The Kinematics and Conservation of Motor Patterns in the Larval Zebrafish, *Danio rerio*

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By

Leslie J. Day

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ABSTRACT OF DISSERTATION

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ABSTRACT

The zebrafish has become a model organism with which to investigate sensorimotor integration and the organization of motor control systems. The ultimate goal of such research is to understand the cellular basis of motor acts including locomotor behaviors. Quantitative kinematic analysis, and focus upon the physical maneuvers that comprise locomotor behaviors, should enhance the utility of the zebrafish model.

The larval zebrafish exhibits two distinct forward swimming patterns: a slow swim and a burst swim. These swimming patterns differ in terms of tail-beat frequency, bend angle, bend location and swim velocity. Using a high-speed camera, we analyzed the motor patterns exhibited by larval zebrafish in response to whole-field illumination changes and during rheotaxis and optomotor behaviors. These behaviors were compared to the slow and burst swimming patterns, and based on a variety of measurements, we conclude that a conserved slow swim motor pattern is used during diverse navigational behaviors. While certain kinematic variables, such as tail-beat frequency, bend amplitude and slip may vary to adjust swim speed and position in the environment, the similarities in the kinematic features suggest a common premotor circuit that can be stimulated by diverse sensory inputs. Conserved movement patterns were also used during diverse navigational turning behaviors, including the optomotor and dark-evoked turns. All of these locomotor patterns appeared to employ a “slow” motor system that is distinct from the high-frequency, short latency motor system used during larval C-start escape behaviors.
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CHAPTER 1

INTRODUCTION:
NEURAL CONTROLS OF LOCOMOTION
PROBLEM OF LOCOMOTOR CONTROL

The brain is made of billions of neurons that make trillions of synapses to form neural circuits. A central goal of neuroscience is to understand how these circuits decode and process sensory information to control locomotor behavior, such as walking, swimming or flying in vertebrates. How the intricate neuronal networks function in order to elicit a variety of movements, from the simple reflexes to the more complex networks of voluntary limb movement, is still mostly unknown. The rhythmic motor patterns of locomotion reflect a set of commands in which some are prearranged before a behavior begins and are sent to the muscles with correct timing for a sequential pattern of muscle contraction and movement. Therefore, a given neural output pattern produces a specific motor output and behavior. In some lower vertebrates, due to the limited number of neurons present in a spinal cord [Bernhardt et al. 1990; Hale et al. 2001], a descending motor system must somehow be able to be reorganized and activate spinal neurons to produce a variety of diverse behavioral motor patterns that allow the animal to move and survive in the environment. Motor pattern diversity is probably greatest in humans, as well as other mammals. Efforts to understand the descending neural control in mammals is complicated by the fact the descending motor control system is just one part of an extensive and highly interconnected locomotor control system. One possible way to answer the neural control problem is to look closely at the motor patterns produced by the underlying neural circuits. Anatomical and functional evidence indicates the presence of distinct spinal circuits being activated in distinct locomotor movements [Bernhardt et al. 1990; Soffe 1993; Ritter et al. 2001; Stein and Daniels-McQueen 2002]. Certain kinematic features are likely to be closely correlated to the underlying neural activity. For example, the frequency of the underlying neural oscillator and the recruitment and intensity of motoneuron firing migh be seen to increase tail beat frequency, bend amplitude and propulsive forces. Many lower vertebrates exhibit a variety of distinctive, discrete locomotive behaviors [Ayers et al. 1983; Budick and O’Malley 2000; Earhart and Stein 2000; Ijspeert 2001; Abdeljalil et al. 2005]. By looking at the different locomotor patterns, one can start to understand the neural circuits that could control such a behavior.
A lower vertebrate model with a relatively simpler anatomical network is appealing for research into the relationships between descending motor control system (DMCS) activity and behavior. Zebrafish (Danio rerio) provide a simplified and optically transparent model for understanding motor control system. The zebrafish has been used as a model in vertebrate biology because many of its characteristics make it easier to study than some other vertebrates [Vascotto et al. 1997]. The zebrafish is a common, small teleost that is easily breed and maintained. All of the development of the fish is done outside the womb in an optically clear embryo, which facilitates investigations in development and neurobiology. The approximately 300 neurons that descend into the spinal cord to control locomotor behaviors arise from the hindbrain and midbrain. Most of these neurons have been morphologically categorized based on dorsal-ventral and rostral-caudal axis location of the soma and the dendritic and axonal aborization [Kimmel et al. 1982; Kimmel et al. 1985; Metcalfe et al. 1986]. The transparency of the larval zebrafish central nervous system and the emerging developmental genetic information allows for accurate identification of neurons in vivo. Also the availability of mutant animals with locomotor deficits makes the zebrafish an attractive model to study the neural control of locomotion [Granato et al. 1996]. In fact, some studies have already been conducted on the genes that modify behaviors such as the escape response [Granato et al. 1996; Lorent et al. 2001]. Once neurons are identified they can be coupled with the known motor patterns and behaviors [Orger et al. 2008]. Larval zebrafish produce a diversity of locomotor patterns by 5 days post-fertilization, some of which include forward swimming, turning, prey capture, escape response and struggling [Fuiman and Webb 1988; Liu and Westerfield 1988; Budick and O’Malley 2000; Borla et al. 2002; Muller and van Leeuwen 2004; McElligott and O’Malley 2005]. In addition to the diversity of behaviors, each behavior has its own nuances, including aspects that can be modulated. This diversity, together with sensorimotor transformations performed, give insight into the underlying neural circuits. While such inverse analysis does not provide precise answers to the neural circuits that control locomotion, such as anatomical details of the circuits, it does provide a framework within which to analyze neural outputs.
**SPINAL NEURAL CIRCUITS OF LOCOMOTION**

Regardless of which swimming behavior is being analyzed, the answer for how behavior is generated necessitates some description of the spinal networks that activate the axial trunk muscles, which is the focus of this dissertation. Research suggests that there are 2 motor patterns and potentially central pattern generators (CPG’s) responsible for 2 different forward swimming behaviors in the larval zebrafish: one for slow swims and one for burst swims [Budick and O’Malley 2000; Thorsen et al. 2004; Masino et al. 2005]. Recent evidence has been presented both on a muscular and a neuronal level. Slow swims are characterized as mild caudal bending of the tail resulting in a slow velocity swim [Budick and O’Malley 2000; Buss and Drapeau 2001; Muller and van Leeuwen 2004]. The pectoral fins are used in alternation to assist in the forward swimming [Thorsen et al. 2004]. The slow swim is sharply contrasted to the burst swim which involves rapid, large and more rostral bends of the trunk with the pectoral fins adducted, resulting in high velocity swim with large degree of yaw [Budick and O’Malley 2000; Thorsen et al. 2004]. The slow red muscle fibers are active during sustained swimming, such as the slow swim, and are controlled by small motorneurons; while the escape response, which contains the burst swim, utilizes fast white muscle fibers controlled by large motorneurons [Myers 1985; Westerfield et al. 1986; Liu and Westerfield 1988]. On a spinal level, interneurons known as the MCOD’s are active during slow swims [Ritter et al. 2001] but are actively inhibited during the burst swim [Masino et al. 2005; McLean et al. 2007]. This evidence suggests that the two forward swimming patterns are have different underlying neural circuits. When looking at the different swim patterns, a continuum between the slow swim frequency and the burst swim frequency has not been reported. In *Clione*, there is a distinct change from slow swims to fast swims that is thought to be caused by the additional activation of interneurons resulting in recruitment and/or dropping out of motor units [Satterlie 1991]. This categorization of behaviors into slow and fast swimming provides a framework for understanding many different larval behaviors.

**LARVAL DESCENDING MOTOR CONTROL SYSTEM**

For the most part, the neural circuits for generating locomotor patterns are confined to a relatively small and evolutionary conserved part of the central nervous system, the
spinal cord. Although sensory feedback is undoubtedly important, much of the timing and activation of muscle contraction seems to be the work of these self-oscillating networks of interneurons of as little as two or three vertebral segments in the spinal cord that produce cyclic pattern of signals [Grillner 1985; Downes and Granato 2006]. These networks are controlled by a variety of factors, including the neurons from the brain that send descending axons to the spinal cord. The vertebrate descending motor control system (DMCS) is an intricate network of a vast number of neurons of varying cellular diversity that receives inputs from numerous areas of the brain including the basal ganglia, mesencephalic locomotor region (MLR), cerebral cortex and cerebellum, as well as from ascending spinal pathways and sensory modalities (visual, vestibular, lateral line).

The mesencephalic locomotor region (MLR) is a conserved region of the brain responsible for giving rise to locomotion via stimulation of the reticulospinal network [Shefchyk and Jordan 1985; Garcia-Rill and Skinner 1987; Le Ray et al. 2003; Dubuc et al. 2008; Grillner et al. 2008]. Increasing stimulation of the MLR results in increased firing of the descending motor command [Brocard and Dubuc 2003]. For instance, in salamander a low frequency stimulation results in stepping and a high frequency stimulation results in a transformation into swimming [Cabelguen et al. 2003]. In lamprey, the MLR activation of different sections of hindbrain were responsible for the initiation and maintenance of slow swim frequency. Other more caudal areas of the brainstem were recruited at high frequency of swimming [Brocard and Dubuc 2003]. They suggested a linear control by the MLR through the reticulospinal neurons such that a higher locomotor frequency could be achieved by increasing excitatory drive to the spinal CPG. This corresponds with the widespread activation of reticulospinal neurons in larval zebrafish during burst swimming [Gahtan et al. 2002].

