 5 different distances from the substrate using the tripod’s sliding center column. The height above the surface of the substrate was determined by the probe scanning the area for a solid surface. The lowest height used is consistent with the height of larger benthic organisms and their sensory appendages. Velocity measurements within each location were taken at a rate of 25 Hz for a period of 5 min. These specific sampling rates were chosen to provide enough signal resolution to resolve the inertial subrange of each habitat type.

**Odor Plume Characterization**

In order to characterize the odor plume structure within the river, we measured a chemical tracer, dopamine, using an electrochemical detection system (Epsilon, Bioanalytical Systems Inc., West Lafayette, IN). A 30 µm triple carbon fiber microelectrode was attached to the ADV probe using electrical tape, where the tip of the electrode was level with the tip of the ADV probe. The electrode was calibrated using a series of dopamine concentrations (50-350 µmol L⁻¹). The dopamine was added to Maple River water to create known concentrations. Calibration of the electrode showed linearity across all dilutions ($r^2 = 0.935$). The regression equation, $y = 7E^{-10}X - 5E^{-7}$ ($r^2 = 0.94, n = 7, P < 0.001$) obtained from the calibration allowed us to convert the current recorded from the Epsilon system to the concentration of dopamine contained in the odor filaments, where $y =$ current of raw record and $X =$ concentration. Dopamine gelatin was prepared by dissolving 6.8 g of dopamine and 14 g of Knox® unflavored
gelatin into 356 ml of water from Douglas Lake, Pellston, Michigan, USA. Gelatin was placed in a baking dish and allowed to solidify overnight in a refrigerator. Gelatin was cut into 3 x 3 x 1 cm cubes for in situ odor plume measurements. Two pieces of 0.1 mol L$^{-1}$ dopamine gelatin were placed in a mesh bag (1 mm$^2$ holes) that was subsequently placed in the river at each measurement site. The mesh bag was held in place with a lead weight attached to the bottom of the bag as well as with small rocks from the river. Gelatin blocks diffused slowly as water flowed over the mesh bags allowing for a passive continuous release of the dopamine tracer into the river. Through inspection of the blocks between measurement sites no evidence of pieces having been ripped off was found. Epsilon recordings were taken at a rate of 25 Hz for a period of 5 min. The collection rate and time was chosen to span the range of frequencies animals are able to perceive and respond to odorant information.

**Sampling Protocol**

Simultaneous odor and flow measurements were performed over gravel, sand and transition habitats. To facilitate measuring flow and odor simultaneously the Epsilon microelectrode was secured to the shaft of the ADV with only the carbon fiber tip of 30 µm placed within the sampling volume of the ADV. Vertical profiles for each habitat were measured for odor and water velocity with the following ADV sampling volumes 6.01 cm ±0.03, 4.98 cm ±0.02, 3.96 cm ±0.02, 2.98 cm ±0.01, and 1.96 cm ±0.02. For purposes of simplicity the sampling volumes will be referred to 6, 5, 4, 3, and 2 cm from the substrate surface. Odor plume and velocity measurements within each habitat type were measured at three distances from the odor source 30, 60, and 90 cm. Instruments were kept at one measuring location while we moved gelatin blocks to the different distances. Vertical profiles were performed at each distance from the source giving a total of 15 measurements per site.
Data Analysis

Odor signals recorded from the three physically different habitats were analyzed using an in house basic program to evaluate various temporal aspects of the chemical signal (Moore et al. 1989; Moore and Grills 1999). The temporal characteristics of the chemical signal that were evaluated were intermittency between pulses, length of pulse, rise time, maximum height, and the absolute and maximum slopes of the odor pulses (Fig 2). These measurements are important because these parameters can influence how the nervous system and organism respond to sensory information. The nervous system encodes these temporal parameters providing organisms with specific spatial and temporal information on the location of a particular goal (Vickers et al. 2001; Fields and Weissburg 2004). These odor plume parameters were analyzed for each substrate type and distance from the probe using a factorial MANOVA and Tukey HSD post hoc test. Time series analysis was performed on the epsilon data using a commercial statistical package (Statistica, Statsoft Inc. Tulsa, OK).

Three dimensional velocity data from the ADV system were analyzed using the commercial software program, ExploreV (Nortek AS, Rud, Norway), to obtain average velocities and spectral densities. Average velocity measurements for each flow dimension were analyzed using a one-way ANOVA to compare changes in velocity over habitat type at each depth measurement. Differences between habitat type and distance were determined with a Tukey HSD post-hoc comparison test.

Time-series data was evaluated with a univariate spectral analysis using the Fourier Transformation auto-covariance function and smoothed using Tukey’s weighting function. Spectral analysis of velocity measurements in the three physically different habitats followed standard practices set forth by (Sanford 1997). The energy dissipation rate $\varepsilon$: 
\[ \varepsilon = U^3 (\kappa^{-1} x z^{-1}) \]  

was estimated using the 5/3 rule on the power spectra calculated in Explore V. The energy dissipation rate for each flow velocity measurement was used to calculate the Kolmogorov (\( \eta \), equation 2) and the Batchelor microscales (\( \eta_s \), equation 3; Sanford 1997). The Kolmogorov scale is the measure of the smallest eddy size and the Batchelor scale is the measure of the smallest distance for differences in chemical concentrations:

\[
\eta = 2\Pi \left( \nu^3 x \varepsilon^{-1} \right)^{1/4}
\]  

(2)

\[
\eta_s = 2\Pi \left( \nu x D_s^2 \varepsilon^{-1} \right)^{1/4}
\]  

(3)

where \( D_s \) is the molecular diffusivity coefficient of dopamine (2 \( \times \) 10\(^{-5} \) cm\(^2\) s\(^{-1}\)). Following calculation of both the Kolmogorov and Batchelor microscales for each of the three physically different habitats, they were analyzed using a one-way ANOVA. Differences between groups were determined using an LSD post-hoc comparison test.

Cross-spectral analysis was performed using the raw flow and odor plume data to obtain squared coherency values across a frequency range of 0-12.5 Hz using Statistica. The squared coherency function indicates the level of influence or relatedness two time series data sets have on each other (Bendat and Piersol 2000). The coherency is a function that relates the input of \( x(t) \) and the output \( y(t) \) with each other. The closer the squared coherency is to unity the more related the two series are to each other (Bendat and Piersol 2000).
RESULTS

Hydrodynamics

*Within Habitat Measurements*

There was an overall significant effect of habitat type on the three dimensional velocities (One way-ANOVA: $F_{42,84} = 88.1 \ p < 0.001$). The stream-wise direction showed significantly faster average velocities in the gravel habitat ($67.8 \pm 2.0 \ \text{cm s}^{-1}$) compared to the sand ($30.6 \pm 0.3 \ \text{cm s}^{-1}$) and transition habitats ($30.5 \pm 0.46 \ \text{cm s}^{-1}$) (Fig. 3A; Tukey HSD; $p < 0.001$). The cross-stream velocities showed no significant differences between gravel, sand and transition habitats ($11.7 \pm 0.5 \ \text{cm s}^{-1}$; Fig. 3B). Negative values equate to differences in flow direction but overall average velocities were not different. In the vertical dimension, average velocities in the gravel ($-3.9 \pm 0.31 \ \text{cm s}^{-1}$) and transition habitats ($-3.0 \pm 0.08 \ \text{cm s}^{-1}$) were significantly higher compared to the sand habitat ($-0.93 \pm 0.6 \ \text{cm s}^{-1}$) (Fig. 3C; Tukey HSD; $p < 0.001$).

*Vertical Profile Measurements*

There was an overall significant difference in vertical velocity profiles within each habitat type (One way-ANOVA: $F_{42,84} = 88.1 \ p < 0.001$). In the stream-wise dimension velocity profiles within the gravel habitat showed significant decreases in velocity ($60.2 \pm 1.8 \ \text{cm s}^{-1}$ to $-0.52 \pm 1.77 \ \text{cm s}^{-1}$) as distance to the substrate decreased (Fig. 3A; Tukey HSD; $p < 0.003$). Velocity in the sand habitat at the 2 cm distance ($23.1 \pm 0.69 \ \text{cm s}^{-1}$) was significantly slower compared to all other distances ($30.6 \pm 0.3 \ \text{cm s}^{-1}$; Tukey HSD; $p < 0.03$). The transition habitat exhibited a significant decrease in velocity between the 4, 5, and 6 cm distances ($30.5 \pm 0.46 \ \text{cm s}^{-1}$) compared to the 3 ($18.8 \pm 1.1 \ \text{cm s}^{-1}$) and 2 cm distances ($4.2 \pm 0.2 \ \text{cm s}^{-1}$) (Tukey HSD; $p < 0.01$).
There were no significant difference in cross-stream velocities within the gravel (13.2 ± 0.82 cm s$^{-1}$) or sand habitats (12.3 ± 0.25 cm s$^{-1}$) (Fig. 3B; Tukey HSD; $P = 0.99$). The transition habitat showed a decrease in velocity between the 4, 5 and 6 cm distances (12.30 ± 0.17 cm s$^{-1}$) and the 3 (8.25 ± 0.63 cm s$^{-1}$) and 2 cm distances (0.17 ± 0.29 cm s$^{-1}$) (Tukey HSD; $P < 0.01$). There was also a significant decrease in cross-stream velocity in the transition habitat between the 3 and 2 cm distance (Tukey HSD; $P < 0.001$).

The velocity profile within the vertical flow dimension showed significant decreases in velocity within the gravel habitat between the 4, 5, and 6 cm distances and the 2 cm distance (Tukey HSD; $P < 0.001$). There were no significant changes in velocity within the sand habitat (Tukey HSD; $P = 0.99$). In the transition habitat, the 3, 5, and 6 cm distances showed no significant differences (Tukey HSD; $P = 1.00$), but there were significant differences between these distances and the 4 and 2 cm distances (Tukey HSD; $P < 0.001$).

In addition to the average velocity, there was an overall effect of substrate type and distance from the substrate on the power spectra (Fig. 4). As the distance to the substrate decreased there were higher power spectra at higher frequencies. The gravel substrate exhibits higher power spectra at the 2 cm distance compared to both transition and sand.

Microscale Measurements

By calculating the Kolmogorov and Batchelor microscales we were able to ascertain the size of the smallest eddies present in the three habitats as well as the smallest distance needed for a change in the concentration of chemical signals within the three different river habitats. Minimum eddy size (Kolmogorov) was significantly larger in the sand habitat compared to the gravel or the transition habitats at the 2 cm distance (Table 1; One-way MANOVA; $F_{28,58} = 16.55$, LSD; $P < 0.001$). The minimum distances over which concentration changes (Batchelor)
were also significantly larger in the sand habitat compared to gravel and transition (Table 1; One-way MANOVA; $F_{28,58} = 16.55$, LSD; $P < 0.03$). The Kolmogorov and Batchelor scale measurements in the gravel and the transition habitats were not significantly different from each other (LSD; $P = 1.00$).

*Fine-scale Odor Plume Distribution*

Temporal and spatial distribution of odor signals through the three physically different habitats showed qualitative differences in the fine-scale distribution of odor plumes within the river (Fig 5). The odor signal in the gravel habitat exhibited higher spectra across all frequencies. These high energy spectra indicate that there are more fluctuations in the concentration or number of odor pulses at specific frequencies. As the substrate surface is approached, the odor signals in the gravel habitat exhibit higher power spectra (i.e. more fluctuations) across all frequencies compared to the transition and sand habitat (Fig 5). As distance to the odor source is increased the frequency at which fluctuations in the signal is also increased.

*Within Habitat Odor Distribution*

Detailed peak analysis showed that the dopamine sources located in physically different habitats had significantly different temporal structures when compared between the three habitats (MANOVA; $F_{28,17768} = 243.45$, $P < 0.001$). The peak heights (e.g. concentration of dopamine) of the odor pulses within the gravel habitat were significantly larger compared to the transition or sand habitats (Fig. 6A-C; Tukey HSD; $P < 0.001$). Peak height within the transition habitat were significantly higher compared to the sand habitat at the 30 and 90 cm distances from the odor source (Tukey HSD; $P < 0.02$), but not at the 60 cm distance (Tukey HSD; $P = 1.0$).

The intermittency between odor pulses (i.e. the time between each peak) was considerably altered due to habitat type. Odor signals in the sand habitat had significantly longer
intermittency times compared to the transition and gravel habitats at all distances from the source (Fig. 7A-C; Tukey HSD; \( P < 0.001 \)). At the 30 cm distance the intermittency was not significantly different between the transition and gravel habitats (Fig 7A; Tukey HSD; \( P = 0.06 \)). Intermittency was significantly longer in the transition habitat compared to the gravel habitat at the 60 cm distance from the source (Fig 7B; Tukey HSD; \( P < 0.01 \)). There were no significant differences between gravel and transition at the 90 cm distance (Fig 7C; Tukey HSD; \( P < 0.99 \)).

The slope of odor pulses (e.g. the sharpness of the rise to the maximum concentration) were significantly greater in the gravel habitat compared to the sand and transition at all distances from the odor source (Fig. 8A-C; Tukey HSD; \( P < 0.01 \)). The sand and transition habitats showed no significant differences in slope in the 30, 60 and 90 distance from the source.

**Vertical Profile of Odor Distribution**

Peak height within the gravel habitat was significantly higher at the 2 and 3 cm distances from the substrate compared to the 4, 5, and 6 cm distances (Fig. 6 A-C; Tukey HSD; \( P < 0.001 \)). Peak height within the sand habitat did not change significantly with distance to the substrate (Tukey HSD; \( P = 1.00 \)). Odor profiles within the transition habitat showed a significant decrease as distance to the source decreased (Tukey HSD; \( P < 0.001 \)).

Intermittency between pulses showed no significant changes within gravel, sand or transition habitats as distance to the substrate changed (Fig. 8; Tukey HSD; \( P = 0.99 \)). Slope of odor pulses within the gravel habitat significantly increased as distance to the source decreased (Fig. 9; Tukey HSD; \( P < 0.001 \)). There were no significant changes in slope within the sand habitat as distance to the substrate changed (Fig. 9; Tukey HSD; \( P = 0.99 \)). The transition habitat exhibited significant decreases in slope as distance to the substrate decreased (Tukey HSD; \( P < 0.001 \)).
The influence of flow within each habitat on the chemical signal was assessed through performing a cross-spectral density analysis to obtain coherency functions for each distance from the odor source within each habitat type (Fig. 11). Coherency analysis indicated higher frequencies exhibit higher coherency between the flow and the concentration of odor pulses within each habitat. In addition, the gravel habitat exhibited higher coherency at the 30 and 60 cm distance compared to the sand and transition habitats.

DISCUSSION

The results of the present study illustrate that there are habitat specific hydrodynamic characteristics as well as habitat specific temporal and spatial distributions of chemical signals. At our study site in the Maple River, substrate changes throughout the streambed create flow microhabitats. As a result, different substrates (e.g. gravel vs. sand) produce unique areas of flow (microhabitats) that can be characterized by fluctuating turbulence structures (Figs. 3, 4). Turbulence fluctuations alter the dynamics of chemical signals as they are transported through different habitats. For example, odor passing over the gravel habitat had significantly higher peak heights and slopes and lower intermittencies between pulses than those odors passing over sand or transition areas (Fig. 6, 7, 8). The distribution of chemical signals is highly influenced by turbulent flow as shown in the resulting coherency between flow and odor concentration. At low frequencies, odor pulses are not altered by flow fluctuations, indicating molecular diffusion as the main influence (Fig. 9). Conversely, at high frequencies, odor pulses and flow fluctuations are occurring on the same temporal scale, indicating turbulent advection (Fig. 9). These results indicate that specific microhabitats can be distinguished by the fine-scale spatiotemporal distribution of chemical signals within each habitat.
The behavioral response of organisms to chemosensory stimuli is dependent on the information contained in the fine-scale temporal and spatial distribution of signals within microhabitats. Consequently, chemosensory mediated behaviors will be influenced by the physical characteristics of the habitat in which these organisms reside. Changes in the local turbulence structure of the Maple River can be attributed to changes in the roughness elements (i.e., substrate) within local microhabitats of the river. Areas of the Maple River that consisted of gravel and a combination of gravel and sand (transition) substrate had more turbulent energy than areas consisting of just sand substrate (Figs. 3, 4). While the mean velocities in the stream-wise dimension ranged from -0.51 to 67.77 cm s\(^{-1}\), the instantaneous values varied from -60.0 to 88.34 cm s\(^{-1}\) due to turbulent fluctuations. These mean velocities were site specific, meaning that both substrate and other factors such as bathymetry and debris obstruction influence how the river is flowing in localized areas and show that flow in these natural systems are not necessarily unidirectional.

As chemical signals encounter turbulence, they are mixed and stirred as they move downstream from the original source. Mixing results in the formation of odor filaments that vary in concentration, duration, and frequency as it travels through the environment (Moore et al. 1989). The distribution of our chemical tracer, dopamine, through three physically different microhabitats was markedly altered because substrate differences induced different turbulence structures and local circulation patterns. The odor signal traveling through the gravel habitat exhibited higher peak concentrations accompanied by shorter intermittencies and larger slope between individual filaments (Fig. 6, 7, 8). This indicates that as a signal moves through the more turbulent environment of the gravel substrate there are a greater number of pulses being
generated by the roughness elements of this habitat. This increase in pulse concentration along with larger slopes, could provide more sensory information to a foraging benthic organism.

Work performed in artificial streams illustrates that available odorant information changes as odor plumes move over physically different habitats indicating that there are habitat specific chemical signal structures that can be utilized by organisms (Moore et al. 2000). Foraging behavior in crayfish has been found to be influenced by the spatial and temporal characteristics of chemical signals (Moore and Grills 1999; Wolf et al. 2004). As our dopamine tracer was transported through microhabitats that contained more chaotic flow (i.e. non-unidirectional flow) the temporal and spatial information contained in each odor filament was altered into a more complex (i.e. more temporal fluctuations) signal. The idea that there are habitat specific chemical signals leads to questions on how habitat specific chemosensory information affects ecological interactions within streams.

Increases in the temporal complexity of chemical signals facilitate ecological responses (Moore and Grills 1999; Keller et al. 2001). As the fine-scale spatial and temporal aspects of chemical signals (frequency of pulses, rise time, etc.) change and become more heterogeneous, responses to information become more efficient (Moore and Grills 1999, Keller et al. 2001, Wolf et al. 2004). This fine-scale distribution is heavily dependent upon the turbulent energy within a given habitat. In the Maple River we would expect crayfish to be faster at locating food, mates or predators in the more temporally complex environment. There is more turbulence and greater fluctuations in the chemical signal through the gravel and transition habitats providing more potential information in which to orient. Wolf et al. (2004) found that when intermittency between odor filaments was low, crayfish increased walking speeds and decreased turning angles
as a function of distance from the odor source. In natural streams information is altered between microhabitats so organisms must adapt to successfully acquire resources.

Since ecological interactions are mediated through sensory information, it is possible that having different sensory microhabitats within a flowing environment will influence the distribution of organisms or the types of behavioral strategies found within those microhabitats. Organisms have adapted to extract information from the patterns of stimulus encountered in their habitats. It has been proposed that in order to extract information from the environment, organisms have a “matched filter” system that allows the animal to perceive and extract information most relevant to them (Wehner 1987). The water bug, *Notonecta*, has visual receptors that respond to a certain size of moving objects that are within the range of their visual predatory response (Weise 1974; Schwind 1980). This example shows the importance of investigating, simultaneously, both the behavior and sensory properties of the organism and the sensory world that is present in the organism’s habitat. Organisms do not respond to sensory information that we can measure with our senses, but to the sensory information that is altered and filtered both by the habitat and by the sensory capabilities of the organism. The present study shows how chemical signals important in chemosensory mediated behaviors change over varying natural aquatic habitats. With knowledge of how signals change over physically different habitats, we will be able to use signals that are relevant to a particular organism to understand underlying behaviors both at the neurophysiological and behavioral levels.
CHAPTER III

SPATIAL ARRANGEMENT OF ODOR SOURCES MODIFIES THE TEMPORAL ASPECTS OF CRAYFISH SEARCH STRATEGIES

INTRODUCTION

Crayfish must distinguish between complex odor signals and respond appropriately to locate mates, find food and prey, to avoid predators, and to select a habitat (Bouwma and Hazlett 2001; Keller et al. 2001; Tomba et al. 2001). Here, we define complexity as an increase in range of temporal or spatial fluctuations within an odor plume. This complexity challenges the abilities of organisms to extract the necessary information to locate the source of an odor signal (Hazlett 1999; Keller et al. 2001; Tomba et al. 2001).

In aquatic systems, various strategies are employed to orient toward an odor source. Blue crabs are thought to use an odor-gated rheotaxis to orient towards an odor source, i.e., they use the flow of water as spatial information and the concentration differences within and on the edges of the plume as temporal information to guide their movements (Weissburg and Zimmer-Faust 1993; Zimmer-Faust et al. 1995; Finelli et al. 2000). Lobsters seem to rely more on a chemotactic strategy, i.e., using both the temporal and spatial components of an odor plume to orient (McLeese 1973; Moore et al. 1991b; Atema 1996). Crayfish appear to use a chemotactic strategy that is guided by spatial information within the odor plume (Moore and Grills 1999; Keller et al. 2001; Tomba et al. 2001; Kraus-Epley and Moore 2002). Therefore, the spatial or temporal distribution of the odor signal is critical for orientation behavior.

The fine-scale structure of chemical signals is important for its perception by organisms in their environment (Mafra-Neto and Carde 1995a; Finelli et al. 1999; Moore et al. 2000). Three major factors could influence the spatial and temporal distribution of chemical signals: 1) the rate of release of a chemical signal (Zimmer et al. 1999), 2) the hydrodynamic characteristics of the environment (Westerberg 1989), and 3) the spatial location of odor sources in the habitat (Westerberg 1991; Keller et al. 2001). Changes in odor plume structure have a strong effect on the efficiency of organisms to orient toward an odor source (Weissburg and Zimmer-Faust 1993; Moore and Grills 1999). For example, increases in turbulence as well as changes in pulse rate of the chemical signal altered plume variables, such as concentration within the patches and intermittency between the patches (Westerberg 1989; Moore et al., 1994, 2000; Zimmer et al. 1999; Finelli et al. 2000).

Orientation behavior in crayfish becomes more efficient with increase of complexity of the odor signal (Moore and Grills 1999; Keller et al. 2001; Tomba et al. 2001). Crayfish walk faster and spend more time moving toward the odor source with a wider range of temporal fluctuations in odor plumes. Changes in the physical habitat, i.e., substrate of the stream, alter the complexity of odor signals (Moore et al. 2000), and the spatial distribution of odor sources can alter orientation behavior of crayfish (Keller et al. 2001). The present study attempts to elucidate how changes in spatial-temporal aspects of an odor signal due to odor source placement alter the spatial-temporal orientation behavior of crayfish.
MATERIALS AND METHODS

Animals

Male and female *Orconectes virilis* were collected from Maple Bay in Burt Lake during the summer of 2001. Crayfish were housed in flow-through outdoor metal troughs located at the University of Michigan Biological Station Stream Research Facility. Crayfish were allowed to acclimate to their new surroundings for at least 24 hr and fed on detrital material that accumulated in the holding tanks. All crayfish were released after testing into Burt Lake, downstream of the capture site, to prevent recapture on subsequent sampling trips.

Artificial Stream Set-up

An artificial stream (16 x 1 x 0.2 m) was constructed of concrete cinder blocks and 4-mm plastic sheeting (Figure 12). The flume was built to recognized standards in order to form an equilibrium benthic boundary layer (Nowell and Jumars 1987). Stream water was used directly from the Maple River resulting in a background concentration of natural odors that was consistent throughout all experimental trials. The stream contained an 11.7-m flow conditioning section in front of the 2.7-m working section and 1.6-m outflow section. The water exited the artificial stream and re-entered the Maple River approximately 200-m downstream of the intake for the stream lab. The end of the stream was covered with a 1.5 x 0.35 m board that contained 2.54-cm diameter holes evenly spaced throughout to maintain a constant depth and flow rate. Collimators consisting of 3 sheets of plastic egg crating (1.7-cm² holes) covered with fiberglass sheeting (1-mm² holes) were placed in the upper part of the stream to laminarize the incoming flow. The working section was partitioned off from the rest of the stream with 2 sheets of plastic egg crating spaced 2.7 m apart.
Water was pumped in from the Maple River into a 1-m mixing area ahead of the 11.7-m stream section. In order to avoid excessive particulate matter build-up, the flow through pipes were equipped with nylon stockings secured to the ends of the flow pipe to filter out the fine particulate organic matter and macroinvertebrate fauna.

Free stream velocity (5.0 ± 0.3 cm/sec) was measured at the beginning of each day by a Marsh-McBirney flow meter. Depth measurements ensured a uniform water depth of 20 ± 0.3 cm. The bottom of the stream was lined with cobble stones (4.5 ± 0.2 cm, N = 24) collected from the Maple River. To facilitate orientation responses of animals, the entire working section of the artificial stream was covered with tarps to reduce ambient light conditions.

*Hydrodynamic Characteristics*

The hydrodynamic characteristics of the artificial stream were determined by measuring the in-stream flow using an acoustic doppler velocimeter (ADV, Nortek USA). Measurements were taken at two downstream positions (1.1 and 1.3 m) within the working section of the stream. At each downstream position, three cross-stream sites and 2 depths, for a vertical profile, were measured. The cross-stream sites were 51 cm from the right wall (midpoint of the stream) and 25 cm to the right and left of center of the stream (Figure 12).

For each of the vertical measurements, the probe tip was suspended at 5.9 cm and 6.5 cm above the substrate. The probe of the ADV was activated to determine the exact height above the substrate and then adjusted to the desired height. At each of the 6 sites (2 downstream x 3 cross-stream each at 2 heights), three-dimensional flow velocities were taken for 180 sec at a sampling rate of 25 Hz. Velocity profiles and calculation of hydrodynamic variables were performed offline (Explore package, Nortek).
Stimulus Preparation

Fish gelatin blocks were used to simulate the effect of slowly diffusing carrion odors, which crayfish feed on in natural systems. Fish gelatin was prepared by mixing 45 g of homogenized frozen ocean perch (*Perca sp.*) fillets with 28 g of Knox unflavored gelatin and 0.71 L of boiling water. After mixing, the hot gelatin was placed in a baking pan lined with plastic wrap and refrigerated overnight until solidified. Solidified gelatin was cut into 3 x 3 x 1.5 cm cubes. Gelatin blocks were quartered before each trial to allow for a uniform surface area, and placed in mesh bags (1-mm² holes) with weights attached to the bottom to keep the bags in place during trials. As controls, empty mesh bags were placed in the same configuration as the bags containing the gelatin blocks.

Orientation Trials

Four male and 36 female *O. virilis* were used only once in orientation trials. Previous studies have shown that there are no differences in orientation strategies between male and female crayfish (Moore and Grills 1999; Keller et al. 2001; Tomba et al. 2001). Crayfish were marked with reflective tape on the back of the carapace and placed inside the stream for a 20 min acclimation period. Reflective tape facilitated observations of animals in subsequent motion analysis. Following a 20 min acclimation period, the mesh bags containing the fish gelatin blocks were placed in an up-stream position, 15-cm from the top grating. Mesh bags were also marked with reflective tape to allow for visual confirmation of the crayfish finding the source. Trials were defined as successful when the crayfish came within 10 cm of the source or touched the bag itself. Trials in which the crayfish did not move, walked along the walls, or did not locate the source were discarded. In the present study, 60 crayfish were used in the experimental trials where 4 in the single 1X, 15 in the single 2X and 11 in the dual presentations were discarded.
Trials were run until the animal located the odor source or a maximum of 15 min. Trials were conducted during the months of June and July 2001 between 0800 and 1700 hours at the University of Michigan Biological Station Stream Research Facility in Pellston, Michigan. All trials were video taped from above using a Cannon XL1 3 CCD Digital Video Camcorder. Orientation trials consisted of three experimental treatments and one control treatment.

1) Single concentration; single spatial source: One piece of fish gelatin was placed in a mesh bag at a single location within the stream \(N=10\). Hence referred to as single 1X.

2) Double concentration; single spatial source: Two pieces of fish gelatin were placed in a single mesh bag at a single location within the stream \(N=10\). Hence referred to as single 2X.

3) Double concentration; dual spatial source: Two pieces of fish gelatin were placed in two bags (one in each bag), one 30 cm behind the other in an upstream position. One bag was placed 15 cm downstream of the working section grating, the other was placed 15 cm upstream behind the grating \(N=10\). Hence referred to as a dual source.

4) Control treatments consisted of an empty mesh bag \(N=10\).

Crayfish were able to reach the more downstream source only. Crayfish were used only once in the behavioral experiments to avoid the influence of experience on orientation behavior.

**Odor Characterization**

To characterize the odor plume structure, gelatin was laced with a chemical tracer (0.1 M dopamine). Dopamine (3.4 g) was mixed with 7 g of Knox gelatin in 0.178 L of boiling water. Dopamine gelatin was placed in a baking dish and refrigerated over night to solidify. The solidified gelatin cubes (3 x 3 x 1.5 cm) were quartered, placed in mesh bags and, placed into the stream in the same positions as the fish gelatin.
An electrochemical detection technique was used to characterize the effects of different spatial arrangements of the odor source. The temporal distribution of dopamine was sampled at a rate of 10 Hz using the In Vivo Electrochemistry Computer System (IVEC-10). Electrochemical measurements were made with a 30-µm diameter carbon fiber electrode calibrated using four concentrations of dopamine ranging from 0-30 µM. Electrodes were calibrated using the above concentration range in a 50 ml beaker. These concentrations were used only for calibration of the electrode. Electrode readings showed linearity over this concentration range (coefficient of determination: $r^2 > 0.97$). Five min electrochemical recordings using 0.1M dopamine gelatin were made 40 cm from the odor source with each odor source arrangement to measure the temporal and spatial distribution of the chemical double, hence food odor distribution (Figure 12).

**Data Analysis**

Flow data obtained through measurements using the ADV system were analyzed by the commercial software program provided with the ADV system (Explore). Hydrodynamic characteristics were estimated from the ADV data following standard practices (Sanford 1997). The turbulent energy dissipation rate was calculated from equation 1 below (Sanford 1997):

$$\varepsilon = (U^3) / \kappa z$$

(4)

where $U$ is the free stream velocity, $\kappa$ is von Karman’s constant (0.41) and $z$ is the height above the stream bed.

From the values obtained through equation 1 we were able to calculate the Kolmogorov microscale ($\eta$, equation 2) and the Batchelor microscale ($\eta_s$, equation 3; Sanford 1997). These microscales are the measurements of the smallest eddy sizes, Kolmogorov, and the smallest distances for differences in chemical concentrations, Batchelor.
\[ \eta = 2\pi(\nu^3 / \varepsilon)^{1/4} \quad (5) \]
\[ \eta_s = 2\pi(\nu D^2_s / \varepsilon)^{1/4} \quad (6) \]

The coefficient \( D_s \) is the molecular diffusion coefficient of dopamine \((2 \times 10^{-5} \text{ cm}^2/\text{s})\). A spectral analysis of the velocity values was performed using the ADV Explore program at a 95% confidence level using Tukey HSD comparison test.

IVEC-10 data were analyzed using an in house basic program. Spatial and temporal components of the odor plume such as, maximum height of the odor pulse (highest concentration of chemical within the odor pulse), absolute slope (from threshold to highest concentration within an odor pulse/rise time), maximum slope (highest increase of concentration between 2 measurements), and rise time (from threshold to the highest value within an odor pulse), number of peaks (number of odor pulses in the entire trial), intermittency (time between peaks of two odor pulses), and spectral density (energy of the signal as a function of frequency) were measured for each arrangement of the odor sources. The beginning of odor pulses was determined as the concentration rises above a threshold and the end was determined when the concentration dropped below 30% of the previous peak height (Moore and Atema, 1991). Values obtained were averaged for each spatial position and analyzed using a Kruskal-Wallis ANOVA by ranks test. Tests for normality of the data showed that the data were not normally distributed. Spectral analysis of odor signals (FFT and spectral densities) was performed using a commercial statistical package (Statistica by Statsoft). For each odor source measurement there were 3432 data points. These data points were subsequently divided into 5 subsamples for each of the odor arrangements. The subsequent subsamples were then used in the spectral analysis for each odor placement (Moore et al. 2000). For statistical analysis only, the energy in these odor spectra were binned in 0.5 Hz bins. A 2-way factorial ANOVA, with odor arrangement and frequency bin as
the factors, was performed using the 5 subsample (686 data points each) spectra from each odor arrangement. A Tukey HSD post hoc test was used to determine individual differences. The resulting spectra from the 5 subsamples of each odor placement were averaged and plotted against frequency.