In larval zebrafish it is thought that the descending motor control neurons include the reticulospinal pathway, which are segmentally organized neurons in the hindbrain; vestibulospinal pathway, a cluster of neurons near the ear that have ipsilateral and contralateral projecting axons; and the nucleus of the medial longitudinal fasciculus (nMLF), located in the caudal midbrain. It is believed that some of the sensory information (vision, lateral line) is funneled into the DMCS, directly or indirectly, to elicit locomotor responses [Coombs et al. 1998; Montgomery et al. 2003; Roeser and Baier 2003; Gahtan et al. 2005].
Zebrafish can provide a variety of movements which vary in speed and strength. It is thought that much of the variation in movement arises from changes in the descending drive from the brainstem to spinal cord [Lee and Eaton 1991]. From calcium imaging studies, it is known that many of the reticulospinal (RS) neurons in the brainstem are active during the escape response [Gahtan et al. 2002], a component of which is a vigorous burst swim. Many of these neurons likely overlap those used in the slow swim response due to the fact there are not many neurons left after the elimination of those neurons in the escape response. Which of the RS neurons are involved in slow swims and what exact role each of those neurons play in controlling the behavior is not known. The best known connection between circuits and behavior is the Mauthner cell (M-cell) and its segmental homologues, MiD2cm and MiD3cm [Faber et al. 1989; O'Malley et al. 1996; Svoboda and Fetcho 1996; Bhatt et al. 2007]. The M-cell has a large diameter axon that extends the entire length of the spinal cord making numerous connections on motorneurons and spinal interneurons and is therefore well suited for its role in the escape response [Fetcho and Faber 1988; Bhatt et al. 2007]. Upon laser ablating of the M-cell and its homologues, Liu and Fetcho [1999] found an increased escape latency elicited by both head and tail taps. However, if just the M-cell was ablated, there was only an increased in latency on tail taps, but not to head taps [Liu and Fetcho 1999]. This demonstrates that it is possible for one cell to initiate distinct components of a behavior. Other ablations of the neurons in the brainstem have shown analogous results. Upon ablation of the nMLF, parts of prey capture were disrupted [Gahtan et al. 2005], although this study failed to show whether it was the whole behavior that was lost or just some alteration of the kinematics. Research on other brainstem neurons, ventromedial neurons in the hindbrain, showed a disruption in turning in response to a turning-evoked stimuli, the optomotor response [Orger et al. 2008].

**BEHAVIORAL DIVERSITY EMERGING FROM CONSERVED LOCOMOTOR CONTROL**

Most fish swim by rhythmic contraction of muscles which results in an exertion of force on water around them which propels the fish forward. This locomotion can be accomplished in two major ways: (1) Body and/or caudal fin locomotion which is characterized by undulatory bending of axial portion of the body that produces a propulsive wave or (2) by median and/or paired fin locomotion [Webb 1994]. Larval zebrafish are
considered to use the subcarangiform mode of swimming, in which there is a side-to-side amplitude wave that is smaller anteriorly, in combination with a labriform mode of swimming which is achieved by oscillatory movements of the pectoral fins [Thorsen et al. 2004]. The propulsive wave is powered by muscles and the differences in kinematics may provide useful information into the underlying neural controls.

Larval zebrafish have an extensive repertoire of locomotor patterns that include routine and escape turns, and forward swims. More complex locomotor patterns include the prey capture swim, which appears to be more computationally demanding due to the dynamic modulation of in tail-beat frequency and more complex trunk bending patterns. This dissertation concerns the forward swimming patterns and the variety of sensory modalities that elicit the slow forward swimming motor pattern. The forward swim patterns can be classified into either burst or slow due to the distinct differences, including speed, yaw and bend pattern of the tail.

BURST SWIM: During the burst swim the larval zebrafish can obtain speeds greater than 100 mm/s with a more rostral high-angular bend of tail and considerable degree of yaw. The burst swim in fish can be reliably elicited as part of the C-start escape response which is stimulated by taps on the head or tail [Foreman and Eaton 1993; O'Malley et al. 1996; Liu and Fetcho 1999; Eaton et al. 2001], by auditory stimuli [Zottoli 1977]or visual stimuli [Zottoli et al. 1987; Guthrie and Banks 1990; Preuss et al. 2006]. In adult zebrafish the C-start escape response was elicited by a looming object (an object that grows in size) [Dill 1974], which can be replicated by a passing shadow [Kimmel et al. 1974] or one black segment on a rotating drum [Li and Dowling 1997].

SLOW SWIM: In contrast, the slow swim in freely moving larvae are differentiated by their mild caudal bends of the tail at low frequency (25-40Hz) and little yaw [Budick and O’Malley 2000; Muller and van Leeuwen 2004]. The quiet period following this brief swim might be followed by another swim bout. This dissertation will show that slow swims can be seen in a variety of behavioral contexts. The spontaneous slow swim is a locomotor pattern that occurs due to no overt stimulus. This spontaneous slow swim has been speculated to be kinematically similar to that seen during visual responses of prey tracking [McElligott and O’Malley 2005]. During prey tracking the larval zebrafish uses a series of J-turns and slow swims to align and bring itself closer to the prey. The prey tracking slow swims are a part of
a sequence of 3 to 5 maneuvers that generally cumulate in a capture swim, whereas the spontaneous slow swims are more isolated bouts.

Although certain behavioral analysis of slow swim bouts used during prey tracking have been described [McElligott and O’Malley 2005] and compared to the spontaneous slow swims, no detailed kinematic evaluation has been done on the type of rhythmic pattern used by freely moving larvae in response to whole-field illumination or in response to moving stripes, an optomotor eliciting stimulus. Furthermore, there has been no systematic comparison of the various slow swim bouts. This dissertation will compare the slow swim behaviors seen in response to different sensory modalities and draw a conclusion about the conservation of motor patterns in larval zebrafish.

**NEURONS TO BEHAVIOR**

Although the morphology of descending neurons in larval zebrafish hindbrain has been well documented [Kimmel et al. 1980; Kimmel et al. 1982; Metcalfe et al. 1986; Gahtan and O'Malley 2001; Gahtan and O’Malley 2003] and some of the distinct locomotor gait patterns have been described, little is understood about relation between the neurons and the behaviors. What is known comes from research in a variety of animals such as lampreys, fish and mammals [Faber et al. 1989; Koch et al. 1992; Deliagina et al. 2000; Gahtan et al. 2002; Dubuc et al. 2008]. Motor neurons can contribute to a motor behavior over a broad range of speeds and strength by either being recruited from a quiescent state [Masino et al. 2005] or by increasing in firing rate [Henneman 1957; Cope and Sokoloff 1999].

The C-start escape response, which utilizes the burst swim, has been the target of much research in teleosts in order to understand the neural circuits that produce behaviors [Korn and Faber 1996; O'Malley et al. 1996; Liu and Fetcho 1999]. The escape response is mediated by a pair of commissural hindbrain neurons known as the Mauthner cells (M-cell) [Zottoli 1977; Eaton et al. 1981], although the M-cell activity is not required for an escape response to occur, as demonstrated after ablation of the cell [Eaton et al. 1982]. However, escape response without the M-cell firing are rare [Eaton and Bombardieri 1978]. When one M-cell fires it inhibits the other cell from firing [Hackett and Faber 1983] and elicits a contralateral contraction of muscles [Jayne and Lauder 1993] that results in a high-velocity, high angle turn away from the stimuli [Eaton and Emberley 1991]. The M-cell is responsible for the short latency periods [Liu and Fetcho 1999] and is sufficient to initiate the initial C-
bend [Nissanov et al. 1990]. Most of the synapses of the M-cell are on motor neurons and some interneurons, such as the commissural ipsilateral descending neurons (CiD) [Faber et al. 1989; Hale et al. 2001]. CiD have been shown to be active during escape response and high tailbeat frequencies with synaptic connections to primary motorneurons [Fan and Hale 2005; Kimura et al. 2006]. The increased firing of the CiD leads to an increased firing rate in motor neurons and a stronger escape response [Bhatt et al. 2007]. This finding of non-recruitment of spinal interneurons contradicted previous research in which hindbrain Mauthner homologues neurons were recruited during a stronger escape response [O'Malley et al. 1996; Liu and Fetcho 1999]. However, this conflict might be resolved by the possibility that the hindbrain neurons that were recruited just increased the firing level of the already active interneurons [Bhatt et al. 2007], rather than the previous idea of recruitment of inactive neurons. It appears that many of the reticulospinal neurons in larval zebrafish are active during the escape response [Gahtan et al. 2002].

The understanding of the neural circuits that control the escape response, in part, was due to the strong understanding of the kinematics of the escape response. Little research has been done on the slow swim patterns, even though the slow swim seems to be the most diversely used pattern; which is the aim of the this dissertation.