Video taped trials were digitized one frame/sec using the Peak Motus System to obtain X, Y spatial coordinates of crayfish movements throughout each trial. An array of behavioral parameters were analyzed including success rate, time to locate odor source, linearity of path, walking speed, walking speed toward source, heading angle, heading angle relative to source and relative to upstream, and turning angle. These are explained and defined in our previous publications (Moore and Grills 1999). A detailed temporal analysis was performed on walking speeds, turning angles and heading angles. These were analyzed statistically using a MANOVA and Fisher LSD post hoc test. The behavioral parameter of question (i.e., walking speed, turning angle and heading angle) was averaged over a ten centimeter bin at different distances from the odor source for each individual animal. These individual binned averages were then used in either a 2\textsuperscript{nd} (walking speed) or 3\textsuperscript{rd} (turning angle) order polynomial regression analysis (Moore et al. 1991b). Each of these regressions was statistically significant ($P<0.05$). The coefficients for each of the individual regressions were averaged for the entire population and were used to generate the population level regressions displayed on the figures in this manuscript. Only those animals that did not contact the side walls during any part of the orientation trial were included in the regression analysis. Following regression analysis, walking speeds were analyzed in blocks of distance from the odor source; 100-150 cm, 50-100 cm, and 0-50 cm, using a 2-way ANOVA and unequal N HSD post hoc test.
RESULTS

Hydrodynamic Characterization

Measurement of the hydrodynamic structure of the artificial stream used in this study showed that the boundary layer was in equilibrium throughout the working section of the stream (Table 2). Spectral analysis of both the 1.1-m and 1.3-m sites at each vertical point indicated a uniform consistency of flow speed (Figure 13), i.e., the flow speed did not change significantly over the course of the working section.

Odor Characterization

Spectral analysis of the dual source arrangement using .1M dopamine gelatin showed changes in the temporal patterns of the odor plume. The dual source arrangement exhibited a wide range of signal fluctuations across a wider range of frequencies (Figure 14). Analysis of the spectra of the different odor sources showed that there was a significant interaction between the placement of the odor sources and the frequency of the peaks (2-way ANOVA: $F_{18}=14.9$, $P<0.001$). Post hoc analysis showed that the dual source had statistically more energy at the lower frequency peaks compared to the single 1X and single 2X sources (Tukey HSD: $P<0.001$). There were no significant differences between the single 1X and single 2X sources ($P=.99$).

Spectral analysis of both single source arrangements showed lower frequency signals (<0.15 Hz) with a steady decrease in signal energy as frequency increased. There was an overall significant difference in the intermittency of peaks within the 3 odor arrangements (Kruskal-Wallis: $H_{2,1004}=90.91$, $P<0.05$). Figure 15 shows that the intermittency between pulses was smaller for the dual source compared to the single 2X arrangement, but the single 1X arrangement had shorter intermittency than the other spatial arrays.
Table 3 shows additional changes in the odor plume structure as a result of altered spatial arrangement. The concentration or maximum height of the chemical tracer within each odor patch increased significantly with the dual source arrangement (Kruskal-Wallis: \( H_{2,1004} = 55.72, P<0.05 \)). The rise time decreased significantly with the dual source arrangement (Kruskal-Wallis: \( H_{2,1004} = 19.1, P<0.05 \)). Additionally, the absolute slope (Kruskal-Wallis: \( H_{2,1004} = 149.3, P<0.05 \)) and maximum slope (Kruskal-Wallis: \( H_{2,1004} = 108.13, P<0.05 \)) of the odor patches increased as a result of the dual source.

**Quantitative Analysis of the Temporal Aspects of Orientation Behavior**

The temporal and spatial aspects of odor tracking behavior revealed differences in the strategies used to orient upstream (Figure 16).

As crayfish moved upstream there was an overall significant effect of odor placement on spatial orientation (MANOVA: Rao’s \( R_{12,87} = 2.83, P<0.05 \)). Turning angles of crayfish as they moved toward the source were significantly smaller when presented with the dual source (46.76 ± 6.20 degrees) than control crayfish (67.01 ± 6.71 degrees) (LSD: \( P<0.05 \)). Compared to the single 1X and single 2X source arrangements, there were no significant differences when crayfish were presented with the dual source (LSD: \( P=0.16 \)). There were no significant differences between control crayfish and either single source arrangement (LSD: \( P=0.65 \)). Crayfish presented with the empty bag controls did not locate the empty bags in the 15 min time frame allowed. As for the heading angle and the net-to-gross measurements, there were no significant differences between the odor placements.

Analyzing walking speeds and turning angles as a function of distance to the source revealed significant regressions (\( P<0.05 \)) and different regressions for the different odor treatments. Walking speeds of crayfish as a function of the distance to the source showed a three
phase pattern. This general pattern was identical between the two single location odor sources (single 1X and single 2X source treatments). Under the dual odor treatment the temporal aspects of orientation behavior were altered (Figure 17). Crayfish presented with the dual source walked significantly faster when they were 100-50 cm away compared to the single 1X, single 2X, or control presentation (HSD: $P < 0.05$). As crayfish approached the odor source, between 0-45 cm, the differences in walking speeds under the three treatments were not maintained (Figure 18). This suggests that the placement, not the concentration, of the odor sources had an affect on temporal aspects of orientation behavior.

Turning angles of animals as a function of the distance to the source showed a similar result, in that animals started searching using identical turn angles far away from the source and then increased their turning angles sharply as they approached the source (Figure 19). Both single source arrangements showed a similar pattern in turn angles with relation to distance from the source, whereas the dual source arrangement had a much different pattern particularly at a greater distance from the odor source (Figure 19). Turn angles of crayfish presented with the dual source were more constant as they moved up current as opposed to the single source arrangements.

**DISCUSSION**

In order to understand how crayfish respond to complex signals we must first know how those signals are distributed in a flowing environment. In the present study, IVEC measurements showed that spatially separated odor sources caused the odor plume to have higher frequency components and more energy in the lower frequency components (Figure 14). We have defined the higher frequency components and increased energy as an increase in the “complexity” of the
odor signals perceived by the crayfish during orientation. The spatial and temporal structure of the odor plume was more “complex” in the dual source arrangement compared to both single odor source arrangements. This “complexity” was further reflected in other temporal aspects of the odor signal such as mean peak height and intermittency (Table 3, Figure 16). The dual source arrangement presented crayfish with larger concentration fluctuations in shorter pulses than the single 2X source even though they were presented with similar surface area and concentration. The pattern in intermittency with the different spatial arrays showed that the single 1X source had more peaks and very small intermittency between these peaks than either of the double sources.

Receptor cells of lobsters have been shown to act as temporal filters to extract spatial information from fluctuations in odor plumes (Gomez and Atema 1999). These receptor cells vary in their temporal filtering capabilities leading to differences in response to differing temporal fluctuations in odor plumes (Gomez et al. 1994; Gomez and Atema 1996b; Gomez et al. 1999).

The behavioral data in the present study indicates the differences in the temporal complexity of the odor signal resulted in more differences in the temporal aspects of orientation behavior (walking speed, and speed toward source) as opposed to the spatial aspects of orientation behavior (turning and heading angles). In general, crayfish located the odor source in all of the treatments. The paths of crayfish were similar to previously published studies and were absent of any zig-zag patterns found in male moth orientation studies (Mafra-Neto and Carde 1996). In addition, orientation paths looked similar to those published for lobsters (McLeese 1973; Moore et al. 1991b). There were periods in the paths where animals stopped, moved sideways and backwards, as well as toward the odor source (Figure 17). From a qualitative view,
the orientation paths looked similar between the treatment groups. The orientation paths showed that animals exhibited a fairly straight course toward the source with a few course corrections as they moved up current.

By linking the IVEC results with the behavioral results, we suggest that the temporal dynamics of odor fluctuations were guiding up-current movement and turning behavior of crayfish. By altering the spatial arrangement of the odor sources we were able to alter temporal characteristics of the signal structure without altering the temporal dynamics of hydro-mechanical signals. Thus, changes seen in crayfish orientation strategies were a result of changes in the spatial arrangement of a food source as opposed to an increase in concentration of the individual food sources, or any alteration in mechanical information. None of the behavioral measurements quantified in this system were altered by changes in the concentration of the odor (single 2X vs. single 1X). When one source was split and separated in space, crayfish maintained a faster walking speed as well as exhibited more consistent turning behavior as they moved up current (Figure 17, Figure 18, Figure 19). In the current design, as crayfish reached approximately 50 cm from the odor source turning angles increased sharply and walking speeds remained constant regardless of source placement. This change in behavior may indicate the point where animals switch to a local search strategy, possibly using more proximate cues, such as visual or tactile, to locate a close source. This general theme has been demonstrated in other studies with lobsters (Moore et al. 1991b). As they approach a certain distance to the source a local search strategy takes over. The exact distance at which the local search occurs will change with changes in design paradigms, such as flow speed, turbulence or concentration of the source.

Ecologically, aquatic organisms are faced with several problems which they must solve in order to perform life sustaining behaviors that are triggered by chemical information. In
typical habitats, these animals experience multiple odor plumes that are mixing over a multitude of physical habitats (Finelli et al. 2000; Moore et al. 2000). Faced with this immense complexity, animals must not only recognize and distinguish predator from food, mate from food, or food from other odor signals, they must be able to have some special awareness of these multiple odor sources, i.e. the ability to discriminate where these sources lie in relation to each other. With even a primitive ability to distinguish locations of odor sources relative to each other, animals will be able to make choices regarding foraging, avoiding predation, or mating. Studies with crayfish showed that they can effectively locate the spatial relationship of conflicting odor cues (Tomba et al. 2001). The results of the present study suggest that crayfish alter their behavioral output in response to increased temporal stimulation, which in turn, is a result of the changed spatial arrangement of food sources.

The way crayfish orient toward an odor source is affected by changes in intermittency and intensity of the signal within each odor patch arriving at the antennules (Kozlowski et al. 2003). In studies with moths, the fine-scale structure, the flux of the stimulus, and the wind direction are important in determining the response of males to female pheromone (Vickers and Baker 1992; Vickers and Baker 1994; Mafra-Neto and Carde 1996). Changes in the rate of release and the fine-scale structure of the plume altered male flight characteristics in response to these changes (Murlis and Jones 1981; Vickers and Baker 1992). For blue crabs, the structure of the plume, as well as the direction of water flow are important in orientation to odor sources (Zimmer-Faust et al. 1995; Finelli et al. 2000). This result suggests that blue crabs orient by utilizing both the spatial and temporal aspects of an odor plume. Although it has been claimed that these animals are using an odor-gated rheotaxis, a recent model results make it hard to pinpoint an exact orientation mechanism for blue crabs (Weissburg and Dusenbery 2002). These
findings lend support to the idea that temporal stimulation of the animal may play an important role in orientation behavior. We hypothesize that the presence or absence of the odor signal mediates movement towards the odor source. By altering the temporal and spatial dynamics of odor plumes we see changes in the temporal and spatial behavioral patterns of crayfish. Thus, we suggest that some spatial aspects of the odor plume are critical in changing the turning angles exhibited by crayfish during orientation behavior.

In summary, both the spatial and temporal aspects of orientation behavior in crayfish were altered by changes in odor plume dynamics. With an increase in the temporal complexity of odor signals, crayfish responded with higher walking speeds and smaller turning angles. By combining the chemical characteristics of the odor plume with hydrodynamic and behavioral measurements, we hypothesize that these crayfish were using a combination of the classically defined kinesis and taxis. Some aspects of crayfish orientation behavior can be termed a kinesis in that walking speeds were controlled by the stimulus distribution, while other aspects were similar to a taxis in that turning angles were decreased in the presence of increased temporal stimulation. It does appear as if the crayfish in this study were not performing an odor-gated rheotaxis that has been reported for other benthic crustaceans (blue crabs, (Weissburg and Zimmer-Faust 1994).
CHAPTER IV

CENTRAL NERVOUS SYSTEM (CNS) PROCESSING OF PULSE DURATION IN THE CRAYFISH \textit{PROCAMBARUS CLARKII}: IMPLICATIONS FOR ENCODING TURBULENT ODOR PLUMES

INTRODUCTION

Sensory systems play an important role in gathering environmental information from which behavioral decisions necessary for survival and reproduction can be made. These systems have properties that have evolved to extract relevant information from noisy environments in order to elicit appropriate behavioral responses (Endler and Basolo 1998). For example, moth receptor neurons show differential responses to changes in the temporal presentation of female pheromone (Grant, et al. 1997). As the time between odor pulses increased, the response of receptor neurons to subsequent pulses decreased. Moths use the temporal pulses of pheromone plumes to locate females and this is correlated to the temporal tuning of receptor neurons (Vickers et al. 1999). Bat echolocation behavior relies on the animal’s ability to filter out background noise during sonar reception when locating prey. The frequencies utilized for echolocation signals depend on the habitat and foraging behavior exhibited by particular species (Schnitzler et al. 2003). These examples illustrate the ability of an organism’s sensory system to be tuned to sensory information that is behaviorally relevant.

For aquatic organisms, chemical signals are the primary source of information used for a variety of tasks that include habitat selection (Pawlik 1992; Tamburri et al. 1996), predator avoidance (Hazlett 1985), mate choice (Dunham and Oh 1992; Giri and Dunham 2000), and foraging for food (Dunham et al. 1997; Moore and Grills 1999). Copepods utilize sheared
feeding currents to entrain chemical signals, thus allowing detection of predatory and food odors from a longer distance (Moore et al. 1999b). Foraging blue crabs are more successful in different variations of turbulent odor plumes (Keller and Weissburg 2004). The available chemical information within the environment plays an integral role in shaping how these and other organisms use distant sources.

Organisms employ a variety of strategies in order to locate the source of a chemical signal. These strategies differ mainly in the relative roles that mechanical, visual, and chemical signals play in guiding behavioral outputs. Odor-gated rheotaxis in blue crabs is reliant on flow to provide directional information to guide movement of animals upstream (Weissburg and Zimmer-Faust 1994). This behavior is controlled by the detection of odor cues from the surrounding medium. Many insects, particularly moths, make use of optomotor-anemotaxis which displays two different behavioral patterns: direct upwind flight stimulated by the presence of chemical signals and guided by the visual flow field, and a casting behavior of cross wind flight that is performed in the absence of odor stimulation (Vickers et al. 1992; Cardé 1996). The switch between upwind flight and casting behavior of insects is mediated by the frequency of the intermittent pulses of individual odor filaments arriving at the moth antennae. Lobsters and crayfish determine distance to an odor source by using a chemotactic strategy that relies on the spatial and temporal fluctuations of individual odor filaments (McLeese 1973; Reeder and Ache 1980; Keller et al. 2001; Wolf et al. 2004). An increase in the temporal complexity of turbulent odor plumes (i.e. increased flux) provides information that allows the crayfish and lobsters to forage more efficiently. The common feature in all these strategies is the detection and filtering of the temporal information from turbulent odor plumes. The strategies used by moths and
crustaceans alike are determined by the spatial and temporal distribution of chemical signals through a moving fluid.

The temporal fluctuations of the duration, intermittency and concentration of individual odor filaments within a turbulent odor plume provide information on the location of a particular odor signal (Moore et al. 1991a; Moore et al. 1994). This temporal information found within a turbulent odor plume predictably changes with distance to an odor source. As distance to a source changes, the duration, the concentration, and the intermittency of individual odor filaments will increase as the source is approached and decrease as the animal moves away from the source (Moore et al. 1994). The predictable change in the fine-scale structure as changes in distance occur, provides information on the location of a chemical signal to an organism’s sensory system. If the nervous system can encode the fine-scale fluctuations seen in natural odor signals, it could provide a possible mechanism for the switch in behavior we see as an animal approaches an odor source.

There is a small body of work that has investigated the mechanisms which nervous systems use to filter natural temporal fluctuations in olfactory information. Lobster olfactory receptor cells function as frequency filters by adapting their response over time to dynamic signal presentations (Gomez et al. 1994; Gomez and Atema 1996a; Gomez and Atema 1996b). The physiology of chemoreceptors on insect and lobster antennae indicate that olfactory receptors can encode pulsed information between frequencies of 2-4 Hz in lobsters and 10 Hz for moth receptor cells (Rumbo and Kaissling 1989a). Temporal tuning and frequency coding has been shown in projection neurons and central olfactory neurons of moths (Christensen and Hildebrand 1988; Heinbockel et al. 1999; Vickers et al. 2001). These studies indicate that certain projection neurons (PN) can modulate their firing rate in time with instantaneous changes in odor
plume distribution (Christensen and Hildebrand 1988; Vickers et al. 2001). The frequency ranges at which the receptor cells can distinguish changes in information are matched to the natural distribution of turbulent odor plumes. But, in order to fully understand the influence these temporal odor dynamics play in orientation behavior we need to also know how temporal odor dynamics are further encoded within the central nervous system.

Research to date has rarely focused on the central encoding of stimulus dynamics and its link to specific orientation behavior. The present study was designed to determine how odor plume dynamics, specifically pulse duration, is encoded in the crayfish’s central olfactory brain. We hypothesize that the olfactory lobe of the crayfish can encode pulse durations that occur in natural stream habitats. By encoding pulse duration faithfully, the crayfish olfactory system could provide the neural mechanisms needed to determine relative distance to an odor source that ultimately leads to orientation behavior.