AIMS OF DISSERTATION

The goal of this dissertation is to examine the possibility of conservation of slow swim motor pattern in larval zebrafish. By examining the kinematics of the motor response to different sensory modalities, this dissertation aims to determine whether the same motor pattern and therefore possibly the same neural circuit is responsible for a behavior that can be elicited by completely different sensory modalities. First, this dissertation will look at two different visually evoked behaviors. Chapter 3 will explore the detailed kinematics of the optomotor response (OMR), locomotion elicited by whole field visual motion. Chapter 4 will look at a different visuomotor transformation not previously described before, axial movements in response to changes in whole field illumination. Chapter 5 will look at the effects of flow environment and swim velocity on the slow swim pattern and look to see if it is possible to elicit a burst swim from the same stimuli that elicits a slow swim pattern. In addition, this dissertation will examine the relationships between the visual and mechanical stimuli used with the aim of better understanding the sensorimotor transformations
performed in the larval brain. Finally, this dissertation will propose a common theme, one of muscle synergies, as a model for sensorimotor integration.
CHAPTER 2

GENERAL METHODS
A. **Animals:** Fertilized eggs are collected from adult zebrafish (Danio rerio) breeding facility and kept in a 28°C incubator solution of Instant Ocean (Aquarium Systems, Mentor OH; 0.12 g/l) [Westerfield 1995]. The larvae were kept on a 14/10 light/dark cycle and fed paramecium starting at 5 days post-fertilization (dpf). These conditions did not change during the time of behavioral analysis. Larvae between 5-10 days post fertilization were used in all experiments, an age in which zebrafish larvae have a relatively fixed locomotor repertoire [O’Malley et al., 2004] and good visual acuity [Bilotta, 2000].

B. **Recording of behaviors:** All recordings were done using the same conditions as described in Budick and O’Malley (2000). In short, larvae were placed in a small plastic petri dish filled with 10% Hank’s solution to a 2mm depth. The initial water temperature was 28°C (from the incubator). Although water temperature has been shown to drop 2°C during testing, this did not show a significant different in the behaviors collected [Budick and O’Malley 2000]. The dish will then be placed under a dissecting microscope that is connected to a MD4256 high-speed digital camera (EG&G Reticon, Sunnyvale, CA, USA) that stores images at 400-1000 frames s⁻¹ onto a PC. The images were analyzed at a later time using Image J (NIH) and Matlab Software (Mathworks).

C. **Analysis of Behavior:** All behaviors (incl. OMR, LES, rheotaxis, labeled-lesion) were analyzed either using NIH Image J, Matlab or by hand. The variables that were analyzed are described below and are comparable to those described in Budick and O’Malley [2000] and Tytell [2004]. Using a Matlab program, 16 points down the dorsal midline of the fish were marked with the first point was at the tip of the snout, the second point was at the rostral end of the swim bladder, the 3rd point at the caudal end of the swim bladder and the 16th point was at the tip of the tail. Points 4 through 15 were evenly placed online the dorsal midline of the body. A matlab program was created to create a cubic smoothing spline with the 16 points for each of the frames that were digitized. From this spline the following calculations were completed in a matlab based program. Unless otherwise stated as instantaneous, all calculations are expressed as an average over one bout of swimming and lengths were normalized to body length of the larval zebrafish.
(i) **Mean Tail Beat Frequency (TBF)** is the reciprocal of the amount of time it takes for the tip of the tail to complete a cycle of movement i.e. from the left to the right and back to the left.

(ii) **Mean Bend Angle** is the average of the maximal bends of the tail that occurs during a bout of swimming. This was calculated by drawing a line tangent to the tip of the tail and a line tangent to the midline of the fish at the swim bladder. The angle created by the two lines is the head-tail angle. This angle was then subtracted from 180° thereby allowing for small bend angles to represent little movement of the tail.

(iii) **Bend location** is the site along the midline of fish that corresponds to the maximal bend point. This was calculated by determining the closest point on the midline of the fish to the vertex of the head-tail angle. This point was expressed in fraction of length of the fish from the head so that the higher fraction would be a caudal bend location.

(iv) **Mean maximum yaw** is described as the angle between the direction the fish is traveling, mean path of motion, and the direction the head is pointing at each maximal bend angle. The mean path of motion was calculated as the regression line through the 3rd and 4th points (the center of mass) throughout the entire swim bout. The direction the head was pointing was calculated by a line created between points 1 and 2. The angle that results from the mean path of motion and head angle is the yaw.

(v) **Swim velocity** was recorded by dividing the distance traveled by the center of mass (point 3) between each frame digitized and the duration between the frames. Swim velocity and distance was normalized to the body length of the fish, the distance from point 1 (snout) to point 16 (tip of the tail). The relative swim velocity was calculated by adding the flow speed of the water to the average swim velocity for the fish during the entire individual bout.

(vi) **Rest periods and bout duration** was calculated for a series of swim bouts responses by using slow video play back. The time between the last visible movement of one swim bout and the first visible movement of the next swim bout was recorded as the rest period. The bout duration was recorded from the time of first visible movement to the end of bout.
(vii) **Stride length** is the distance traveled forward by the larva during one tail beat cycle. This was calculated by dividing the instantaneous swim velocity by the instantaneous TBF. The **relative stride length** was calculated by using the relative swim velocity divided by the mean of the instantaneous TBF.

(viii) **Wave velocity** is the speed at which the propagating wave travels down the length of the larva. Following the description by Shadwick and Gemballe [2006], successive midlines along the horizontal axis were superimposed and the distance between peaks of lateral displacement of the midline were measured in fraction of body length. This measurement was divided by the progression time to obtain wave velocity. **Wave length**, the length the propulsive wave of contraction travels down the body during steady swimming, was calculated by dividing wave velocity by instantaneous TBF.

(x) **Slip** of the larva’s body in water is the ratio of swimming velocity to body wave velocity. As slip approaches 1, the swimming is more efficient as expressed by propulsive or Froude efficiency ratio.
CHAPTER 3

OPTOMOTOR BEHAVIOR
INTRODUCTION

The optomotor response (OMR) is a visually-triggered locomotor or orienting behavior of apparent ethological significance, given its widespread use by both vertebrate [Pronych et al. 1996; Abdeljalil et al. 2005; Douglas et al. 2005]; and invertebrate animals [Srinivasan et al. 1999; Dürr and Ebeling 2005; Zordan et al. 2005; Glantz and Schroeter 2006]. In fishes the OMR is a navigational behavior that can be readily elicited by moving a pattern of stripes within their visual surround [Shaw and Sherman 1971; Springer et al. 1977; Krauss and Neumeyer 2003; Neuhauss 2003]. This behavior appears to be conserved in diverse teleost fishes and amphibians [Cronly-Dillon and Muntz 1965; Townes-Anderson et al. 1998; Shirakashi and Goater 2002; Kroger et al. 2003], and in teleost species appears to be driven by pretectal nuclei that sit in caudal forebrain in close proximity to the optic tectum [Roeser and Baier 2003]. Pretectal nuclei are also involved in negative phototaxis [Ullen et al. 1997]. During the OMR behavior, fish often attempt to keep pace with their visual surround, which may help them to hold station and avoid being swept into some new and potentially less-suitable environment [Cronly-Dillon and Muntz 1965; Shaw and Sherman 1971; Rock and Smith 1986; Maaswinkel and Li 2003]. The OMR may also contribute to other behaviors such as schooling and might additionally help fish to become less obvious to predators by decreasing their movement relative to the visual surround.

In laboratory settings, the OMR is a swimming motor pattern that will e.g. lead fish to accumulate at the end of a narrow channel, based on the direction of stripe motion [Roeser and Baier 2003; Fleisch and Neuhauss 2006]. Optomotor behaviors have been useful in genetic screens of zebrafish to identify mutations affecting neural development and the functional organization of visuomotor and related neural systems [Brockerhoff et al. 1998; Neuhauss et al. 1999; Maaswinkel and Li 2003; Neuhauss 2003; Guo 2004; Muto et al. 2005]. The zebrafish OMR has been described with regard to visual acuity [Maaswinkel and Li 2003; Neumeyer 2003], and in regards to specific kinds of motion and visual features that will elicit it [Schaerer and Neumeyer 1996; Orger et al. 2000; Krauss and Neumeyer 2003; Orger and Baier 2005]. It should be noted, however, that the OMR is just one of a variety of teleost visual behaviors [Gahtan and Baier 2004; Kitschmann and Neumeyer 2005; Canfield 2006; Fleisch and Neuhauss 2006]; and that it may function independently of the largest primary visual structure in fishes, the optic tectum [Roeser and Baier 2003].
Nonetheless, as a widely-used and potentially conserved behavior, it would be of interest to better understand the OMR’s nature and its underlying neural controls.

The relationship between the zebrafish OMR and other visuomotor behaviors is of interest in regards to understanding visuomotor transformations and the underlying locomotor-control circuitry. But in the case of the larval zebrafish OMR, we lacked information on the specific locomotor maneuvers being used—we only knew the end result of the response: orientation and movement along the stripe-motion axis. Previous OMR studies had not utilized high-speed kinematic analyses, even though the slowest larval behaviors require high-speed imaging to obtain defining kinematic details [Budick and O’Malley 2000]. The aim of this study is analyze the OMR behavior in context of other well known motor patterns.

METHODS

The experimental protocol has been described previously (see Overall methods) and therefore will be described here briefly to show differences in the methods. Behavioral analysis was performed on larva 6-9 dpf. Larva of this age have a relatively fixed locomotor repertoire [O’Malley et al. 2004], good visual acuity [Bilotta 2000] and previous OMR experiment show a positive response to the OMR stimulus at this age [Orger et al. 2000]. Small 14 mm square wells were made in 3mm deep agar in order to observe the locomotor behaviors. The wells were filled with Instant Ocean to a depth of approximately 3 mm and one larva at a time was placed into the well. The Petri dish containing the well was then suspended on a clear plexiglass sheet. To elicit optomotor responses, printed patterns of black and white stripes were moved directly under the plexiglass sheet. This allowed the camera mounted above to capture both the larval behaviors and the stripe motion. The OMR can be elicited by either a rotating drum surrounding the larva [Cronly-Dillon and Muntz 1965; Clark 1981; Bilotta 2000; Krauss and Neumeyer 2003; Maaswinkel and Li 2003; Fleisch and Neuhauss 2006] or by the linear motion of a stripe pattern which may be placed under the animal [Neuhauss et al. 1999; Orger et al. 2000; Kroger et al. 2003; Orger and Baier 2005]. For our purposes, linear stripe motion allowed easy recording of both stripe and larval motion and also facilitated the analysis of OMR precision. The dish was illuminated using a fiber-optic light guide (Fiber-Lite, Dolan-Jenner, Lawrence MA). The locomotor behaviors were recorded at a rate of 600 frames s⁻¹ using a high-speed, MD4256
digital camera (EG&G Reticon, Sunnyvale, CA), mounted on a Zeiss dissecting microscope. All experiments were done at room temperature (22-24°C). Movie sequences, collected in a single recording session of approximately 2 hours, were saved on a hard drive and then converted into AVI files for subsequent analysis. Each larva was allowed to acclimate to the arena for 5 minutes, with a stationary stripe pattern sitting underneath. Subsequently, during periods of relative quiescence, the stripe pattern was moved in a single direction at a uniform speed and the ensuing behaviors recorded. Each high contrast stripe was ~0.5 mm thick and the alternating black and white pattern extended well beyond the recording arena (see pattern in Figure 3.1A). Recordings of either individual OMR movement bouts or a series of movement bouts were saved to disk for analysis. Each larva was tested a minimum of 5 times. We used a spatial frequency of 0.06 cycles degrees\(^{-1}\), which was found in previous work to elicit a robust response in larval zebrafish [Bilotta 2000]. The speed of stripe motion was varied randomly over a series of trials to assess the effect of motion speed on the OMR behavior. We used a range of stripe speeds over which the OMR response was found to be readily elicited [Maaswinkel and Li 2003]. Being able to accurately record the printed stripe motion along with the larval behavior allowed for precise calculation of stripe speed and larva’s response to all directions of stripe motion.

During the same recording session, other behaviors were recorded from the larval zebrafish. These behaviors included a slow swim, escape response and routine turns. These recordings were used to compare and accurately categorize the parts of the OMR behavior.

Behavioral Analysis

As described earlier, the recordings of the desired behaviors were analyzed using IMAGE J (NIH). Some of the variables describe previously that were used included bend location, bend amplitude, yaw and tail beat frequency (TBF). Average swim velocity was calculated over the forward swimming portion of the OMR behavior. To further analyze the optomotor behavior several angular measurements were performed. These included:

(i) **Angular velocity**: the change in orientation of the larva during a turn was divided by the duration from the start of the turn to maximal bend of the turn. The angle of the bend was measured at maximal bend and then divided by the duration.
(ii) **Offset angle**: the angle between the larva and the axis of stripe motion (figure 3.1B). First a vector was drawn parallel to the direction of motion of the stripes. The second vector was a line from the rostral midpoint of the swim bladder to the middle of the tip of the snout. The angle between the 2 vectors is the offset angle. Thus, a larva aligned with the stripe motion axis would have an offset angle of 0°. This angle was measured prior to the start of the larva’s behavior, **initial offset angle**, after completion of the turn, and at the end of the behavior, **final offset angle**. Angular measurements were done using the images magnified in ImageJ (NIH).
RESULTS

High speed imaging of larval responses to OMR-eliciting stimuli (moving stripes) revealed consistent patterns of locomotor maneuvers that caused the larvae to orient towards and swim in the direction of stripe motion. Figure 3.1A illustrates a representative OMR of a 7-day old larva. Shortly after the onset of stripe motion, the larva turns towards the axis of motion (arrow) with a single large bend and continues swimming forward using a mild undulatory swim pattern. The OMR behavior is comprised of multiple, discrete movement bouts similar to the one shown in Figure 3.1A; they are separated by distinct pauses. Figure 3.2 illustrates the extended OMR behavior by plotting both larval velocity and larval orientation over the full duration of an OMR recording. Note that the gaps in the velocity traces correspond to pauses where there were no trunk movements. The top two traces show an example OMR with an initial orienting turn towards the stripe motion axis, followed by further movement bouts that tend to roughly track the direction of stripe motion. Although in this first example subsequent movement bouts take the larva “off axis”, the net result of the subsequent movements, and the OMR behavior more generally, is to track the direction of stripe motion. This is indicated in the second example where two successive turns result in good alignment to the motion axis (see Turning Component below). The OMR is thus a complex behavior in which visual computations are able to generate a series of locomotor bouts that combine purposeful turning and swimming components. The locomotor components of the OMR have strong similarities to two spontaneous larval behaviors: the routine turn and the slow swim (Figure 3.3A, B). These spontaneous behaviors were previously defined using high-speed video and kinematic analysis [Budick and O’Malley, 2000]. While the locomotor components of the OMR behavior (Fig. 3.1A) appeared similar to these spontaneous behaviors, in terms of such features as yaw and angular velocity, we wanted to make a quantitative comparison of the kinematic variables that relate to the underlying neural controls. These comparisons are shown in the next several sections.

Forward Swimming Component

The forward swimming component of the OMR consists of rhythmic, caudal bending of the trunk, low tail-beat frequency and slight yaw, reminiscent of previously described slow swim bouts that last for just a few cycles of swimming. This swim pattern is starkly different from the larval burst swimming pattern, which we shown here in the context of a
larval escape behavior (Fig. 3.3C). The initial turning (C-bend) component of the escape behavior is maximal in frame #2 and is followed by a burst swim bout that is characterized by great yaw, marked bending of the trunk and vigorous forward swimming. The larva’s rapid progress can be appreciated by comparing the difference traveled in the OMR swim (Fig. 3.1A, last 4 frames of row #1) vs. during the burst swim (Fig. 3.3C, last 4 frames). The OMR swimming component is thus quite different from the burst swim pattern and is more like the spontaneously observed slow swim pattern [Budick and O’Malley 2000] which also occurs during prey tracking [McElligott and O’Malley 2005] and in response to a whole-field illumination increase [O’Malley et al. 2004; Burgess and Granato 2007].

The kinematic details of the forward swimming component of the OMR are summarized in Figure 3.4 which shows a low swim velocity, minimal yaw and low tail-beat frequencies (TBFs). Comparing the OMR kinematics with previously measured burst and slow swim parameters from the same age larvae (Fig. 3.5), we see that the slow and OMR swims share similar velocity, yaw and TBF parameters and that they differ markedly (and statistically) from the burst swimming parameters (see figure legend). This is also true for bend location, but the bend amplitudes of the OMR swims are noticeably and statistically larger than the slow swim bend amplitudes and are in fact quite similar to those of the burst swim (Fig. 3.5D). Curiously, this larger OMR bend amplitude does not result in a burst-swim like velocity. This is likely due to the very low OMR TBF—which is much lower than the burst swim TBF and in fact slightly, but statistically, lower than the slow-swim TBF (Fig. 3.5E). In addition, because the bend location of the OMR is quite caudal, like the slow swim, there is less bending of the trunk as a whole. Compare for example the OMR bend at the end of row 2 in Figure 3.1A and the burst swim bend in the second last frame of Figure 3.3C. The net result is that the OMR swim looks much like a slow swim and has similar parameters that result in a similar velocity.

We also evaluated the effect of stimulus speed on the swimming behavior by varying the stripe movement speed over a range of values. The swim kinematics appeared very similar throughout the range of stripe speeds tested ranging from roughly 5 to 50 mm/sec. Plots of the kinematic variables for 50 trials from 12 larvae showed that tail-beat frequency, velocity, bend amplitude and bend location did not change significantly over the range of stripe speeds tested (Fig. 3.6). At the low end of stripe speeds, larvae swam faster than the stripes, while at the fastest speeds (>20 mm/sec) they could not keep up with the stripes.
Kinematic parameters that could potentially increase velocity, such as tail-beat frequency and bend amplitude, did not change. Because these parameters depend upon the spinal oscillator frequency and the locus of descending excitation, this indicates that the change in visual stimulus speed did not materially affect the operation of the locomotor networks, although an increased frequency of bout triggering, which might occur under other conditions, could allow larvae to better maintain position in their environment. Although we did not see such a change in bout frequency within the time duration of our recordings, further experiments are needed to determine if larvae might increase their overall rate of forward motion by e.g. increasing the frequency of forward swim bouts. In any case, the similarity of the swim pattern at all stripe speeds demonstrates that these larvae rely on a unitary, slow-swim pattern for their optomotor response over a broad range of stimulus speeds.

**Usage of Pectoral Fins**

A further feature of the OMR swim pattern concerns utilization of the pectoral fins (Figure 3.7). During the initial turn there is bilateral extension of the pectoral fins (circled in the 3rd frame), but subsequently the fins are used in an alternating pattern. The alternating pattern continues throughout the forward swim and is consistent with the fin movement pattern used during the larval slow swim pattern or gait [Thorsen et al. 2004]—further supporting the idea that the OMR forward movement component is a slow swim. These fin movements were consistently observed in recordings where the pectoral fins were visible. Visibility depends upon nuances of the lighting conditions and so fin movements are difficult to see at low magnification in still images. But at higher magnification (Fig. 3.7B, from frames in Fig. 3.7A with circled fins), one can see both the bilateral fin extension (first frame) and the alternating pattern (next two frames). The use of pectoral fins for holding station has previously been described for fishes in pelagic and benthic environments [Wilga and Lauder 2001]. Conjugate fin extension was previously observed late in the larval zebrafish capture swim bout during a braking maneuver [Borla et al. 2002]. The conjugate fin movement we see during the OMR turn is distinct from other examples of pectoral-fin usage such as the tucking of fins against the trunk during the escape C-bend [Thorsen et al. 2004]. In examining our current routine turn data, we have now noted an initial bilateral
extension of the fins (data not shown)—indicating a parallel between the OMR and routine-
turn behaviors.