MATERIALS AND METHODS

Animals

Male and female *Procambarus clarkii* were purchased from the Atchafalaya Biological Supply Co. (Raceland, LA). At Bowling Green State University, crayfish had their claws banded and were placed in a community holding tank containing de-chlorinated water at 23° C with a 14 h:10 h (light: dark) cycle. Crayfish used in neurophysiology experiments were transported from Bowling Green State University to The Ohio State University, Rothenbueeler Honey Bee Laboratory, Columbus, OH. Crayfish were placed in a bucket of ice water for transport to decrease the stress of traveling. Animals were then placed in a community holding tank held at ambient light and temperature conditions. Animals in both locations were fed 2 pieces of rabbit
pellets 3 times per week. A total number of 4 animals were used in the neurophysiological recordings.

**Preparation of Animals**

An isolated head preparation was employed in neurophysiology experiments. Crayfish were anesthetized by placing them in an ice bath for 15 min. or until there was decreased response to physical stimulation. The carapace of the crayfish was scored using a Dremel® tool with a cutting wheel attachment. After scoring the carapace, the remaining connections were severed with dissection scissors and the head capsule was cleared of excess tissue (Mellon and Alones 1994). The isolated head capsule was subsequently pinned onto a slanted sylgard recording chamber (Fig. 18). The medial antennule was placed into an olfactometer and the medial and lateral arteries were cannulated with chilled oxygenated crayfish saline at a rate of 2 ml/min. Saline was oxygenated through aeration of O₂ gas into a pressurized Nalgene® carboy. Double cannulation was used to keep the olfactory lobe and brain alive for recording (Mellon and Alones 1994). Cannulae were made using borosilicate glass pipettes (1.0 mm OD; Sutter Instrument Co. #BE-100-50-10). The glass pipettes were pulled to a fine tip using a Flaming/Brown micropipette puller model P-87.

**Odorant Delivery**

Presentation of the odors was controlled by a solenoid valve (The Lee Co. Essex, CT # 850720) run by an in house computer configuration file. Freshwater was held in a 10 L pressurized carboy placed above the recording apparatus. Freshwater flowed continuously over the antennule at a rate of 80 ml/min. Ten odor pulses were injected directly into the freshwater flow 10.7 cm upstream of the olfactometer. There was a 1.5 min delay between each odor presentation where fresh water washed over the antennule.
**Stimulus Paradigm**

Crayfish were presented with three odors (glutamine, glycine, and shrimp) at a concentration of $10^{-5}$ M for the glutamine and glycine and shrimp extract (Wardley’s Shrimp pellets). These odors were chosen to represent simple amino acids (glutamine and glycine) and a more complex signal signifying whole food odors (shrimp) that have been shown to be stimulatory behaviorally and in crustacean chemosensory neurons (Corotto and O'Brien 2002; Garm et al. 2005). Presented odors were diluted from an original stock solution of $10^{-3}$ M for the amino acids glutamine and glycine. Shrimp extract was prepared daily by homogenizing 5 g of shrimp pellets per liter of deionized water. Stock solutions of L-glutamine (Sigma G-3126) and L-glycine (Sigma G-7126) were frozen and stored in individual 10 ml scintillation vials until use. Dilutions and shrimp extract were made daily for experimentation.

Odors were presented using 5 different pulse durations (ms) in order to simulate the natural fluctuations of odor filaments seen in turbulent odor plumes. The pulse durations (ms) used in the present study were as follows:

1) 100 ms
2) 500 ms
3) 1000 ms
4) 2500 ms
5) 5000 ms

Ten replicate pulses were presented in random order of odor and duration with an interpulse interval of 5000 ms between each pulse. These durations were chosen because they encompass
the range of pulse durations of odor plumes in the turbulent stream systems inhabited by crayfish (Wolf et al. in review).

**Recording physiological activity in the olfactory lobe (OL)**

Neurophysiology recordings were performed using a 16-channel silicon microelectrode array (University of Michigan Center for Neural Communication Technology) to simultaneously monitor and record spike activity of ≤ 60 neural units or clusters of neural activity within the OL. The sixteen-channel microelectrodes were arrayed in 4 sets of tetrodes (model 2x2tet). The four tetrodes are arranged in a square configuration across two shanks with a distance of 150 µm apart. Probes were inserted into the central portion of the OL under visual control. Neural activity was sampled across all electrodes within a tetrode if they crossed a minimum threshold which was set at a range between 50-159 µV. Data were collected by a Neurolynx hardware/software system using a Data Translations 3010 digital I/O board for A/D conversion. Threshold neural events were sampled at 32 kHZ for a total sample wave of 1 ms (Daly et al. 2004).

Chemosensory activity was verified by stimulating the antennule with shrimp odor and with plain de-chlorinated tap water. Placement of the probe in the OL was confirmed by an increase in cell response to shrimp odor prior to recording. Verification of probe placement was further determined histologically following experimentation using 20 µm brain cryosections stained with crystal violet. Brains were excised and fixed in 4 % paraformaldehyde and cryoprotected using a sucrose gradient: 10 (1 hr), 20 (1 hr) and 30 % (overnight) following neurophysiology recordings (Belanger et al. 2003). Cryoprotected brains were embedded in thermoshandon embedding matrix and were sliced into 20 µm sections using a Microm D-6900 cryostat (Heidleberg, GER). The 20 µm sections were placed serially on standard microscope
slide and frozen until staining. Sections were stained for 1 min using Difco BBL gram crystal violet primary stain (Beckton Dickson & Co. #4312505) and rinsed with cold 0.1 M phosphate buffered saline (PBS). Stained sections were allowed to dry and mounted in Vectashield® (Vector labs, Burlingame, CA). Mounted sections were viewed an Olympus BX51 Photomicroscope with high resolution Nomarski DIC optics (B&B Microscopes LTD, PA). Images were captured using a Spot Insight monochromatic camera (Model # 11.3 three shot color, Diagnostic Instruments Inc. Sterling Heights, MI) attached to the microscope and processed using Spot imaging software (V4.6).

Off-line Spike Sorting

Spike sorting into separate neural units was performed off-line using a two-step, semi-automated process. Raw tetrode data were first processed using the BubbleClust toolbox for Matlab® (Neurolynx, Tucson, AZ) (Daly et al. 2004). BubbleClust evaluates events across all tetrode recording channels using spike wave energy and the first two extracted principle components of the wave forms. BubbleClust then produces a decision tree that separates events from lowest to highest neighbor density. Clusters of nearest neighbor densities were evaluated for stability and unit fidelity through a series of summary statistics that included, peak plots, autocorrelations, cross-correlations, inter-spike interval histograms, waveform summary statistics, and peak by channel by time plots. The clusters of activity that appeared to represent valid and stable waveforms of single neuron or mulit-neuron activity were then exported to the MClust toolbox for Matlab®. Clusters were manually cut and sorted by defining cluster boundaries to exclude events that diverged from the average waveform of a particular cluster.

Following manual sorting, clusters were evaluated and chosen based on the summary statistics listed earlier. There were a total of 60 identifiable neural units selected from the 4
recordings, each from an individual animal. These selected neural units were exported as time-stamped events where the individual recordings were analyzed separately. Time-stamped events were imported into NeuroExplorer (Plexon® Inc., Dallas, TX) for further analysis. Neural units were further selected for by running a spectral density analysis to identify any neural units that contained 60-Hz contamination from background electrical noise. No neural units selected exhibited a sharp peak in the 60-Hz range.

**Statistical Analysis**

Time stamped events imported into NeuroExplorer were analyzed by running peristimulus time histograms for each odor and presentation paradigm. Peristimulus time histograms averaged the response of neural units into 20 ms time bins for each of the 10 pulses presented for each odor and duration. Peristimulus time histograms were performed to obtain unit responses to each repetitive pulse under the experimental paradigm. Out of the 60 neural units isolated from 4 animals, 27, or 45% were used in further analysis.

Binned responses from the peristimulus time histograms were smoothed using an exponential smoothing algorithm to highlight the timing of the peak response of neural units to the presentation of odor under different pulse durations, odor type, and pulse number (Gardner 1985). The response of neural units per 20 ms time bins were summed for the population and smoothed using the above algorithm technique. The response of neural units within the 1st (s) of stimulation was averaged across the population of 27 neural units and statistically analyzed using a Factorial ANOVA. The 1st (s) of stimulation was used because it was determined that this time frame encompassed the time frame of peak response of neural units. Differences between pulse duration, odor, and pulse number were determined using a Tukey HSD post-hoc analysis.
Cumulative sum analysis was performed to determine the change in firing rate of neural units over 10 pulses for each of the different pulse durations (Ellaway 1978). The change in unit response to pulse duration was normalized to the lowest pulse duration time of 100 ms. Normalization consisted of dividing the response of neural units by the increase of duration time over 100 ms. For a duration of 500 ms, the change in response of neural units was divided by a factor of five to account for the 5 fold increase in stimulation time. This procedure was adjusted according to the other three duration times. The resulting normalized responses were averaged for the population of 27 neural units for the 10 pulses, odor types and pulse durations. The population responses were statistically analyzed using a Factorial ANOVA. Differences between pulse number, pulse duration and odor type were determined using a Tukey HSD post-hoc analysis.

RESULTS

Brain Histology

Histological analysis was performed on crayfish brains following each recording session to verify probe placement. The brains slices indicate that the probe was placed in the central portion of the OL during recordings as evidenced by the puncture holes that correspond to the size and spacing of the two shanks of the probe (Fig. 19). The holes are approximately 160 µm in width from the outer edge of one hole to the outer edge of the corresponding hole (Fig. 19). This is consistent with the width of the two shanks which are approximately 150 µm-170 µm.

Cell Response Characteristics

Waveform profiles across the 4 channels of the tetrode were produced to verify the separation of individual neural units (Fig. 20). In general, cells exhibited varied responses to the
presentation of odor type and duration. Figure 21 shows the response properties of a group of cells to pulse duration. There are groups of cells that exhibit excitatory and inhibitory responses due to pulse presentation. The neural units that exhibit an excitatory response do so for the course of the pulse duration.

Neural units showed their highest responses to the presentation of odor between 150-500 ms following stimulus onset (Fig. 22). The time to the highest response was not altered due to pulse duration. In addition to the peak response occurring in the same time frame, neural units also exhibited similar duration of response. The peak response to stimulus presentation lasted approximately 250 ms in length.

There are no differences in the peak response of neural units over the course of pulse repetition (Fig. 23). The peak response of neural units when presented with three different odor types remained between 150-500 ms (Fig. 24). There was an overall significant effect of odor type on neural unit responses within the 1st (s) of stimulation (One-way ANOVA: $F_{2,762} = 61.38; P < 0.002$). When presented with shrimp odor, neural units exhibited more spikes per 20 ms time bins compared to glutamine (Tukey HSD: $P < 0.001$) or glycine (Fig. 25; $P < 0.002$). Similarly, when presented with glycine neural units exhibited more spikes per 20 ms compared to the presentation of glutamine (Tukey HSD: $P < 0.001$).

**Population Responses to Pulse Duration**

There was an overall significant effect of pulse duration population responses (Factorial ANOVA: $F_{(4,334)} = 628.3; P < 0.001$). Neural units exhibit a decrease in the rate of activity in response to increasing pulse duration (Fig. 26). When presented with pulse durations of 100 ms, neural units exhibited a higher change in response (14.3 ± 0.82 spikes per 20 ms) over background compared to the other 4 pulse durations (Tukey HSD: $P < 0.001$). The change in
activity (3.18 ± 0.12 spikes per 20 ms) over background was significantly lower at the 500 ms pulse duration than the 100 ms duration (Tukey HSD: P < 0.001). The change in neural response at the 500 ms duration was significantly higher when compared to 1000 (1.24 ± 0.24), 2500 (0.43 ± 0.37), and 5000 (0.64 ± 0.11) ms durations (Tukey HSD: P < 0.001). There were no significant differences in neural unit activity between the higher pulse durations of 1000, 2500, and 5000 ms (Tukey HSD: P = 0.08, 0.37, 0.95). To summarize, the population of neural units within the olfactory lobe exhibited decreased response properties as pulse duration increased (Fig. 26). The most significant decrease in response of the population occurred as pulse duration increased from 100 ms to 500 ms. There were also decreases in response properties when duration increased from 500 to 1000 ms. The response characteristics of populations did not significantly change with pulse durations between 1000-5000 ms.

Responses of neural units to pulse duration was significantly influenced by the odor type presented (Fig. 27; Factorial ANOVA: F(8,3334) = 4.45; P < 0.001). Neural units showed a significant increase in spike activity (15.74 ± 0.82) when presented with shrimp odor at a 100 ms pulse duration compared to glycine (12.92 ± 0.76; Tukey HSD: P < 0.001). There were no significant differences in the response of neural units between shrimp and glutamine (14.25 ± 0.98) at the 100 ms pulse duration (Tukey HSD: P = 0.38). These effects were seen at the 100 ms duration but not at the 500, 1000, 2500, and 5000 ms pulse durations (Tukey HSD: P = 0.99).

DISCUSSION

The results of the present study found that individual neural units within the OL exhibit peak responses within the first second of stimulation and the maximum response of neural units occurred 150 and 500 ms following stimulus onset (Figs. 22, 23, 24). Population responses exhibited a decrease in spike number due to increasing pulse duration (Fig. 26). Neural units
within the OL can reliably differentiate between pulse durations up to 1000 ms (Fig. 26). Encoding of odor type seems to be influenced by pulse duration as illustrated by the higher responses of neural units to shrimp at the 100 ms pulse duration (Fig. 27). These results illustrate that the OL can encode the temporal stimulus dynamics that are integral in mediating orientation toward a specific goal.

Orientation behavior involves the detection and processing of chemical cues that provide information on the location of a specific item. It has been demonstrated in numerous studies that chemical orientation behavior is dependent on the temporal fluctuations or flux of individual odor filaments that structure turbulent odor plumes (Keller et al. 2001; Keller and Weissburg 2004; Wolf et al. 2004). Specifically, the fluctuations in the concentration, duration and intermittency of odor filaments influence the successes and efficiency of orientation behavior (Murlis and Jones 1981; Finelli et al. 1999). In the present study the crayfish OL is able to encode odor filament duration by exhibiting differential neural responses to changes in pulse duration (Fig. 26) Neural units show significant changes in firing rate due to pulse duration up to 1000 ms, after which longer durations do not show significant differences in response properties. Knowing how the nervous system encodes the temporal dynamics of turbulent odor plumes will provide much needed insight into how complex information initiates and maintains orientation behavior.

The ability of the nervous system to encode pulse duration has profound influences on how an organism orients toward a chemical signal and its ability to locate the source of information in the most efficient way possible. The location of the source of chemical information can be determined by the fine-scale components that structure a turbulent odor plume. The responses exhibited by neural units in the OL show that the CNS can encode pulse durations between 100
and 1000 ms in length. These pulse durations correspond to those seen in naturally occurring turbulent odor plumes that are encountered by organisms (Moore et al. 1992). Because these fine-scale components such as duration, intermittency, and concentration, change predictably with distance to the odor source, the ability to reliably differentiate between naturally occurring fluctuations confers an organism a possible neural mechanism to estimating the location of an odor source (Moore et al. 1992; Finelli et al. 1999).

**Possible Mechanisms for Encoding Pulse Duration**

Neural units within the OL typically exhibited a peak response to the presentation of odor within 500 ms of stimulus onset. The timing of the peak response of neural units in the OL is unaffected by the pulse duration presented to the animal. Gomez and Atema (1996) showed similar results in recordings from olfactory receptor cells located on lobster antennules. Lobster receptor cells show a peak response to the presentation of odor within 220 ms of stimulus onset regardless of pulse duration. In the present experiment the peak response of neural units showed significant differences when the three different odor types were presented to the antennule (Fig. 25). This indicates non-aesthetasc odor encoding in the OL. Previous work indicates that non-aesthetasc chemoreceptors on the medial antennule can invoke responses from certain cell types within the OL, LAN and MAN of the crayfish midbrain (Schmidt et al. 1992; Mellon 1995; Schmidt and Ache 1996; Mellon 1997). Our results support evidence that secondary processing of odorant information occurs in the OL from non-aesthetasc chemosensory information projected from the LAN to the OL (Mellon 1995). Type I and type II cells in the OL of *Procambarus clarkii* show activity in response to the stimulation of the medial or lateral antennule (Personal communication Mellon). Type II cells exhibit multimodal capabilities showing responses to both mechano- and chemo-sensory stimulation.
In natural habitats the location of a chemical odor source can be determined by the spatial and temporal properties of the odor plume. As the distance to an odor source increases the duration of an odor pulse will become smaller. Duration of odor pulses become longer as the distance to the source is decreased (Moore et al. 1994). This predictive element within turbulent odor plumes provides an animal with information needed to determine where a particular odor source is in space. The results of this study illustrate a possible neural mechanism by which an animal could determine the location of a particular goal in relation to itself. Having a central mechanism in place that encodes the temporal aspects of a chemical signal will provide an animal with the spatial and temporal information necessary for goal orientation.
INTRODUCTION

The ability of sensory systems to detect and encode changes in stimulus dynamics is essential in allowing an organism to orient to turbulent odor plumes. Recent investigations have shown that the olfactory system is able to differentiate changes in the pulse duration, which is the amount of time an odor filament is present in the environment, demonstrating the ability of the olfactory brain to encode temporal fluctuations of olfactory stimuli (Wolf et al. In prep-a). The physiology of sensory neurons within the olfactory system can act as a temporal filter allowing for the extraction of relevant stimuli from a noisy turbulent environment (Gomez et al. 1994; Gomez et al. 1999).

Chemical stimuli that are transported through turbulent environments have specific temporal components that are important in mediating foraging behavior. In essence, it is the fine-scale fluctuations in the intermittency, duration and concentration of chemical odor plumes that initiate and maintain movement of an animal toward a goal (Carde et al. 1984; Zanen and Carde 1999; Horner et al. 2004; Ferner and Weissburg 2005). In particular, the intermittency of odor pulses is one feature of natural odor plume dynamics that seems to highly influence organisms’ plume following strategies (Kozlowski et al. 2003; Keller and Weissburg 2004; Wolf et al. 2004). These temporal odor plume components contain potential information for chemoreceptor neurons to detect and process.
There are numerous studies that have investigated how olfactory receptor cells in the peripheral nervous system encode temporal aspects of chemical stimuli (Strausfeld and Kaissling 1986; Rumbo and Kaissling 1989b; Gomez, et al. 1994; Kaissling 1996; Kaissling 1998; Gomez, et al. 1999; Barrozo and Kaissling 2002). Olfactory receptor cells encode temporal stimulus dynamics through processes of adaptation and disadaptation of neural spike activity. It is this regulation of neural activity through adaptation that provides an internal code for processing of sensory information.

The underlying mechanisms regulating neural activity occur at the cellular and neurochemical levels (Yamamoto et al. 2002). Spike frequency adaptation occurs through the opening of calcium-dependent potassium channels ($I_{K(Ca)}$) stimulated by preceding action potentials (Yamamoto, et al. 2002). Though the mechanisms involved in adaptation and the regulation of spike activity is highly important this experiment was not designed to test these types of process. Consequently, we are not concerned with the mechanisms behind adaptation per se, but with the implications adaptation has for filtering turbulent odor plume information. These studies all show that receptor cells are capable of filtering odor signals at frequencies that occur in natural habitats.

Though responses of receptor cells to natural stimulus dynamics has been clearly documented, investigations into how the central olfactory pathway encodes naturalistic temporal stimulus dynamics is limited to just a few studies (Christensen and Hildebrand 1988; Vickers et al. 2001). In order to understand how turbulent odor plume dynamics influence foraging and orientation behaviors the role of the central olfactory system plays in encoding of this information needs to be addressed. In the present study we investigated the olfactory lobe (OL) responses to a series of odor pulses that mimic the turbulent fluctuations found in natural
systems. By understanding the central processing of natural turbulent fluctuations we can begin to elucidate how these signals are utilized in distance orientation.

MATERIALS AND METHODS

Animals

Male and female *Procambarus clarkii* were purchased from the Atchafalaya Biological Supply Co. (Raceland, LA.). At Bowling Green State University, crayfish had their claws banded and were placed in a community holding tank containing de-chlorinated water at 23° C with a 14 h:10 h (light: dark) cycle. Crayfish used in neurophysiology experiments were transported from Bowling Green State University to The Ohio State University, Rothenbueler Honey Bee Laboratory in Columbus, OH. Crayfish were placed in a bucket of ice water for transport to avoid the stress of traveling. Animals were then placed in a community holding tank held at ambient light and temperature conditions. Animals in both locations were fed 2 pieces of rabbit pellets 3 times per week. A total number of 4 animals were used in the neurophysiological recordings.

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cannulated with chilled oxygenated crayfish saline at a rate of 2 ml/min (Mellon and Alones 1994). Saline was oxygenated through aeration of O₂ gas into a pressurized Nalgene® carboy. Double cannulation was used to keep the olfactory lobe and brain alive for recording (Mellon and Alones 1994). Cannulae were made using borosilicate glass pipettes (1.0 mm OD; Sutter Instrument Co. #BE-100-50-10). The glass pipettes were pulled to a fine tip using a Flaming/Brown micropipette puller model P-87.

Odorant Delivery

Presentation of the odors was controlled by a solenoid valve (The Lee Co. Essex, CT # 850720) run by an in house computer configuration file. Freshwater was held in a 10 L pressurized carboy placed above the recording apparatus. Freshwater flowed continuously over the antennule at a rate of 80 ml/min. Twenty odor pulses were injected directly into the freshwater flow 10.7 cm upstream of the olfactometer. There was a 1.5 min delay between each odor presentation where fresh water washed over the antennule.

Stimulus Paradigm

Crayfish were presented with three odors (glutamine, glycine, and shrimp) at a concentration of 10⁻⁵ M for the glutamine and glycine and 5g/l for shrimp extract (Wardley’s Shrimp pellets). These odor were chosen to represent simple amino acids (glutamine and glycine) diffusing off decaying matter and a more complex signal signifying whole food odors (shrimp). Presented odors were diluted from an original stock solution of 10⁻³ M for the amino acids glutamine and glycine. Shrimp extract was prepared daily by homogenizing 5 g of shrimp pellets per liter of deionized water. Stock solutions of L-glutamine (Sigma (G-3126) and L-glycine (Sigma G-7126) were frozen and stored in individual 10 ml scintillation vials until use. Dilutions and shrimp extract were made daily for experimentation.
Odors were presented at 5 different interpulse intervals (ms) in order to simulate the natural fluctuations of odor filaments seen in turbulent odor plumes. The interpulse intervals (ms) used in the present study were as follows:

4) 250 ms
5) 500 ms
6) 1000 ms
7) 2500 ms
8) 5000 ms

The 20 replicate pulses were presented in random order of odor and interpulse interval with a pulse duration of 500 ms for each pulse. The IPIs in the present study were chosen to mimic a naturalistic presentation of turbulent odor plumes that we have found to be influential in mediating orientation behavior in crayfish (Moore et al. 1989; Moore et al. 1992; Moore et al. 2000; Wolf et al. In Review)

**Recording Physiological Activity in the OL**

Neurophysiology recordings were performed using a 16-channel silicon microelectrode array (University of Michigan center for Neural Communication Technology) to simultaneously monitor and record spike activity of \( \leq 60 \) neural units or clusters of neural activity within the OL. The sixteen-channel microelectrodes were arrayed in 4 sets of tetrodes (model 2x2tet). The four tetrodes are arranged in a square configuration across two shanks with a distance of 150 \( \mu \)m apart. Probes were inserted into the central portion of the OL under visual control. Neural activity was sampled across all electrodes within a tetrode if they crossed a minimum threshold which was set at a range between 50-159 \( \mu \)V. Data were collected by a Neurolynx hardware/software system using a Data Translations 3010 digital I/O board for A/D conversion.
Threshold neural events were sampled at 32 kHZ for a total sample wave of 1 ms (Daly et al. 2004).

Chemosensory activity was verified by stimulating the antennule with shrimp odor and with plain de-chlorinated tap water. Placement of the probe in the OL was confirmed by an increase in cell response to the shrimp odor prior to recording. Verification of probe placement was determined histologically following experimentation using 20 µm brain cryosections stained with crystal violet. Brains were excised and fixed in 4 % paraformaldehyde and cryoprotected using a sucrose gradient: 10 (1 hr), 20 (1 hr) and 30 % (overnight) following neurophysiology recordings (Belanger et al. 2003). Cryoprotected brains were embedded in thermoshandon embedding matrix and were sliced into 20 µm sections using a Microm D-6900 cryostat (Heidleberg, GER). The 20 µm sections were placed serially on standard microscope slide and frozen until staining. Sections were stained for 1 min using Difco BBL gram crystal violet primary stain (Beckton Dickson & Co. #4312505) and rinsed with cold 0.1 M phosphate buffered saline (PBS). Stained sections were allowed to dry and mounted in Vectashield® (Vector labs, Burlingame, CA). Mounted sections were viewed an Olympus BX51 Photomicroscope with high resolution Nomarski DIC optics (B&B Microscopes LTD, PA). Images were captured using a Spot Insight monochromatic camera (Model # 11.3 three shot color, Diagnostic Instruments INC. Sterling Heights, MI) attached to the microscope and processed using Spot imaging software (V4.6).

**Off-line Spike Sorting**

Spike sorting into separate neural units was performed off-line using a two-step, semi-automated process. Raw tetrode data were first processed using the BubbleClust toolbox for Matlab® (Neurolynx, Tucson, AZ) (Daly et al. 2004). BubbleClust evaluates events across all
tetrode recording channels using spike wave energy and the first two extracted principle components of the wave forms. BubbleClust then produces a decision tree that separates events from lowest to highest neighbor density. Clusters of nearest neighbor densities were evaluated for stability and unit fidelity through a series of summary statistics that included, peak plots, autocorrelations, cross-correlations, inter-spike interval histograms, waveform summary statistics and peak by channel by time plots. The clusters of activity that appeared to represent valid and stable waveforms of single neuron or mulit-neuron activity were then exported to the MClust toolbox for Matlab®. Clusters were manually cut and sorted by defining cluster boundaries to exclude events that diverged from the average waveform of a particular cluster.

Following manual sorting, clusters were evaluated and chosen based on the summary statistics listed above. There were a total of 95 identifiable neural units selected from the 4 recordings, each from an individual animal. These selected neural units were exported as time-stamped events where the individual recordings were analyzed separately. Time-stamped events were imported into NeuroExplorer (Plexon® Inc., Dallas, TX) for further analysis. Neural units were further selected for by running a spectral density analysis to identify any neural units that contained 60-Hz contamination from background electrical noise. No neural units selected exhibited a sharp peak in the 60-Hz range.

Statistical Analysis

Time stamped events imported into NeuroExplorer were analyzed by performing peristimulus time histograms averaged over all 20 pulses of each odor at each IPI presentation (NeuroExplorer, Dallas TX). Peristimulus time histograms were used to obtain unit responses to each repetitive pulse under changes in IPI and odor type. Records of neural unit responses were binned in 20 ms time increments. The numerical values obtained from the peristimulus time
histograms were used in further statistical analysis to determine change in firing rate of cells due to IPI, odor, and pulse number. Out of 95 neural units isolated from 4 animals, 64, or 67 %, were used in further analysis. Neural units that exhibited responses of < 3 spikes per 20 ms time bins were considered not above background and were not included in further analysis.

*Analysis of Neural Unit Adaptation*

To determine cell responses over the 20 pulse presentation of odor and IPI we summed the response of units for each pulse number. Linear regressions were run on each neural unit to determine the trend of response from the presentation of pulse 1 to pulse 20. The slope value produced from the linear regression was used to determine adaptation of individual units. A negative slope value indicated a decrease in neural response over the repetition of the 20 pulses presented. The values obtained for the 67 units were statistically evaluated using a Factorial ANOVA and differences between IPI and odor were determined using an LSD post-hoc comparison test (Statistica 6.0, Tulsa, OK).

The response of neural units was divided into two categories, high and low activity, based on activity rates obtained from the peristimulus time histograms. Neural units were deemed low activity if the number of spikes produced was between 4-10 spikes/20 ms. High activity units were considered to be neural units that exhibited \( \geq 10 \) spikes/20 ms. The number of spikes produced was averaged over the 20 repetitive pulse train and statistically analyzed for differences in spike activity due to IPI and odor using a Factorial ANOVA and an LSD post hoc comparison test.

An analysis of factor variance was run to compare common response patterns across neural units within the ensemble recordings (Daly et al. 2004). The factor analysis was designed to identify neural units that had correlated response patterns across IPI, odor, pulse number and
time based on principle components analysis. The five best factors were retained and statistically analyzed using general linear modeling. The factors retained had an eigenvalue > 1.

RESULTS

Brain histology

Histological analysis was performed on crayfish brains following each recording session to verify probe placement. The brains slices indicate that the probe was placed in the central portion of the olfactory lobe during recordings as evidenced by the puncture holes that correspond to the size and spacing of the two shanks of the probe (Fig. 19). The holes are approximately 160 µm in width from the outer edge of one hole to the outer edge of the corresponding hole (Fig. 19). This is consistent with the width of the two shanks which are approximately 150 µm-170 µm.

Cell Response Characteristics

Waveform profiles across the 4 channels of the tetrode verified the separation of individual neural units isolated from neural ensemble recordings (Fig. 28). Perievent histograms and raster plots showed varied cell responses across the neural ensemble population (Fig. 29). There were significant changes in neural unit response due to interpulse interval (IPI) and odor type (Fig. 29).

Three types of neural units within the ensemble were identified due to response characteristics. Responses of neural units within the OL were characterized as mechano-responsive, mechano-olfactory responsive and olfactory responsive (Fig. 29, Table 4.). The mechano-repetitive properties are evident by the high peak responses seen as the stimulus is turned on and off (Fig. 29; Columns 1, 3). Neural units showed temporally patterned responses
that were dependent on the interactions between time, odor, IPI, and pulse number (Table 4; GLM; $P < 0.01$). For example, the five factors extracted for animal 1 illustrates the variety of individual responses noted above. Factor 1 indicates that the neural activity evoked was altered by mechanical stimulation (Table 4, IPI: $P < 0.01$, Pulse*IPI: $P < 0.01$) and not by odor or a combination of odor and mechanical stimulation ($P =0.08, 0.5$ respectively). Factor 2 shows that evoked neural activity was influenced by combinations of odor and IPI presentations ($P < 0.01$) indicating mechano-and chemo- responsive neural units. Factors 3 and 4 show strong odor influenced neural responses that are in turn influenced by changes in the frequency of pulse presentation ($P < 0.001$). GLM results for factors 3 and 4 indicate that neural activity is evoked primarily by odor. Finally, factor 5 has strong implications that evoked neural activity is both mechanically and odor responsive ($P < 0.01$). Overall, changes in IPI had a significant effect on the evoked responses of neural units to mechanical and odorant stimulation.

### Population Responses to IPI

There was an overall significant effect of changing IPI and odor type within the ensemble population (Factorial ANOVA; $F_{8,2370} = 2.95$, $P < 0.003$). Neural unit responses were divided into high and low activity based on the number of spikes per 20 ms time bins. Within these cell types, the average responses of neural units were significantly higher at the 250 ms intermittency compared to intermittencies at 500 ms and above (LSD; $P < 0.001$; Fig. 30A-B). Response of cells was odor dependent, with shrimp odor having significantly higher activity than glycine or glutamine (LSD; $P < 0.001$ Fig. 30A).

The series of 20 pulses presented at each IPI and odor showed significant changes in neural responses due to IPI (Factorial ANOVA; $F_{4,885} = 2.95$, $P < 0.04$). Neural units showed
decreased in response between the 1st pulse and 20th pulse at all IPI presentations except the 5000 ms IPI (Fig. 31). The 250 ms IPI had significantly more decrease in activity over the 20 pulses presented than the 1000, 2000, or 5000 ms IPI (LSD $P < 0.02$). The response of neural units at the 5000 ms IPI showed that responses of neural units over the 20 repetitive pulses did not decrease (Fig. 31). In general, neural responses showed decreasing activity when presented with fast IPI over the 20 pulse train (Fig. 32).

DISCUSSION

The results of this study illustrate three main points. First, the response characteristics of neural ensemble units within the central olfactory system are temporally patterned indicating that populations of units are responding in concert to changes in IPI and odor type (Table 4). Second, response properties of single units within the olfactory lobe are dependant on pulse intermittency and odor type (Fig. 29). Third, neural unit responses exhibited decreases in activity to repetitive pulses at high IPI frequencies (Figs. 31, 32). When the intermittency between pulses was small, < 500 ms, neural units decreased their response activity to repeated stimulation (Figs. 30, 31, 32). The temporally patterned response in conjunction with differences in adaptation due to IPI, suggests that neural units within the OL can act as a temporal filter to encode turbulent odorant information.