Turning Component of the OMR Behavior

Perhaps the most important aspect of the OMR as a navigational behavior is that the
larva must perform a visual computation in order to orient to the direction of stripe motion.
**Figure 3.8** illustrates that the OMR is indeed a directionally selective behavior: four OMR
responses are shown in which the larva turns towards the axis of stripe motion, which is to
the right in these examples. Turns in the “correct” direction were in fact made in 52 of the
60 turns analyzed. Note that in each case the larva uses a single
large bend to turn and then continues forward in this new direction using the mild
undulatory swim pattern described above. We next analyzed the magnitude of the turns
made by larvae exhibiting OMR responses. **Figure 3.9** shows the change in orientation for
32 turns and it should be noted that a very large majority of the turn angles are less than 90
degrees (95% of the turns analyzed). No turns greater than 180 degrees was observed. This
indicates that with the OMR, larvae do not make very large angle turns—in contrast to e.g.
the larval escape behavior in which turns of 90 degrees or larger are often observed. That
most turns are less than 90 degrees indicates that larvae use the OMR to make only a limited
course correction.

The observation that larvae make 90 degree-or-less OMR turns, and that these turns
are almost always towards the direction of stripe motion, has an important behavioral
consequence: it means that larvae are responding only to visual stimuli that indicate that
they are being “swept backwards” relative to their visual surround. They do not respond to
stimuli indicating that they are being “swept forwards” (i.e. to backwards stripe motion),
either by turning towards their “forward motion axis” or by making large angle turns to
orient themselves into this visually-suggested “current”. To assess the precision of the
visuomotor computations that they do make, we plotted the angular size of the “correct-
direction” turns against their initial angular offset from the stripe-motion axis (**Fig. 3.10A**).
At the largest offset angles (around +/- 90 degrees; absolute values are shown), the initial
turn angle of the OMR is maximized. The bulk of the orientation change is accomplished by
a single large bend (in contrast to the more gradual turns used e.g. during larval prey-
tracking). While the initial turn roughly accomplishes the task of orienting towards the
stripe-motion axis, it does not accomplish this with much precision: after the initial response there is a sizable residual offset (Fig. 3.10B). Indeed, even after a series of turns, these larvae are not precisely oriented with the motion axis. The turning component of subsequent OMR movement bouts can actually take larvae off-axis (Fig. 3.2), but the overall outcome of the behavior is a roughly correct direction of swimming along the stripe-motion axis. These results indicate that this behavior relies on a relatively coarse visuomotor control system and further suggests that the OMR may have an obligatory turning component, since turns occur even when larvae are closely aligned with the stripe motion axis.

The initial turning component of the OMR appears similar to a routine turn (Fig. 3.3A) that occurs spontaneously in this age larvae [Budick and O’Malley 2000]. The spontaneous routine turn is characterized by a substantial, single bend that encompasses much of the trunk and is followed by a mild to negligible slow-swim pattern. The turning component of the OMR (Figs. 3.1 and 3.8) has these same features. The OMR turn is decidedly unlike the initial turn of the C-start escape behavior (Fig. 3.3C). OMR turns are also unlike the J-turns used in larval prey-tracking [McElligott and O’Malley 2005]. To compare OMR turns with routine and escape turns, we focused on turn parameters that relate to the underlying neural controls (Fig. 3.11). In comparison to earlier measurements of routine and escape turns, we find that for OMR turns their turn angles, bend durations and counter-bend angles closely overlap the corresponding routine-turn measurements but are strikingly different from values for the escape turns (Fig. 3.11A, B, C). In the case of angular velocity, the OMR turn velocities are so small that they are found at the extreme opposite end of the spectrum from the escape-turn angular velocities (Fig. 3.11D). In conjunction with the fact that a routine-turn pattern of pectoral fin usage is exhibited during the OMR, this establishes that the larval OMR uses a routine-turn like locomotor maneuver.
DISCUSSION

The high-speed recordings provided above reveal the locomotor maneuvers used by larval zebrafish as they respond to a specific OMR-eliciting stimulus, namely a moving stripe pattern. While larvae of this age exhibit a surprising diversity of turning and swimming patterns [Kimmel et al. 1974; Budick and O’Malley 2000; Borla et al. 2002; Thorsen et al. 2004; McElligott and O’Malley 2005; Burgess and Granato 2007], the larval OMR behavior is created from just two locomotor patterns or maneuvers: routine turns and slow swims. The turning aspect of the behavior has direct implications regarding the visuomotor transformations being performed. In response to specific visual inputs, zebrafish are able to compute motor trajectories that enable them to compensate (within limits) for the optic flow of their environment. These transformations are implemented via descending motor pathways that engage specific spinal central pattern generators (CPGs), as best evidenced by the forward slow-swimming pattern. We consider each of these processes and conclude with a discussion of the possible conservation of neural circuitry underlying this widely used visuomotor transformation.

Engagement of Spinal Central Pattern Generators

The OMR is comprised of a routine turn followed by a rhythmic forward-swimming bout that falls in the slow swim category. The forward swimming component is most distinct from the larval burst swimming pattern, which is primarily associated with the C-start escape behavior [Eaton et al. 2001; Gahtan et al. 2002]. Instead, the OMR swim velocity, tail-beat frequency and bend location are all similar to the values typical of larval slow-swim patterns. The previous parsing of both swimming and turning behaviors into slow and fast clusters, had previously led us to suggest that the larval spinal cord possesses two functionally distinct CPGs: for slow and fast behaviors respectively [Budick and O’Malley 2000]. Growing evidence in support of a 2-CPG spinal architecture includes the differential activation of red and white muscle during different swimming patterns [Buss and Drapeau 2002] and the de-recruitment of an inhibitory interneuron when larvae transition from the slow to burst swim pattern [Masino and Fetcho 2005]. In addition, the coordination of pectoral fin usage with the slow swim pattern has led to the slow swim being characterized as a distinct locomotor gait, with a different neural basis than the burst swim pattern [Thorsen et al. 2004]. All evidence provided herein on the OMR behavior, including
the usage of pectoral fins, the slow swim pattern and the use of a “slow” turning pattern fit with the suggestion that the OMR relies on a slow muscle system and slow spinal CPG.

This suggestion accords with the idea that the OMR is a “more routine” type of behavior that relies on the slower red muscle system and its underlying CPG. The relatively mild and caudal activation of presumably red muscle by a specific population of motorneurons [Liu and Westerfield 1988; Buss and Drapeau 2002] and the low angular velocity routine-turn component of the OMR, should involve much less energy consumption than the powerful C-start escape turn and burst swim. This may help explain a curious observation, which is that the OMR swim velocity did not change over a 10-fold change in stripe velocity. The larval swimming pattern was faster than the lowest stripe speeds, but not fast enough to keep up with the highest stripe speeds. Larval zebrafish are clearly capable of swimming at much faster speeds than they exhibit in the OMR (as documented in Figure 3.3C), but they do not do so in response to this stimulus. One interpretation is that burst swimming is reserved for more acutely life-or-death situations such as avoiding a predator. Adult fishes, including zebrafish, are better able to keep up with an OMR stimulus (see e.g.[Maaswinkel and Li 2003]), but the energy cost to larval zebrafish may be excessive. The net result is that zebrafish larvae use the OMR primarily as an orienting and crude navigational response. This said, we should note that these larvae are not experiencing actual current flows along their lateral line, nor are they experiencing the angular velocities (and vestibular sensations) that their being rotated in a current would generate. Absent these additional stimuli, we have to keep open the possibility that these larvae may exhibit only a limited response that does not fully reflect the behaviors that would be generated by optic flow in the context of a realistic hydrodynamic environment. While not seen in our experiments, other labs do show faster net larval movements for certain OMR stimuli, that appear to be a result of a higher frequency of OMR swim bouts like those seen here (Engert lab unpublished data).

**Visuomotor Transformation**

The turning component of the OMR is distinguished as a routine turn based on its form and angular velocity: these fall squarely within the “spontaneous routine turn” category described in 2000 [Budick and O’Malley 2000]. A similar kind of turn may also occur in response to a whole-field illumination increase [described in chapter 4 of this thesis and
Burgess and Granato 2007]. OMR turns are most unlike escape turns given the high angular velocities and large counter-bends that characterize escape turns. Previously, we could only speculate about the ethological salience of routine turns, but their use in the OMR behavior indicates at least one functional role and also raises the possibility that in those earlier experiments some of the prior “spontaneous” routine turns may have been triggered by environmental cues not apparent to us (although they were conducted in a generally still visual environment). While the kinematics of the spontaneous routine turns are conserved in the OMR response, these are two distinct behaviors. Spontaneous routine turns were observed quite infrequently, whereas the onset of stripe motion elicits the OMR behavior in a relatively short period of time and multiple turn/forward swimming bouts occur in relatively rapid succession. In addition, routine turns can occur in complete darkness, as revealed by IR imaging of spontaneous behaviors [McElligott and O’Malley 2005].