The temporal filtering properties of chemoreceptor cells encode changes in instantaneous concentration fluctuations, intermittency, and duration of odor filaments through the adaptation and disadaptation of cell responses to repeated stimulation (Kaissling et al. 1987; Moore and Shao 2000). In computer model receptor cells, two types of adaptation were exhibited. First, the response to a single stimulus pulse declined under prolonged pulse duration. Second, responses
of cells declined as a result of multiple pulse presentations (Moore and Shao 2000). The response of model receptor cells to repeated stimulation was decreased as the frequency of pulses was increased. In the present study, neural units within the OL exhibited adapted responses to repeated stimulation. In addition, adaptation was more pronounced as the intermittency between pulses was increased (Fig. 32). The differences in adaptation are illustrated by the decline in cell response when stimulated repeatedly at 250 and 500 ms IPIs (Fig. 32). The adaptation properties of neural units in the OL and in chemoreceptor neurons act as a temporal filter and flux detector. Neural units encode the changes in intermittency observed in turbulent odor plumes as distance to the source is altered. Encoding of fine-scale fluxes of turbulent odor plume characteristics could provide location information to orienting crayfish.

Changes in the fine-scale distribution of odor filaments within a turbulent odor plume are integral in mediating orientation toward odor sources (Murlis and Jones 1981; Wolf et al. 2004). In order for an organism to take advantage of the available information in the environment they must be able to differentiate between relevant odorant information from background noise (Gomez et al. 1994; Gomez et al. 1999). Specifically, changes in the duration, intermittency and concentration of individual odor filaments can guide how an organism moves toward or away from a particular source (Kozlowski et al. 2003). As an odor source is approached the duration and concentration of odorant in the pulse increase (i.e. pulses are present longer and at higher concentrations at the animals sensory appendages) and the intermittency between pulses decrease (Moore et al. 1991).

The ability to filter relevant information from the environment lies with the sensory systems functional properties (Wehner 1987). By having a sensory system that is tuned only to the most dominant properties of stimuli enables organisms to detect and process only the relevant
information and ignore the noise in the environment (Hovarth and Wehner 1999). In the crayfish, the most important information used in orientation behavior is the temporal fluctuations of a turbulent odor plume (Moore and Grills 1999; Keller et al. 2001; Kozlowski et al. 2003). The dominant temporal fluctuations in natural aquatic systems have been shown to be below 4 Hz (Moore, et al. 1992). It has been demonstrated that lobster chemoreceptor cells are able to reliably track signal fluctuations up to 4 Hz showing the “matched filter” properties of the peripheral receptor cells (Gomez et al. 1994).

In the present study we stimulated olfactory organs with intermittencies that ranged in frequencies between 0.2 to 4 Hz. The central olfactory pathway of crayfish is temporally tuned to changes in pulse intermittency (Table 4). As the frequency of pulses approaches 4 Hz (i.e. faster intermittencies) the cells within the OL adapt their rate of activity (Fig. 31, 32). As the frequency of pulses decrease (i.e. slower intermittencies) cells show less adaptation to repeated stimulation. The adaptation rates associated with differing intermittencies could provide information on how far away an odor source is from the animal. By using natural stimulus dynamics in characterizing how the nervous system processes sensory information we can begin to understand how organisms actually perceive their environment and determine what aspects of complex information are needed for behavioral tasks.
INTRODUCTION

Chemoreception mediates many types of behaviors that are necessary for survival. Chemical signals provide information that is utilized by organisms to select preferential habitats (Pawlik 1992; Tamburri et al. 1996), avoid predation (Hazlett 1985), select mates (Dunham and Oh 1992; Giri and Dunham 2000), and to forage for food (Dunham et al. 1997; Moore and Grills 1999). Oyster larvae are induced to settle from flowing water into benthic habitats by the presence of chemical cues produced from the presence of benthic organisms (Turner et al. 1994). The chemical cues needed to induce settlement are concentrated over substrates by the hydrodynamic conditions of benthic habitats. Odor plumes dispersed by wind mediate upwind flight in moths allowing males to locate available females to mate with (Murlis et al. 1992). Orientation toward food resources by lobsters and crabs is controlled by the presence and absence of chemical cues in moving water (Mackie 1973; Weissburg et al. 1994; Atema 1996). Behavioral responses mediated by specific chemical signals are dependent on the fine-scale distribution of chemical information through turbulent environments.

Natural flowing systems are turbulent environments by which chemical signals are structured and dispersed. Turbulent eddies are created as a moving fluid encounters obstructions and flows over substrates that are comprised of increasing roughness elements (Tennekes and Lumley 1992; Davidson et al. 1995). As a consequence of turbulent flow, odor plumes released
from a source are broken into fine-scale odor filaments by the action of the smallest eddies present in the flow (Murlis et al. 1992; Sanford 1997). Odor filaments created by turbulent advection consist of varying concentrations of odor molecules that are present at varying temporal scales. It is the fine-scale fluctuations in the concentration, intermittency, and duration of individual odor filaments that provide information on the location of a goal (Moore et al. 1991a; Moore et al. 1994). The fine-scale structure of information predictably changes with distance to an odor source. As distance to a source decreases, the concentration, and the duration of individual odor filaments increase while the intermittency between filaments decrease (Moore et al. 1994). These predictable changes in the fine-scale structure of chemical odor plumes provide critical information on the location of a chemical signal that is necessary for orientation behaviors.

Organisms employ a variety of strategies that enable them to orient toward or away from an odor source. These strategies mainly differ in the relative importance of mechanical, visual and chemical information in initiating and maintaining directed movements toward a source. For example, moths and blue crabs utilize visual and mechanical information respectively to guide directed upstream movements (Baker and Carde 1979; Weissburg and Zimmer-Faust 1994). These movements are controlled by the temporal and spatial fluctuations of turbulent odor plumes (Elkington et al. 1987; Carde and Knols 2000; Weissburg et al. 2003; Keller and Weissburg 2004). In crayfish and lobsters, it is the spatial and temporal fluctuations of turbulent odor plumes that seem to guide directed upstream movements (McLeese 1973; Moore and Grills 1999; Wolf, et al. 2004). These studies illustrate the importance of detecting, encoding, and filtering the temporal fluctuations of odor filaments contained in turbulent odor plumes for the mediation of orientation behavior.
Investigations into how nervous systems encode naturally occurring fluctuations in olfactory information are few in number. Lobster, olfactory receptor cells function as a frequency filter by adapting their response over time to dynamic signal presentations (Gomez et al. 1994; Gomez and Atema 1996a; Gomez and Atema 1996b). Lobster chemoreceptor cells showed differential abilities to resolve pulses with frequency ranges between 2-4 Hz (Gomez et al. 1994). Temporal tuning and frequency encoding by peripheral olfactory receptors and central projection neurons have been shown to occur in certain moth species through phasic and tonic cell responses (Christensen and Hildebrand 1988; Rumbo and Kaisling 1989a; Vickers et al. 2001). Pheromone receptor neurons and central olfactory neurons are able to accurately encode odor pulses up to 10 Hz (Christensen and Hildebrand 1988; Rumbo and Kaisling 1989). The frequency ranges at which the receptor cells can encode information are matched to the frequency ranges of odor plumes encountered in nature by both moths and lobsters (Murlis and Jones 1981; Moore et al. 1992).

The central encoding of natural stimulus dynamics and its link to orientation behavior has not been extensively investigated. The present study, combined with the previous related studies (Wolf et al. In prep-a; In prep -c) was designed to determine how odor plume dynamics, and specifically pulse concentration in this study, is encoded in the crayfish’s central olfactory brain. We hypothesize that the olfactory lobe of the crayfish can encode pulse concentrations that occur in natural stream habitats. By encoding pulse concentration faithfully, the crayfish olfactory system could provide the neural mechanisms needed to determine an odor source location allowing for the specific behavioral responses seen as an animal approaches an odor source.
MATERIAL AND METHODS

Animals

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Odors were presented at 5 different concentrations (M) in order to simulate the natural fluctuations of odor filaments seen in turbulent odor plumes. The concentrations for glycine and glutamine were as follows:
Shrimp concentrations were determined as a percentage of the 5 g L$^{-1}$ concentration. The percentages of shrimp extract used were as follows:

1) 100 %
2) 10 %
3) 1.0 %
4) 0.1 %
5) 0.01 %

The 20 replicate pulses were presented in random order of odor and concentration with pulse durations of 500 ms and interpulse intervals of 5000 ms for each pulse. These concentrations were chosen to represent the range of concentration changes seen in natural stream systems (Wolf et al. In Review).

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Following manual sorting, clusters were evaluated and chosen based on the summary statistics listed above. There were a total of 64 identifiable neural units selected from the 3 recordings, each from an individual animal. These selected neural units were exported as time-stamped events where the individual recordings were analyzed separately. Time-stamped events were imported into NeuroExplorer (Plexon® Inc., Dallas, TX) for further analysis. Neural units were further selected for by running a spectral density analysis to identify any neural units that contained 60-Hz contamination from background electrical noise. No neural units selected exhibited a sharp peak in the 60-Hz range.
Statistical Analysis

Time stamped events imported into NeuroExplorer were analyzed by running peristimulus time histograms for each odor and presentation paradigm. Peristimulus time histograms averaged the response of neural units into 50 ms time bins for each of the 20 pulses presented for each odor and duration. Peristimulus time histograms were performed to obtain unit responses to each repetitive pulse under the experimental paradigm. Out of 64 neural units isolated from 3 animals, 19 (30 %) were used as inhibitory units and 28 (44%) were used as excitatory neural units in further analysis.

Binned responses from the peristimulus time histograms were smoothed using an exponential smoothing algorithm to highlight the timing of the peak response of neural units to the presentation of odor under different pulse concentration, odor type, and pulse number (Gardner 1985). The response of neural units per 50 ms time bins were summed for the population and smoothed using the above algorithm technique. The response of neural units within the 1st (s) of stimulation was averaged across the population of 28 excitatory neural units and statistically analyzed using a Factorial ANOVA. The 1st (s) of stimulation was used because it was determined that this time frame encompassed the time frame of peak response of neural units. Differences between pulse concentration, and odor, and pulse number were determined using the Fischer LSD post-hoc analysis.

Cumulative sum analysis was performed to determine the change in firing rate of neural units over 20 pulses for each of the different pulse concentrations (Ellaway 1978). Neural units were divided into two response types: excitatory (positive cumsum) or inhibitory (negative cumsum). Responses were averaged for each cell type over the 20 pulses. The population responses were statistically analyzed using a One-way ANOVA to evaluate differences between
odor type concentrations. There were no significant differences in response over the 20 repetitive pulses thus allowing us to combine the responses over the 20 pulses for further statistical analysis. Differences between odor pulse concentrations were determined using the Fischer LSD post-hoc test.

RESULTS

Brain Histology

Histological analysis was performed on crayfish brains following each recording session to verify probe placement. The brains slices indicate that the probe was placed in the central portion of the olfactory lobe during recordings as evidenced by the puncture holes that correspond to the size and spacing of the two shanks of the probe (Fig. 19). The holes are approximately 160 µm in width from the outer edge of one hole to the outer edge of the corresponding hole (Fig. 19). This is consistent with the width of the two shanks which are approximately 150 µm-170 µm.

Cell Response Characteristics

Neural units show similar time to peak response to stimulation regardless of pulse concentration. Time to peak response occurred typically around 500 ms following stimulus onset with a return to baseline response around 500 ms after stimulus termination (Fig. 33). The response of neural units to the first pulse of odor stimulation showed no significant effect of odor concentration on the peak response of cells. In addition, there were no significant differences in neural response over the 20 repetitive pulse presentations. Individual cell responses showed variable responses to odor type and concentration as exhibited by the perievent histogram and raster plots (Fig. 34). This indicates that there are differential responses across different cell types within the neural ensemble.
Population Response to Odor Concentration in the OL.

There was an overall significant effect of odor concentration on the change in response of both excitatory and inhibitory neural units (One-way ANOVA: $F_{14,9865} = 6.38$, $P < 0.001$ and $F_{14,6265} = 12.68$, $P < 0.001$ respectively). Neural responses to changing odorant concentration were dependant on the complexity of the odorant presented. Neural units showed a decrease in the change in response to decreasing glycine concentration, but there was an increase in the change in response as glutamine concentration was lowered. When presented with a complex odorant, shrimp, neural unit responses exhibited a Gaussian distribution with an increase in neural responses to the middle concentration values and decreases in response at the high and low ends of the spectrum. The specific changes in response of neural units within each odor type are outlined below.

Glycine

There was a significant decrease in response of excitatory neural units as odor concentration was reduced from $10^{-3}$ to $10^{-7}$ M (Fig. 35A). There were no significant differences in the change in activity between concentrations of $10^{-3}$, $10^{-4}$, and $10^{-5}$ M (LSD; $P = 0.11$ and $0.050$ respectively). In addition, the change in response of neural units to the $10^{-6}$ and $10^{-7}$ M concentrations was not significantly different (LSD; $P = 0.28$). Neural units were able to differentiate between concentrations of $10^{-3}$ and $10^{-6}$ & $10^{-7}$ M (LSD; $P < 0.001$) as well as between $10^{-4}$, $10^{-5}$, and $10^{-6}$ M (LSD; $P < 0.003$). There were no significant changes in response of neural units between $10^{-4}$, $10^{-5}$ and $10^{-7}$ M concentrations (LSD; $P = 0.06$ and $0.19$ respectively).

Inhibitory neural units showed a higher inhibitory rate of change at higher concentrations (Fig. 36A). The change in activity of neural units at the $10^{-3}$, $10^{-4}$ and $10^{-6}$ M concentrations were...
not significantly different from each other (LSD; $P = 0.53$, and $0.14$ respectively). The $10^{-7}$ M concentration showed the smallest change in activity compared to all concentrations (LSD; $P < 0.001$).

**Glutamine**

Excitatory neural units exhibited lower changes in response activity when stimulated with higher concentrations of glutamine odor (Fig. 35B). Stimulation with $10^{-3}$ M glutamine showed significantly lower change in activity when compared to concentrations of $10^{-5}$, $10^{-6}$ and $10^{-7}$ M (LSD; $P < 0.01$, 0.04, and $0.046$ respectively). Neural units showed a significantly smaller change in response at $10^{-4}$ M compared to all other concentrations (LSD; $P < 0.04$, 0.001, 0.001, 0.01 respectively). There were no significant changes in response activity between concentrations of $10^{-5}$, $10^{-6}$, and $10^{-7}$ M (LSD; $P = 0.85$).

Neural units that exhibited inhibitory responses to glutamine showed a significant change in response activity between the $10^{-7}$ M concentration and all other concentrations (Fig. 36B; LSD; $P < 0.05$). The other concentration values showed no significant changes in response activity (LSD; $P = 0.96$).

**Shrimp**

Neural units that exhibited an excitatory response to the presentation of shrimp odor showed a general Gaussian distribution in the change of activity due to odor concentration (Fig. 35C). Neural responses showed the highest increase in activity when stimulated with the $10^{-4}$ and $10^{-5}$ M concentrations (LSD; $P < 0.006$, 0.001, and 0.001) when compared to $10^{-3}$, $10^{-6}$, and $10^{-7}$ M concentrations respectively. Whereas the $10^{-3}$, $10^{-6}$ and $10^{-7}$ M concentrations showed no significant changes in response activity when compared to each other (LSD; $P = 0.18$, 0.14, and 0.24 respectively).
Inhibitory responses to stimulation by shrimp odor showed the largest decrease in
response at the $10^{-6}$ M (or 0.1%) concentration (Fig. 36C; LSD, $P < 0.001$). The smallest
decrease in response was seen at the $10^{-7}$ M (or 0.01%) concentration. This was significantly
smaller compared to the $10^{-4}$ (10 %), $10^{-5}$ (1 %) and $10^{-6}$ M (0.1%) concentrations (LSD, $P <
0.02$). There were no significant changes in response between the $10^{-3}$ M (100 %) and the $10^{-4}$
(10 %), $10^{-5}$ (1 %), and $10^{-7}$ M (0.01 %) concentrations (LSD, $P = 0.25$).

DISCUSSION

Our results clearly show that the CNS has both excitatory and inhibitory responses to the
presentation of odor pulses (Fig 34). Additionally, the response of neural units showed that
changes in odorant concentration are encoded within the OL of crayfish (Figs. 35, 36). Finally,
changes in odorant concentration are encoded in the CNS differently for simple amino acids and
complex odor stimuli (Fig. 35, 36).

The ability of the crayfish olfactory brain to encode stimulus concentration was evident
from the results of the present study. The effect of presenting different concentrations of odor
pulses on neural unit responses was shown to be dependent on the odor type presented. Each
odor type showed a different pattern of response to changing concentration. Neural units within
the OL exhibited increased activity when presented with higher molar concentrations. At lower
concentrations there was a smaller change in activity to glycine presentation (Fig. 35A, 36A).
Neural unit responses showed an increase in activity at the three lowest concentrations of
 glutamine (Fig. 35B, 36B). Exposure to shrimp odor elicited larger changes in neural activity at
the concentrations of $10^{-4}$ and $10^{-5}$ while the highest concentration and two lowest concentrations
exhibited smaller changes in activity (Fig. 35C, 36C). These results illustrate that neural units have differential responses to changing concentration due to odor type.