In regards to the visual computations, 3 features are evident. First the behavior is directional in that a large majority of turns (87%) were made towards the direction of stripe motion. Secondly, the behavior occurs selectively in response to stimuli that would produce a perceived slippage or backwards motion. Thirdly, the orientation correction is relatively coarse grained: they do not precisely align their direction to the motion axis on the first turn or on subsequent turns. An obvious candidate for providing directional information to the larval brain is the direction-selective retinal ganglion cell which appears to be present in all vertebrate animals [Chiao and Masland 2003; Fried and Masland 2007]. When the larva’s orientation is perpendicular to the stripe-motion axis, this will most strongly activate ganglion cells with a preferred direction along the medial-lateral axis. As this produces the largest observed turns, a reasonable conjecture is that these visual signals are conveyed to visual centers in the brain that ultimately generate an asymmetric motor command at the level of spinal cord. Interestingly, at offset angles greater than 90 degrees we see relatively few OMR responses which means that directional signals showing rearward movement of the stripes (or apparent forward movement of the larvae) may inhibit the generation of OMR turns. There are other sensory processes that may normally contribute to the OMR including hydrodynamic signals from the anterior neuromasts and lateral line [Voigt et al. 2000; Pichon and Ghysen 2004; Chagnaud et al. 2007] as well as visual signaling of second-order motion [Orger et al. 2000] possibly via Y-type retinal ganglion cells [Demb et al. 2001], but
the basic direction-selective mechanisms common to vertebrate animals appear to provide visual information sufficient to drive the observed optomotor responses.

While the OMR is a robust response in terms of prompt initiation of the behavior and general direction of motion, it is not nearly so precise as e.g. the saccadic eye movements of mammals [Hu et al. 2007], nor is it as precise as the larval zebrafish prey-tracking behavior [McElligott and O’Malley 2005]. This raises questions of the spatial precision of the visual centers that are performing such visuomotor transformations in the larval zebrafish. The largest and perhaps highest-precision central visual structure in non-mammalian vertebrates is the optic tectum. The tectum appears to play a major role in prey tracking and capture behaviors, as indicated in studies of zebrafish [Gahtan et al. 2005], goldfish [Herrero et al. 1998], salamander [Dicke and Roth 1994], toads [Tsai and Ewert 1988], frogs [Marin et al. 1997] and the pallid bat [Fuzessery et al. 1993]. Interestingly, the OMR behavior is not substantially impaired by photoablation of the retinal inputs to the optic tectum in zebrafish [Roeser and Baier 2003] and a related behavior, negative phototaxis, is similarly unaffected by lamprey tectal lesions[Ullen et al. 1997]. In the lamprey study it was shown that a more rostral pretectal nucleus was necessary for the phototactic behavior. Thus potentially conserved diencephalic regions may mediate visuo-locomotor behaviors in a broad swath of the anamniotic vertebrates. The findings of a “coarser” turn control system for OMR contrasts with the larva’s prey-tracking system, wherein sophisticated, visually-guided J-turns are used to track objects with “paramecium-level” resolution [McElligott and O’Malley 2005]; this behavior is impaired by optic tectum lesions[Gahtan et al. 2005]. To perform the OMR, one does not need such precise visual sampling. Rather, a coarse sampling of just portions of the visual field using relatively sparse sensors (or sparse relays), should provide sufficient information to enable larvae to accomplish the basic task of maintaining orientation and a roughly correct direction of swimming. While these larvae may be too small to hold station, this behavior would at least minimize the forces of currents acting to sweep them into new and potentially undesirable environments.

Conservation of the Optomotor Response

The OMR, because of its reliable and easy induction, and because of its reliance on neural systems spanning the pathway from sensory receptors to motor output, has been widely used to explore the functional organization of the nervous system. Drosophila in
particular has been used to discover genes involved in this pathway\cite{Gotz1970} and to explore diverse visuomotor transformations and their associated neural controls\cite{Srinivasan1999, Kern2001}. In the case of vertebrate animals, optomotor behaviors have been used extensively in genetic screens of zebrafish (an animal amenable to saturation mutagenesis) \cite{Li1997, Brockerhoff1998, Muto2005, Fleisch2006}. While many of the molecular mechanisms underlying phototransduction and neurotransmitter signaling are conserved across much of the animal kingdom, the tremendous differences in neural architectures between flies and vertebrate animals make it difficult to determine if some remnant of an ancestral optic-flow calculator has persisted in these diverse lineages. What is clear is that similar strategies are used, as e.g. the use of optic flow to drive an essential orienting response in both flies\cite{Srinivasan1999} and, as shown here, in fishes.

Within the vertebrate sub-phylum, many likely components of the OMR behavior (e.g. retinal direction-selective mechanisms, pretectal visual nuclei, descending reticular fibers and spinal CPGs) are all present throughout the group. But even amongst the anamniotes it is difficult to determine if conserved signaling pathways and conserved visuomotor transformations underlie the OMR. At the input level of the retina, the starburst amacrine cells that excite the directionally-selective ganglion cells are well conserved across the gnathostome vertebrates\cite{Tauchi1984, Brandon1991, Nguyen2000, Vigh2000, Yamada2003}. At the output level, it is believed that a common mechanism is used in the spinal cord to generate the basic alternating motor pattern that results in undulatory swimming \cite{Fetcho1992, Buchanan2001, Hale2001, Kimura2006, McDearmid2006}. But to show a conserved mechanism for the visuomotor transformation occurring in the brainstem would require the establishment of detailed circuit-level features that are difficult to obtain even in the transparent and relatively simple brain of the larval zebrafish—and even using a powerful array of available experimental techniques \cite{O'Malley1996, Liu1999, Gahtan2001, Brustein2003, Gahtan2003, O'Malley2004, Niell2005, Ramdya2006}. What might be more easily obtained across teleost species are those kinematic details of the OMR that help define the underlying neural circuitry. Does the OMR include an obligatory turning component, like that seen in larval zebrafish? Is it selective for perceived slippage against the visual surround? Does the OMR behave as
only a “rough orienting system?” To the extent that such details hold true for teleosts and related vertebrates, it would suggest that conserved neural mechanisms underlie visuomotor transformations in these animals.
Figure 3.1. Kinematic details of the larval OMR response. (A) The larval zebrafish initiates a turn of about 90 degrees shortly after the pattern of black and white stripes begins moving to the right (as indicated by arrow). This is followed by rhythmic slow swimming in the direction of the arrow. From the larva’s perspective, the stripes might reflect a stationary bottom, and their forward motion would suggest that the larva is being swept backwards. As such, a turn towards the arrow combined with a forward swim would help larvae to maintain their current location. Images were acquired at 600 frames per second. Every 5th frame is shown beginning with the frame just prior to the onset of larval movement. (B) Head-tail angle. One line was drawn from the tip of the snout through the middle of the caudal swim bladder and a second line was drawn as a tangent to the end of the tail. The angle between these two lines (head-tail angle) was then subtracted from 180° to determine the bend amplitude. (C) Offset angle is defined as the angle between the axis of stripe-motion and the midline of the larva as defined by a line drawn from the tip of the snout to the middle of the caudal swim bladder. Offset angle was used to determine the accuracy of the OMR response. Note that the arrow shows only the general direction of stripe motion; stripe motion is always perpendicular to the stripes’ long axis in this and other figures. Larvae used in this study were all close to 4 mm in total length.
Figure 3.2. Discrete swim bouts make up the OMR. Analysis of extended high-speed recordings of two different larvae reveals that brief, individual locomotor bouts comprise the larval optomotor response. Larval velocity (A, C) and the larva’s offset angle (B, D) are plotted over a 2.5 sec recording of these OMR behaviors. The larva spends the bulk of its time resting, interrupted by 3 or 4 brief swim bouts in the general direction of stripe motion. In (B) the initial turn reduces the offset angle to near zero and the next turn results in a direction change away from the stripe motion axis. In (D), the second turn results in the best alignment to stripe motion. In this and subsequent turns, the larva ends up in orientations that are in the general direction of stripe motion (+/- 45 degrees). These turns are always accompanied by mild forward swimming which results in motion generally along the axis of stripe motion.
Figure 3.3. Examples from the zebrafish locomotor repertoire. Two spontaneously observed larval behaviors are the routine turn (A) and the slow swim (B). (A) Larvae use low angular velocity routine turns to orient in a new direction. This turn is typically less than 90 degrees and followed by a very mild forward swimming bout [Budick and O’Malley, 2000]. (B) Spontaneous slow swims are characterized by low velocity, small angular bends of the trunk and minimal yaw (side-to-side movement of the head during forward swimming). (C) A gentle tap to the larva’s head often elicits a C-start escape behavior comprised of a high angular velocity C-bend and a vigorous burst swim with substantial yaw. All 3 videos were collected at 600 frames/sec and every 4th frame is shown.
**FIGURE 3.4**