Changes in neural response characteristics have implications for how organisms use information from turbulent odor plumes to orient toward a goal. In moving fluids odorant information is transported and made available to organisms’ sensory appendages through two processes: turbulent advection and molecular diffusion. Though molecular diffusion plays an important at the level of individual receptor cells, it is the role turbulent advection plays on the distribution of signals through the environment that is critical in guiding orientation toward a goal (Carde et al. 1984; Keller and Weissburg 2004; Ferner and Weissburg 2005). Fine-scale fluctuations in concentration, duration, and intermittency provide valuable directional information to orienting organisms (Mafra-Neto and Carde 1995; Mafra-Neto and Carde 1998; Wolf et al. 2004). In the present study, changes in odorant concentration were encoded by both excitatory and inhibitory responses in the OL (Fig. 35, 36). Neural units showed a decrease in response to decreasing glycine concentration and an increase in response to decreasing glutamine and shrimp concentrations. Neural encoding of fluctuations in odorant concentration has implications for how organisms perform orientation behaviors by providing an underlying mechanism for the processing temporal and spatial information.

The ability of a nervous system to encode fluctuations in odor filament concentration can influence how an animal orients to a chemical signal and how it locates an odor source in natural turbulent environments. The location of an odor source is encompassed within the fine-scale structure of odor filaments within a turbulent odor plume. The response activity of neural units in the OL indicates that the olfactory system can encode odor concentration through changes in response activity; these changes in activity are odorant specific. The changes odorant
concentrations the nervous system is able to detect are similar to what is experienced by the animal in natural stream systems (Moore et al. 1994). Since fluctuations in the fine-scale components of odor plumes such as concentration, duration, and intermittency change predictably with distance to an odor source, having the ability to detect changes in naturally occurring fluctuations of turbulent plumes imparts an organism with a neural mechanism to determine the location of a goal.

The present study on the neural processing of concentration changes taken in conjunction with our previous two investigations on the neural processing of intermittency and duration fluctuations provides an in depth picture on how turbulent odor plumes are encoded in the central olfactory system (Wolf et al. In prep-a; In prep-c). The results from all three of these studies indicate that the central olfactory system can act as a temporal filter or flux detector that extracts the most dominant stimulus feature from the environment (Wehner 1987; Gomez and Atema 1996b; 1996a; Gomez et al. 1999; Moore and Shao 2000). Each aspect of fine-scale stimulus dynamics, intermittency, concentration and duration are encoded by the excitatory and inhibitory response properties of neural units in the central olfactory pathway. Intermittency between pulses is encoded through an increase in the rate of adaptation of neural units to increasing IPI stimulation (Wolf et al. In prep-c). As the duration of pulses increase the ability of neural units to differentiate between long pulse durations is abolished (Wolf, et al. In prep-c). In the present study, increases in odor concentration caused increases and decreases in the change in neural responses to odor presentation. The increase or decrease in neural response was dependant on the type of odor presented (Figs. 35, 36). The combination of these studies suggests that fluctuations in turbulent odor plumes occurring in natural stream systems are temporally matched with the nervous systems ability to detect and further encode instantaneous changes in signal properties
(Christensen and Hildebrand 1988; Gomez et al. 1994; Gomez and Atema 1996b; 1996a; Gomez et al. 1999; Heinbockel et al. 1999; Moore and Shao 2000). Being able to detect and process instantaneous fluctuations in odor plume characteristics provides an organism with the ability to navigate and make behavioral decisions based on complex natural stimuli.

In natural flowing environments, the direction and location of a chemical odor source can be implied from the spatial and temporal properties inherent in a turbulent odor plume. As distance to an odor source changes the concentration, intermittency, and duration of chemical molecules within odor filaments is altered. Odor filaments become less concentrated and are available for shorter periods of time the farther an odor source is from a foraging animal. In contrast, as an animal moves toward a chemical source, odor filaments become more concentrated and the duration an odor filament is present is increased (Moore et al. 1989). These predictive elements provide a gradient by which an animal can locate and estimate the relative distance of a chemical source and respond with an appropriate behavior. Having neural system in place that can encode fluctuations in the temporal aspects of turbulent odor plumes provides organisms with the ability to decipher the spatial and temporal information necessary for distance orientation.
CHAPTER VII
SUMMARY AND GENERAL CONCLUSIONS

This dissertation encompasses 5 independent projects designed to explore the impact of natural sensory stimuli on important behavioral decisions such as foraging for food. This compilation of work illustrates three major findings to help forge the gap of knowledge between how the physical environment influences the distribution of information and the behavioral decisions that are based on that information. First, the physical environment structures the fine-scale temporal characteristics of turbulent odor plumes providing habitat specific chemosensory information to foraging animals. Second, increased fluctuations in temporal odor plume characteristics (i.e. intermittency, duration and concentration) allows for more efficient orientation by crayfish to distant odor sources. Third, the central olfactory pathway of crayfish encodes fluctuations of temporal odor plume dynamics that are present in natural stream systems.

In chapter 2, I have demonstrated that there are habitat specific fluctuations in the temporal dynamics (i.e. duration, concentration, and intermittency) of chemical odor plumes. Natural streams and rivers are comprised of a variety of habitats that are made up of different substrate types such as gravel, sand, cobble, and a mixture of any combination of these elements. Substrates such as gravel and sand can be described as having different roughness elements that create habitat specific turbulence structures within the flow of the stream. Turbulence structures within each habitat cause changes in the temporal distribution of chemical signals as they move across different substrates (Fig 5). This is illustrated by changes in the intermittency, concentration, and duration of odor pulses across three physically different habitats of a natural flowing stream system (Figs. 6, 7, 8). Habitat specific turbulence structures create habitat
specific chemosensory signals that can provide valuable information to foraging animals influencing their orientation strategies.

Crayfish forage more efficiently when presented with temporally complex odorant information (Wolf et al. 2004). In this study crayfish were using a combination of the classically defined orientation strategies kinesis and taxis. Some aspects of crayfish orientation behavior can be termed a kinesis in that walking speeds were controlled by the stimulus distribution, while other aspects were similar to taxis because turning angles were decreased in the presence of increased temporal stimulation. Alteration of the placement of odor sources caused changes in the temporal complexity of odor plume dynamics. By changing odor source arrangement there was an increase in concentration fluctuations in concert with low intermittencies between odor filaments (Figs. 12, 13). This increase in temporal complexity of an odor signal resulted in more efficient crayfish orientation behavior exhibited by higher walking speeds and smaller turning angles (Figs. 15, 17). These results illustrate the influence temporal odor signal dynamics have on behavior of foraging organisms.

The crayfish central olfactory brain, the olfactory lobe (OL) encodes temporal stimulus dynamics through increases and decreases in the excitation and inhibition of neural units to the presentation of pulses with altered duration, intermittency, and concentration (Chapters 4, 5, 6). Neural units within the OL decreased spike activity as the antennules were presented with longer duration pulses (Chapter 4; Fig. 26). OL neural units were able to differentiate between pulses of 100-1000 ms durations. When the intermittency between pulses was altered, OL neural units exhibited temporally patterned responses associated to the change in interpulse interval (IPI) and odor type (Chapter 5; Table 4). The response activity of neural units exhibited a decrease in activity over repeated stimulation (20 pulses) and that the rate of decrease in response was more
pronounced at shorter IPIs (Figs. 31, 32). Lastly, changing the concentration of odor pulses resulted in differential responses of excitatory and inhibitory neural units to odor type (Figs. 35, 36). There was a decrease in the change in response of neural units as glycine concentration decreased from $10^{-3}$ M to $10^{-7}$ M (Fig. 35A). Presentation of different glutamine concentrations resulted in an increase in spike activity as concentration was decreased (Fig. 35B). Altering shrimp odor concentrations caused a larger change in spike activity at the 2 middle concentration values, $10^{-4}$ and $10^{-5}$ M, while the highest and two lowest concentrations exhibited little difference in the change in spike activity (Fig. 35C). These studies show that fluctuations in fine-scale temporal odor plume dynamics alter central nervous system activity that eventually leads to the initiation and execution of orientation behavior.

Implications for ecological interactions

Organisms live in complex heterogeneous environments in which they must detect and decipher critical chemosensory information in order to find food (Zimmer et al. 1999), reproduce (Kaißling and Kramer 1990), locate habitats (Brown and Rittschof 1984; Pawlik et al. 1991; Pawlik 1992), and avoid predation (Chivers and Smith 1993; Chivers et al. 1996). In order to utilize diverse chemical information, organisms use a variety of behavioral strategies to solve the problems they face (McLeese 1973; Kramer 1976; Herrnkind 1983; Keller et al. 2001). Though there are many studies that have investigated several aspects involved in mediating orientation behavior, few have focused on the connection between stimulus generation, mediation of the behavior and the neural coding of environmental stimuli. These three elements encompass what I have termed as the complete behavioral pathway involved in mediating orientation decisions.

Investigating the complete behavioral pathway from stimulus generation to detection and mediation of behavior as was done in this body of work, allows for a greater understanding into
how complex information is perceived and influences behavior. As crayfish forage in their
natural habitats they are challenged with complex information in which they must decipher to
make appropriate behavioral decisions (Keller et al. 2001; Tomba et al. 2001). For crayfish, we
see altered orientation strategies due to changing complexity of the surrounding environment and
changes in the temporal aspects of odor plumes (Moore and Grills 1999; Wolf et al. 2004). This
illustrates behavioral plasticity where by crayfish alter behavioral strategies based on the
information that is available.

In natural stream systems, chemical information is highly complex due to turbulent
fluctuations in the temporal components of the odor plume (Moore et al. 2000). Turbulent
fluctuations in natural streams are habitat specific due to different roughness elements of the
underlying substrate which results in there being habitat specific chemical signals (Hart, et al.
1996; Wolf, et al. In Review). Due to there being habitat specific chemical signals it would be
advantageous for an organism to have a behavioral system in place that is malleable enough to
adjust to situations where the animal faces habitat specific chemical information. If this is the
case you would expect to find altered orientation strategies when an animal is placed in different
types of habitats. Ultimately, how an organism responds behaviorally to complex information
depends on how the nervous system encodes changing information from the environment.

The crayfish main olfactory processing center, the olfactory lobe, encodes a range of
temporal stimulus dynamics that occur in natural stream systems. This range of neural encoding
provides a possible neural mechanism that could allow an animal to estimate the relative location
and distance to an odor source. Behaviorally, organisms such as crayfish show a switch in
behavior as they approach an odor source. Typically, as the distance to the source decreases
animals tend to switch from distance orientation characterized by fast walking speeds and small
turning angles to a local search strategy that is characterized by slow walking speeds and large
turning and heading angles (Moore et al. 1991b; Wolf et al. 2004). Fluctuations in the temporal
aspects of chemical signals, such as intermittency, duration and concentration change predictably
with distance to an odor source (Moore et al. 1989; Moore et al. 1992). These predictive
elements provide important information about the location of an odor source to an orienting
animal.

Due to the predictive nature of the temporal aspects of turbulent odor plumes, I propose
that changes in neural activity provide a basis for altering behavioral strategies over different
types of habitats. The peripheral and central nervous system act as a temporal filter to extract
relevant odorant information from noisy turbulent environments (Christensen and Hildebrand
seen in these organisms are matched to the environment in which these animals inhabit and the
information they encounter. Combining how the olfactory sensory system processes changes in
duration, intermittency and concentration gives a clearer picture on how these organisms are able
to behaviorally switch strategies as they approach an odor source. The work presented in this
dissertation utilizes an interdisciplinary approach to address problems faced by organisms
throughout their lives. Integrating the stimulus dynamics seen in natural environments with
behavior and neural processing allows for a complete picture of how organisms perform
behaviors necessary for survival.

In particular, I have illustrated that it is the natural fluctuations in temporal odor plume
characteristics that influences and mediates orientation behavior. In addition, I have shown that
the nervous system is particularly tuned to processing the temporal fluctuations that have been
found to be influential in structuring orientation strategies. As crayfish orient and forage for food items they exhibit behavioral switches in the way they approach an odor source. This switch in behavior is mediated by changes in the temporal fluctuations of odor filaments that constitute turbulent odor plumes. The olfactory sensory system can encode naturally relevant changes in fine-scale odor plume dynamics providing a neural basis for how crayfish can behaviorally switch strategies as they approach an odor source. This work provides an extensive background of knowledge that can be used in future investigations to further understanding of complex information is processed neurologically resulting in behavioral acts. Future work needs to address the neural connections behind the temporal filtering properties in the central olfactory system and motor control mechanisms that ultimately lead to an animal’s response to a chemical signal.
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Fig. 1. Schematic diagram of our study site in the Maple River, Pellston, MI USA (not to scale). Flow is from left to right. Pictured are the three microhabitats in which both flow and odor plume measurements were performed; sand, transition, and gravel. Asterisks indicate the placement of the probes (ADV and microelectrode) within each habitat. Letters indicate the placement of the Dopamine laced gelatin source as distances from the probes, A—90 cm, B—60 cm, C—30 cm. Measurements were taken at 5 heights above the substrate to obtain a vertical profile of both flow and odor. Heights above the substrate are indicated to the right of the figure.
Figure 2. Representative odor trace showing the temporal parameters measured for odor signal distribution over the gravel, sand, and transition habitats. Peaks (asterisk) are defined in Moore et al. 1994. Peak height (µM) is concentration over a baseline level. Peak slope (µMs⁻¹) is the maximum slope during the rising phase of any peak. Decay is the slope during the falling phase of any peak and peak length (sec) is the time it takes for the rise and fall of the peak from baseline.
Figure 3. Average velocity (± SEM) of the Maple River over three different substrate types; gravel, transition and sand and 5 distances from the substrate. A—stream-wise \((n = 3)\), B— cross-stream \((n = 3)\), and C—vertical \((n = 3)\) flow direction. Columns with the same letters are not significantly different. One-way ANOVA was run to compare within flow dimensions, not between flow dimensions. Significance was set at \(P < 0.05\).
Figure 4. Times series analysis of flow data from the Maple River over three different substrate types. Vertical profile is depicted in separate graphs: A—6 cm above substrate, B—5 cm above substrate, C—4 cm above substrate, D—3 cm above substrate, E—2 cm above substrate. Power spectra were smoothed for clarity using an adjacent averaging smoothing technique.
Figure 5. Time series analysis of the odor plume distribution over three different habitat types, gravel, sand and transition over three distances from the odor source and 5 distances from the substrate surface. Distance from the odor source is depicted from top to bottom with the 6 cm distance from the substrate located in the top row of graphs. Distance from the odor source is depicted from left to right with the 30 cm distance located in the first column of graphs. Spectral densities were smoothed for clarity using a Hamming weighting function.
Figure 6. Peak height (±SEM) of odor pulses within three physically different habitats at three distances from the odor source and 5 distances from the substrate surface. Distances from the odor source are depicted separately in three graphs; A—30 cm, B—60 cm, and C—90 cm. Columns with the same letters are not significantly different. A 2-way factorial MANOVA was run to compare differences between habitats and distances from the sensor. Significance was set at $P < 0.05$. 
Figure 7. Intermittency of odor pulses within three physically different habitats at three distances from the odor source and 5 distances from the substrate surface. Distances from the source are depicted separately in three graphs; A—30 cm, B—60 cm, and C—90 cm. Columns with the same letter are not significantly different. A 2-way factorial MANOVA was run to compare differences between habitats and distances from the source and substrate. Significance was set at $P < 0.05$. 
Figure 8. Absolute slope (±SEM) of odor pulses within three physically different habitats at three different distances from the odor source and 5 distances from the substrate surface. Columns with the same letters are not significantly different. A 2-way factorial MANOVA was run to compare differences between habitats and distances from the sensor. Significance was set at $P < 0.05$. 
Figure 9. Squared coherency of odor plume and velocity measurements over three different habitats and three distances to the odor source. The coherency between the flow and odor measurements are depicted for the 2 cm distance from the substrate. Columns depict the distance the odor source was from the microelectrode. Distances are from left to right with the 30 cm distance representing the left hand column of graphs, 60 cm the middle and 90 cm the right hand column. Rows depict the habitat type from which the measurements were obtained; A—Gravel, B—Sand, and C—Transition. The closer the coherency functions are to unity the more coherent the two time series data are to each other.
Figure 10. Artificial stream setup at the UMBS Stream Research facility located in Pellston, MI. Water was pumped in continuously from the nearby Maple River. △—ADV measurement sites, ▲—ADV and IVEC-10 measurement site, ★—Odor source placement. Width of the stream is projected 4 times its real width relative to the length. The inset shows the working section. Odor sources are 30 cm apart with the most upstream source behind the working section grating. The ADV recording sites were 1 and 1.3 m from the top of the working section and the IVEC sampling site is 40 cm from the odor source.
Figure 11. Spectral analysis of water velocity in the artificial stream at two points within the working section of the stream. The solid black line represents measurements taken 1.1 m from the top of the working section and the light solid line represents measurements taken 1.3 m from the top of the working section. The vertical profile of the stream velocity at both distances shows the water velocity has reached equilibrium and that the boundary layer is 5.9 cm above the substrate.
Figure 12. Spectral analysis of the odor plume structure of the single 1X ($N=1716$), single 2X ($N=1716$), and dual ($N=1716$) odor sources using .1 M dopamine gelatin as an odor source. The light solid lines represent the single source arrangements and the black solid line represents the dual source arrangement.
Figure 13. Average time (± SEM) between signal peaks (intermittency) of .1M dopamine gelatin. The dual odor source arrangement had less time between odor pulses than the single 2X source. Columns with the same letter are not significant different from each other. Significant differences were determined by a Kruskal-Wallis ANOVA by ranks and multiple comparisons: single 1X (N=453) vs. dual (N=350) (Z=4.32, P<0.001), single 2X (N=200) vs. dual (Z=2.96, P<0.009).
Figure 14. Representative orientation paths of 3 crayfish when presented with, A—single 1X, B—single 2X, C—dual, and D—control. Direction of flow, the relative position of the odor sources, and crayfish starting and ending positions are shown in A. Tracks were recorded 1/sec.
Figure 15. Polynomial regression of walking speeds of crayfish as a function of distance to the odor source. • — single 1X (N=7); ● — single 2X (N=5); ▲ — dual (N=7); ○ — control (N=10).
Figure 16. Comparison of walking speeds of crayfish (± SEM) when presented with the different odor placements in blocks of 50 cm distances from the odor source. N=10 for each treatment. Columns with the same letters are not significantly different from each other. A 2-way ANOVA for distance to the odor source and odor placement was performed ($F_{2,2} = 31.5, 4.0$). Unequal N HSD: $P<0.05$. 
Figure 17. Polynomial regression of turning angles of crayfish as a function of distance from the odor source. • — single 1X (N=7); ○ — single 2X (N=5); ▲ — dual (N=7); ◆ — control (N=10).
Figure 18. Isolated head preparation recording chamber. This is a slanted sylgard lined chamber where the head of the crayfish is inserted and the pinned on to the slanted floor. The medial antennule is placed into the red tube where odor will be administered.
Figure 19. Crystal violet staining of sagittal 20 µm brain sections. A- bright field image of the olfactory lobe (OL) and accessory lobe (AL). B- DIC image of the OL. Placement of the recording probe (p) is indicated in the circled area.
Figure 20. Waveform profiles of three individual units represented form two of the four recordings. Waveform profiles (a) and (b) are from recordings taken on 04/03/03 and waveform profile (c) is from a recording made on 03/12/03. The traces shown in each profile represent the average waveform across all four recording channels of a particular tetrode for a single unit of activity.
Figure 21. Perievent histograms and rasters of three odor presentations at three different pulse durations. Perievent histograms are in 20 ms time bins with a fixed numerical maximum for all graphs. Time on the x-axis is from -0.5 s to 5 s. Maximum value for the y-axis is fixed at 8 counts/bin, which is the maximum count per bin seen in any one of the histogram plots. Highlighted sections indicate the duration of odor presentation. Light gray represents glutamine, gray indicates glycine, and dark gray indicates shrimp odor.
Figure 22. Population responses to different pulse durations of shrimp odor. Responses of 27 individual units were summed for the 1st pulse of odor and grouped in 20 ms time bins. Curves were smoothed using an exponential smoothing algorithm for display clarity and comparison. The first 1.5 s of stimulation is displayed.
Figure 23. Population responses to different pulse durations of one odor type, shrimp. Responses of all 27 units were summed for each of the 10 pulses and grouped in 20 ms time bins. Curves were smoothed using an exponential smoothing algorithm for display clarity and comparison. The first 1.5 s of the 10 s recordings are displayed.
Figure 24. Population response to different odor types presented at 5 different pulse durations. The response of all 27 units were summed for the 1st pulse and grouped in 20 ms time bins. The curves were smoothed using and exponential smoothing algorithm for display clarity and easy comparison. The first 2 s of recording time are displayed. Note the difference in response due to odor type under all pulse durations presented, reflecting odor type responses.
Figure 25. Average population response (±SEM) to three different odors. Response of all 27 units were summed for the first pulse and grouped in 20 ms time bins. The response of units in the 1 s time period of the first pulse and all pulse durations were averaged for each odor type. Letters indicate significant differences of each column, $P < 0.001$. 
Figure 26. Change in firing rate over background to different pulse durations. Cumulative sum analysis was used to obtain the change in response over 10 pulses for the different pulse durations. Responses were normalized to 100 ms pulse duration to account for increased time lending to counting more spikes. Responses of all 27 units were summed for 10 pulses and odor type. Points with the same letter are not significantly different, $P < 0.01$. 
Figure 27. Duration by odor population responses of units in the OL of crayfish. Cumulative sum analysis was used to obtain the change in response over 10 pulses for the different pulse durations. Responses were normalized to 100 ms pulse duration to account for increased time lending to counting more spikes. Responses of all 27 units were summed for 10 pulses and odor type. Columns with the same letter are not significantly different from each other, $P < 0.01$. 
Figure 28. Waveform profiles of four individual neural units from two of the four recordings when exposed to differing IPI. The traces shown in each profile represent the average waveform across all four recording channels of a particular tetrode for a single unit of activity.
Figure 29. Perievent histograms and raster plots representing three odors presented at three different IPI lengths. Perievent histograms are in 20 ms time bins. Columns represent four individual cells from one of four recordings. Rows represent the three odors and the IPI at which they were presented to the antennule. Stimulus onset is at 0 (s) and stimulus termination occurs at 0.5 (s).
Figure 30. Average response of neural ensemble units per 20 ms time bins. Responses are divided into two cell types based on activity rates; A—High activity (≥ 10 spikes/20 ms) and B—Low Activity (≤ 10 spikes/20 ms). Statistical comparisons are between odors and IPI within activity type. Columns with the same letters are not significantly different (ANOVA, \( P < 0.05 \)).
Figure 31. Average change in number of spikes over 20 repetitive pulses. The adaptation rate of neural units is represented by odor type over the IPI presentation. Points with the same letter are not significantly different (ANOVA; $P < 0.05$).
Figure 32. Raw traces of neural response over 20 repetitive pulse presentations of 5 different IPI lengths. Traces were smoothed using a simple exponential smoothing function. Linear fit of the traces was run to illustrate the adaptation of cell responses over the 20 pulse train. Note the decrease in adaptation as the IPI length is increased from 250 ms to 5000 ms.
Figure 33. Population response to different odor types presented at 5 different concentrations. A—Glycine, B—Glutamine, and C—Shrimp. The response of all excitatory neural units were summed for the 1st pulse and grouped in 50 ms time bins. The curves were smoothed using and exponential smoothing algorithm for display clarity and easy comparison. The first 1 s of recording stimulation is displayed.
Figure 34. Perievent histogram and raster plots of neural units representing 3 odors presented at 3 different concentrations. Perievent histograms are in 50 ms time bins averaged over 20 repetitive pulses. Raster plots represent each of the 20 pulses for odor and concentration. Columns represent 5 individual neural units taken from one recording. Rows represent the 3 odors at three different concentrations. Note the differences in response of units to odor type and concentration.
Figure

35. Average change in spike activity of excitatory neural units stimulated with 5 different concentrations. A—Glycine, B—Glutamine, and C—Shrimp. Responses over all 20 pulse presentations were averaged for each odor and concentration. Columns with the same letter are not significantly different (ANOVA; $P < 0.05$)
Figure 36. Average change in spike activity of inhibitory neural units stimulated with 5 different concentrations. A—Glycine, B—Glutamine, and C—Shrimp. Responses over all 20 pulse presentations were averaged for each odor and concentration. Columns with the same letter are not significantly different (ANOVA; P < 0.05)
Table 1. Hydrodynamic characterization of gravel, transition and sand habitats at 5 distances from the substrate surface in the Maple River, Pellston, Michigan, USA. Microscale calculations for each habitat were statistically analyzed using a one-way ANOVA and Tukey HSD post hoc test. Values with different letters indicate significant differences ($F_{28,58} = 16.55, P < 0.001; \text{LSD}: P < 0.01$).

<table>
<thead>
<tr>
<th>Distance From Substrate</th>
<th>Dissipation Rate $\varepsilon$</th>
<th>Kolmogorov Scale $\eta$</th>
<th>Batchelor Scale $\eta_s$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gravel</td>
<td>Sand</td>
<td>Transition</td>
</tr>
<tr>
<td>6</td>
<td>0.942$^a$</td>
<td>1.80$^{a,c}$</td>
<td>6.35$^{a,c}$</td>
</tr>
<tr>
<td>5</td>
<td>1.11$^a$</td>
<td>1.74$^a$</td>
<td>8.50$^{a,c}$</td>
</tr>
<tr>
<td>4</td>
<td>1.46$^a$</td>
<td>2.20$^{a,c}$</td>
<td>10.27$^{a,c}$</td>
</tr>
<tr>
<td>3</td>
<td>2.31$^{a,c}$</td>
<td>2.49$^{a,c}$</td>
<td>23.25$^c$</td>
</tr>
<tr>
<td>2</td>
<td>173.13$^b$</td>
<td>9.74$^{a,c}$</td>
<td>129.67$^d$</td>
</tr>
</tbody>
</table>
Table 2. Hydrodynamic characteristics of an artificial stream

<table>
<thead>
<tr>
<th>Distance from top of working section</th>
<th>$U_\infty$ (cm/s)</th>
<th>$\varepsilon$ (cm$^2$/s$^2$)</th>
<th>$\eta$</th>
<th>$\eta_s$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1 m</td>
<td>6</td>
<td>0.09</td>
<td>1.76E-05</td>
<td>7.04E-11</td>
</tr>
<tr>
<td>1.3 m</td>
<td>6</td>
<td>0.09</td>
<td>1.79E-05</td>
<td>7.17E-11</td>
</tr>
</tbody>
</table>

$^a$Abbreviations: $U_\infty =$ free stream velocity, $\varepsilon =$ turbulent energy dissipation rate, $\eta =$ Kolmogorov scale, and $\eta_s =$ Batchelor scale.
Table 3. Characterization odor plume structure of single 1X and Single 2X and dual odor source arrangements.

|                          | Single 1X | Single 2X | Dual  
|--------------------------|-----------|-----------|------
| **Maximum Height**       | 1.87      | 1.91      | 3.05 *  
| **Absolute Slope**       | 5.27      | 4.33      | 8.28 *  
| **Maximum Slope**        | 10.08     | 9.28      | 13.71 *  
| **Rise Time**            | 0.45      | 0.63      | 0.38 *  

*Indicate significant differences (Kruskal-Wallis, $P<0.001$). Valid cases varied from 452 for single 1X, 200 for single 2X, and 350 for dual.
Table 4. Analysis of factor variance evaluating the effect of stimulus intermittency (IPI) on odor evoked responses within the OL. There were three types of units identified based on their responses: Mechano responsive, mechano-olfactory, and olfactory responsive units. There are significant temporally patterned responses of neural units due to time, odor and IPI. GLM; P < 0.001.

| Factor | Time | Pulse | Time * Pulse | Odor | Time * Odor | Pulse * Odor | Time * Pulse | IPI | Time * IPI | Pulse * IPI | Time * Odor | Odor * IPI | Time * Odor | Pulse * Odor | Time * Pulse * IPI |
|--------|------|-------|-------------|------|-------------|--------------|-------------|-----|-----------|------------|-------------|-----------|-------------|--------------|--------------|-----------------|
| Animal 1 | F1 | 0.065 | 0.068 | 0.029 | 0.068 | 0.13 | 0.65 | 0.56 | <0.001 | 0.008 | 0.04 | 0.24 | 0.49 | 0.18 | 0.62 | 0.94 |
|         | F2 | 0.004 | 0.94 | 0.13 | 0.006 | 0.79 | 0.04 | 0.46 | <0.001 | 0.011 | 0.056 | 0.91 | 0.007 | 0.34 | 0.26 | 0.78 |
|         | F3 | <0.001 | 0.01 | 0.84 | <0.001 | 0.32 | 0.53 | 0.61 | <0.001 | 0.003 | 0.007 | 0.19 | 0.0002 | 0.0003 | 0.53 | 0.18 |
|         | F4 | 0.52 | 0.22 | 0.94 | <0.001 | 0.41 | 0.54 | 1.0 | 0.001 | 0.0003 | 0.19 | 0.44 | 0.0002 | 0.0003 | 0.65 | 0.69 |
|         | F5 | 0.029 | <0.001 | 0.32 | 0.004 | 0.39 | 0.064 | 0.21 | 0.0004 | 0.002 | 0.47 | 0.028 | 0.10 | 0.20 | 0.25 | 0.57 |
| Animal 2 | F1 | <0.001 | 0.72 | 0.16 | 0.064 | 0.91 | 0.13 | 0.25 | 0.24 | 0.41 | 0.32 | 0.10 | 0.008 | 0.37 | 0.99 | 0.02 |
|         | F2 | 0.002 | 0.015 | 0.40 | 0.82 | 0.81 | 0.66 | 0.21 | 0.61 | 0.081 | 0.70 | 0.69 | 0.089 | 0.71 | 0.25 | 0.92 |
|         | F3 | <0.001 | 0.001 | 0.64 | 0.17 | 0.21 | 0.047 | 0.26 | <0.001 | 0.49 | 0.25 | 0.54 | <0.001 | 0.47 | 1.0 | 0.061 |
|         | F4 | <0.001 | 0.15 | 0.78 | 0.84 | 0.49 | 0.57 | 0.0034 | 0.057 | 0.20 | 0.28 | 0.27 | 0.032 | 0.86 | 0.53 | 0.52 |
|         | F5 | 0.89 | 0.30 | 0.21 | 0.045 | 0.49 | 0.099 | 0.84 | 0.35 | 0.61 | 0.014 | 0.22 | 0.023 | 0.544 | 0.35 | 0.10 |
| Animal 3 | F1 | 0.019 | 0.52 | 0.031 | 0.56 | 0.019 | 0.26 | 0.94 | 0.54 | 0.25 | 0.75 | 0.39 | 0.83 | 0.90 | 0.15 | 0.53 |
|         | F2 | 0.39 | 0.55 | 0.60 | 0.94 | 0.56 | 0.96 | 0.36 | 0.53 | 0.96 | 0.69 | 0.35 | 0.36 | 0.77 | 0.01 | 0.15 |
|         | F3 | <0.001 | 0.65 | 0.055 | 0.79 | <0.001 | 0.98 | 0.64 | 0.0003 | <0.001 | 0.51 | 0.26 | 0.007 | 0.65 | 0.11 | 0.31 |
|         | F4 | <0.001 | 0.83 | 0.87 | 0.42 | 0.05 | 0.98 | 0.33 | 0.008 | 0.42 | 0.14 | 0.59 | 0.72 | 0.30 | 0.21 | 0.20 |
|         | F5 | 0.94 | 0.86 | 0.84 | 0.80 | 0.77 | 0.96 | 0.19 | 0.005 | 0.47 | 0.42 | 0.82 | 0.09 | 0.59 | 0.67 | 0.45 |
| Animal 4 | F1 | <0.001 | 0.001 | 0.28 | <0.001 | 0.001 | 0.63 | 0.81 | 0.65 | <0.001 | 0.05 | 0.69 | <0.001 | 0.001 | 0.001 | 0.16 |
|         | F2 | 0.03 | 0.065 | 0.30 | 0.13 | 0.79 | 0.71 | 0.77 | 0.15 | 0.003 | 0.87 | 0.70 | 0.006 | 0.16 | 0.44 | 0.78 |
|         | F3 | <0.001 | 0.37 | 0.12 | 0.03 | <0.001 | 0.33 | 0.77 | <0.001 | 0.32 | 0.027 | <0.001 | <0.001 | 0.09 | 0.89 | 0.94 |
|         | F4 | 0.003 | 0.14 | 0.99 | 0.36 | 0.34 | 0.42 | 0.99 | 0.58 | 0.99 | 0.34 | 0.49 | 0.47 | 0.89 | 1.0 | 0.0 |
|         | F5 | <0.001 | 0.001 | 0.55 | 0.655 | 0.12 | 0.057 | 0.46 | 0.003 | 0.0007 | 0.058 | 0.97 | 0.068 | 0.13 | 0.44 | 0.28 |