**Figure 3.4.** Forward swimming component of the OMR response. The histograms show the number of responses observed as a function of 5 kinematic variables. The OMR swim is (A) a low velocity swim (mean 13.5 ± 5.5 mm/s) with (B) relatively little yaw (mean 2.6 ± 1.1 degrees). (C) The maximal bend location is 79.4 ± 2.7 percent of the body length with (D) a mean bend amplitude of 73.2 ± 13.7 degrees. (E) The tail beat frequency was fairly consistent (24.2 ± 2.4). N = 25 larva with 2 to 10 trials.
Figure 3.5. Kinematic comparison of forward swimming patterns. Measurements are shown for three types of forward swimming patterns: spontaneous slow swims, OMR swims and burst swims; the slow and burst swim measurements are from Budick and O’Malley [2000]. The histograms show the mean +/- S.D. for each measured variable. While there are statistical differences between some of the OMR and slow swim parameters, these differences are numerically small and both swim types look dramatically different from the burst swim parameters. (A) The mean swimming speed of the OMR (13.5 ± 5.5 mm/s) was slightly different from that of the slow swim (9.3 ± 3.0 mm/s; p<0.05) but both were markedly lower than the burst swim velocity (101.9 ± 41.1 mm/s; p<0.01). (B) The average yaw in the OMR (2.6±1.1°) was also slightly different from the slow swim’s yaw (1.4 ± 0.08°; p<0.05), but both were again dramatically lower than the burst swim yaw (21.3 ± 5.2°; p<0.01). (C) The average bend location of the OMR (79.4 ± 2.7%BL) was indistinguishable from that of the slow swim (80.1 ± 0.06%BL; p = 0.97) but significantly different from the burst swim’s bend location (61.2 ± 0.01%BL, p<0.01). (D) Where the OMR swim did diverge from the slow swim was in terms of its bend amplitude, which at (73.2 ± 13.7°) was statistically similar to the burst swim bend amplitude (75.4 ± 6.7°; p = 0.54) and quite a bit larger than that of the slow swim (46.3 ± 8.1°; p<0.001). In terms of tail beat frequency (E), the OMR (24.2 ± 2.4 Hz) was slower than the slow swim’s TBF (33.8 ± 2.8 Hz) and dramatically slower than the burst swim’s TBF (101.9 ± 41.1Hz). (mean ± S.D.).
Figure 3.6. The effects of stripe speed on the OMR response. The speed of the moving stripes was varied randomly between trials to determine the effect of stripe speed on the OMR response. Each point on the 3 graphs represents an average of the values made during a single OMR response over the course of the forward swimming component. We found no correlation between stripe speed and (A) mean tail beat frequency, (B) mean swim speed, (C) mean bend amplitude or (D) mean bend location. None of the four regression lines shows a significant correlation between stripe speed and the measured parameter.
Figure 3.7. Pectoral fin usage during the OMR. (A) The larva makes an initial turn to the left, in the direction of stripe motion, and continues to swim slowly forward. During the initial turn the larva has both pectoral fins laterally extended (circled in 3rd frame) and then uses them in alternation during the subsequent swimming response (circled in 7th and 9th frames). Images are 10 ms apart and were collected at 600 frames/second. (B) Selected images from (A) have been enlarged to better illustrate pectoral fin movements. At 20 ms the larva is performing a routine turn and both fins are extended. The frames at 60 ms and 80 ms illustrate alternating use of the pectoral fins during forward swimming. Arrow represents the stripe motion.
Figure 3.8. Directional nature of the OMR. The essential feature of the larval OMR is an initial turn towards the axis of stripe motion, represented by the arrow. Stripes are moving to the right in all frames. While the angles of initial turns vary in amplitude, in each of the 4 examples shown the turn is towards the direction of stripe motion. (A) Large right turn with an initial turn angle of 65°. (B) Small right turn with an initial turn of 21°. (C) Large left turn with an initial turn of 97°. (D) Small left turn with an initial turn of 32°. Each of the turns transitions into a slow swim. Videos were collected at 600 frames/sec and selected frames are shown here to best illustrate the turning behavior.
Figure 3.9. The angle of the initial turn during the OMR response. The OMR is initiate with a turn of varying magnitude. The initial heading of the larva was normalized to 0° to illustrate the range of turn angles. Positive angles represent leftward turns, while negative angles represent turns to the right. No turns were greater than 180°. This plot shows only turns towards the axis of stripe motion (which accounted for the great majority of OMR responses). To not over clutter the graph we show just 32 turns from 10 larvae; turns from the remaining 10 larvae had a similar distribution.
Figure 3.10. OMR turn amplitude is correlated with the initial offset angle. (A) There was a significant correlation between the initial offset angle of the larva, in relation to the motion axis of the stripes, and the angle of the first turn the larva made ($R^2 = 0.639; p < 0.001$). The dotted line represents a 1:1 correlation. (B) By the end of the behavior the larvae were better oriented towards the axis of motion of the stripes than prior to the behavior, but they were still not “precisely” aligned with the motion axis.
Figure 3.11. Comparison of OMR turns to routine and escape turns. The OMR turns (striped bars) were similar to the routine turns (open bars) and significantly different (P < 0.05) from the initial C-bend turn of the escape response (gray bars) in each of these measures: (A) initial turn angle, (B) bend duration, (C) counter-bend angle, and (D) angular velocity.
CHAPTER 4

BEHAVIORAL RESPONSE TO CHANGES IN WHOLE-FIELD ILLUMINATION
I. **INTRODUCTION**

Photic stimulation is known to elicit different motor behaviors in animals. Photic stimulation can elicit blinking in humans [Manning and Evinger 1986] and pupillary constriction in teleosts [Douglas et al. 1998]. Besides light’s affect on the eye, light can elicit different locomotor patterns. Drosophila and desert fleas will change direction and move towards lit areas [Burdelov et al. 2007; Scantlebury et al. 2007] and lampreys and fish will tilt their dorsal side towards the light [Ullen et al. 1997; Nicolson et al. 1998] in order to maintain proper orientation in the water. It is clear that visual stimuli can elicit motor responses, such as the OMR (previous section) and prey tracking [McElligott and O’Malley 2005] in larval zebrafish. It has also been shown that the average locomotor response in larval zebrafish increases briefly to sudden increase in illumination [Emran et al. 2007]. But what motor output pattern arises from the changes in illumination in larval zebrafish was largely unclear prior to the conduction of these experiments. After these experiments had been largely completed and reported [Day et al. 2006], another group has published some of the same results [Burgess and Granato 2007]

The zebrafish’s retina by 5 days post-fertilization (dpf) is mature enough to allow for visually mediated responses [Brockerhoff et al. 1995; Easter and Nicola 1996]. Some well established responses include the optomotor response, optokinetic response and prey tracking, which all require visual detection of movement. The larval zebrafish responds to this detection with different movement patterns that include slow swimming, turning and J-turns [Budick and O’Malley 2000; Muller and van Leeuwen 2004; McElligott and O’Malley 2005; Orger et al. 2008]. Larval zebrafish also respond to changes in illumination using the retina of their eyes, rather than nonretinal tissues such as the pineal gland [Muto et al. 2005; Emran et al. 2007]. Thus the larval zebrafish makes a good model to study the behavioral responses to changes in illumination to help facilitate the underlying neuronal circuits.

What is known about the larval zebrafish’s motor response to light is limited to fictive and restrained preparations. In response to an abrupt increase in whole-field illumination, the partially restrained larval zebrafish will exhibit mild caudal bending at low-frequencies [O’Malley et al. 2004] which appears to be similar to the slow swim described by Budick and O’Malley [2000]. Recordings from paralyzed larval zebrafish’s motor neurons in response to LES show a similar burst frequency (20-63Hz seen in the restrained
larvae [Masino and Fetcho 2005]) to that of freely moving larvae performing spontaneous slow swim (tail beat frequency of 25-40 Hz [Budick and O’Malley 2000]). However, there was a longer mean episode duration in the restrained larvae that lasted on average of 303ms [Masino and Fetcho 2005], compared to the 200ms seen by Brustein and Drapeau [2003] and in experiments done in our lab. But is the response to increase illumination different in a freely moving larva? The following section will explore the similarities that exist between the spontaneous slow swim and the motor pattern seen in unrestrained larvae in response to increase in whole-field illumination.

It has also been shown that adult zebrafish respond to a sudden and short decrease in illumination with rapid body movement [Kimmel et al. 1974; Easter and Nicola 1997] known as an escape response. Li and Dowling [1997] were able to elicit an escape response using similar methods to that in the OMR response. They would place the adult zebrafish in a stationary tank and rotate one large black segment around the tank. This stimulus would elicit an escape response similar to that when encountering a predator. Whether these responses are evident in larval zebrafish is unclear.

**METHODS**

**Behavioral Recordings**

With the use of high speed imaging, the behaviors of 6-9 dpf larvae (N= 14) in response to different light stimuli was obtained. Each larva’s response to 3 different stimuli was record with high-speed camera. The trials were a sudden, long-lasting increase in whole field illumination (light on), a brief decrease in illumination (flicker) or a long-lasting decrease in whole-field illumination (light off). The larval’s naïve behavior of a spontaneous slow swim and routine turns were also recorded and analyzed for comparison. The larval escape response was elicited with a light tap on the right or left side of the head with a small metal probe and resulting behavior saved for comparison.

To record the behaviors mentioned above, individual larvae were placed in a dish set on an infrared LED and under a high-speed camera microscope set up, as described earlier. The entire apparatus, including the larva, were then enshrouded in black tarp to prevent any stray light from entering. It is unlikely that the infrared LED provided any visible light for the fish as the sensitivity to light in larval zebrafish falls off after 620 nm [Brockerhoff et al.
Two visible light LED’s controlled with a high-speed shutter, were also placed in the recording apparatus such that when the light was turned on, the dish the fish was swimming in would be well lit light from all angles. To assure that the sound of the shutter was not the stimulus for movement, we observed the larva during a control experiment in which the shutter was opened but no change in illumination occurred. We observed no visible difference or increased frequency in behavior when the shutter was opened or closed but the light intensity did not change.

For each of the 3 lighting conditions, the larva was allowed to acclimate for 5 minutes to the swimming arena lit with either the IR light from below, for light on, or the visible light from above, for the light off and flicker. The IR light was on throughout all these conditions. During a period of relative quiescence, as determined initially by the experimenter and confirmed by video playback, the natural light was turned on (for light on response - LES), turned off (dark evoked – DET) or turned off and on quickly with a 200 ms dark period using a high-speed shutter (for flicker). A high-speed video camera collected the resulting behaviors at 400 frames/second. Video recording was stopped after the second behavior and a video was saved starting from 300ms before the stimulus was initiated.

In order to rule out recordings of spontaneous slow swims, a latency period and frequency of occurrence for each of the behaviors were measured. Responses that began less than 50ms after the stimulus were excluded as this appears to be too short of a time for visuomotor transformation [Niell and Smith 2005; Canfield 2006]. Those responses that take longer than the normal range of latencies (> 2 s) were excluded due to the fact they were probably not induced by the light stimulus.

**Analysis and Statistics**

As described in an earlier section (Methods: Chapter 2) the midline of the larvae were digitized using a customized Matlab program (see Fig. 2.1). Each of the midlines were then analyzed using Matlab to determine 8 different variables: bend angle, bend location, tail-beat frequency (TBF), bout duration, swim velocity, yaw, stride length and slip. All lengths are normalized to body length. Turning behaviors were analyzed using Image J (NIH), as previously described, for bend angle and angular velocity.

All statistics were performed in SPSS 16 (SPSS, Chicago, IL,USA). Comparisons between LES and slow swim pattern for each of the variables were done with either a two-
tailed independent t-test assuming equal variance. The different turns were compared in SPSS 16 based on angular velocity and bend angle using ANOVA, with Bonferroni correction factor due to multiple analysis and unequal variance. All variables were tested for homogeneity of variance with the Levene’s test. A 0.05 probability level was used a criterion for statistical significance. All values expressed are means ± s.d., unless otherwise noted.
RESULTS

Increase in Whole Field Illumination

Multiple high-speed behavioral recordings from 6-9 dpf larvae (N=14) were captured for a light on response (LES) and spontaneous slow swim (slow swims). From these individuals 23 trials of LES and 27 slow swims were analyzed to compare forward swimming kinematics. In order to determine whether the response to an increase in illumination was similar to a slow swim bout, the individual kinematic variables (bend angle, bend location, yaw, tbf, stride length, slip, swim velocity, bout duration) from the subjects were compared using a paired-T-test. Upon comparison there was little significant difference between the slow swim pattern, previously described in Budick and O’Malley (2000), and the swimming pattern seen in response to a whole-field increase in illumination (LES).

Both the LES and slow swim exhibit a mild caudal bending of the tail that resulted in a slow swimming velocity (Figure 4.1; Figure 4.3 and see figure 3.3 for a slow swimming example). The only significant difference seen between LES and slow swim patterns was the bout duration (Figure 4.2). The bout duration for LES (116.96 ± 28.31ms) was shorter than that of the slow swim motor pattern (152.40 ± 40.11ms; p <0.01). Although the LES was shorter, the other variables remained unchanged (Figure 4.3). The mean swim velocity, tailbeat frequency, stride length and slip seen in LES was the same as what was seen in the same larva’s slow swim pattern and comparable to previously described slow swim behavior in Budick and O’Malley [2000]. The mean bend angle, bend location, and yaw also remained unchanged from slow swimming to the LES motor pattern. Budick and O’Malley [2000] described a significant difference between slow swimming and burst swimming in the larval zebrafish, which was also seen in our study. When LES was compared to burst swimming elicited from a tactile escape response, there was a significant difference in each of the variables analyzed (Figure 4.3).

Turn Response

Upon observation, some of the responses to the increase in illumination started with a turning behavior while others did not. To look further into this behavior, 46 trials from 14 larvae of the LES were further analyzed. Out of the 46 trials only 45% included a turning component. While reviewing the videos of the turning behavior, we noticed turns were not
always of the same direction (i.e. some were to the right, others to the left). Since fish prefer well lit areas [Brockerhoff et al. 1995], we wondered if the direction the light influenced the turning behavior. A different set of larva (N=9, 86 trials total) then previously used, were tested for response and directionality of turn when influenced by direction of the light. In this group, larva responded 57% of the time with a turn. The trials were binned into 8 categories according to the larval’s initial orientation to the light such that a 0° angle would represent the larvae facing into the light. The frequency of the turning responses (both towards and away from the light) and of slow swimming only bouts were measured and graphed (figure 4.4). Larvae that were facing the light within ± 22.5° showed no preference in motor behavior response, with an equal number of turning behaviors and slow swimming only behaviors. As the larvae’s initial orientation was directed further away from the light, the larvae were more likely to respond to the increased illumination with a turn response towards the light. This seems to suggest that the light evoked turn and swim response we saw is being used to orient the fish into well lit areas.

Touch-evoked escape responses in zebrafish have a latency of <5 ms [Liu and Fetcho 1999]. To determine the LES behavior response time, and potential for use of the same neural circuits in the escape response, latency of response from the stimulus (increase illumination) was measured. The latency of the light-evoked response averaged 234 ± 128ms, with a wide range of 80 to 595 ms (figure 4.5). We also compared turning angular velocities seen in LES to that of routine turns and c-bends in an escape response. The angular velocity of LES (5.3 ±1.4° ms⁻¹; N=21) was similar to that of the routine turn (5.1±0.92° ms⁻¹; N=17; p=0.14; Figure 4.6). Due to the low angular velocity, long latency turn towards the stimulus, it does not seem that this LES turn would be useful for predator avoidance. Further analysis and comparisons of these turns are discussed below.

**Response to decrease in whole field illumination.**

Previous research reported that 3 month old zebrafish respond to a decrease in illumination with an escape response [Dill 1974]. However, the research did not perform fine high-speed kinematic analysis of the data to determine the resulting motor pattern. Here we evaluated the larval zebrafish’s (N=13) response to three different stimuli. The three stimuli we used were a long-lasting decrease in illumination, called light off in this paper (N
a short-term decrease in illumination, called a flicker (N=37) and tactile escape-eliciting stimulus (N=12), which was used for turn comparisons.

First we compared the two responses to a decrease in illumination: the short (flicker) and the long-lasting (light off) decrease in illumination. Larvae responded to light off much in the same way as to the flicker stimulus (Figure 4.7). Both responses were high angle bends (flicker = 184±40°; light off = 180±32° mean±s.d. p>0.05) with a low angular velocity (flicker = 6.0±2.2° ms⁻¹; 5.8±2.9° ms⁻¹; mean±s.d. p>0.05) (Figure 4.6). A small proportion of the time (30%) the larva would perform a second bend of similar angle and velocity (data not shown). Occasionally (10/44 times), the larvae began the dark-evoked response with a reverse wave that resulted in an S-shape body position prior to the turns described above (Figure 4.8). Sixty-five percent of the time following the high angular turn, the larva would perform a slow swim. The other times the larva would either perform just a half of a tail-beat or just let the tail unfold.

Due to the nature of the neural circuit including the Mauthner cell and the urgency of the behavior, the escape response has a very low response latency of 4 ms [Liu and Fetcho 1999]. The latency response for both the light off (379±289ms) and flicker (415±315ms), which were not significantly different (p<0.05), were considerably higher than that for the escape response. The latency of the dark-evoked turns also showed a very large variability (Figure 4.9). More importantly, the dark-evoked and flicker stimuli did not result in a substantial movement away from a possible strike location, but more frequently resulted in a turn response towards where the light was extinguished (data not shown, see also [Burgess and Granato 2007]).

Although, the escape response did visually show similarities to the light off and flicker response, detailed analysis showed significant differences between the escape response and the dark evoked turns (see Figure 3.3, Figure 4.6, 4.7). These differences will be explained in the next section.

**Comparison of different larval turning behaviors**

The 5 behavioral responses we studied here (Flicker, Light off, Light On –LES, Routine turn, escape C-bend) all contained a turning component. To determine if there were differences in these behaviors, we measured the bend angle, duration and the angular velocity of the turn as well as the resulting change in orientation due to the turn. Based on
those variables, when the results were compared with ANOVA and post hoc tests, we found 3 different types of turns; a low angle, low velocity turn, high angle low velocity turn and a high angle high velocity turn.

The escape response had a significantly high angular velocity (20.7±5.0 ° ms⁻¹) compared to the other 4 conditions (p<0.001). The light on, light off, flicker and routine turn all showed similarly low angular velocities (p>0.05) (Figure 4.6B). The bend angle varied between the 5 groups (Figure 4.6A). The dark evoked turns due to light off or flicker stimulus were similar with a high angular turn that resulted in the larva completely turning around. These turns had the similar high bend angles with the light off turns averaging 180±32° and the flicker response 184±40° (p=1.0). The two dark evoked turns had a significantly higher turn angle than the LES, routine turns and escape turns (p<0.001). The mean bend angle of the routine turn (125±16°) was similar to that of the LES turn (140±37°). As previously reported [Budick and O’Malley 2000], the escape turn bend angle was different than that of the routine turn bend angle (p<0.01). What was unexpected was the bend angle of the LES turn was similar to that of the escape turn (mean=144±9°). This is discussed in chapter 6 in the context of the sensorimotor transformation being performed.

The resulting change in direction due to the turn varied amongst the 5 behaviors (P<0.001; Figure 4.6C). Both routine turns and light on turns resulted in similar changes in orientation of less than 90° (P=0.110). The escape c-bends, light off and flicker turns all showed large changes in orientation that sometimes exceed 180°. However, when direction change was compared to angular velocity, escape c-bends were quite different from those of the light off or flicker turns (Figure 4.6D). This leads us to believe that there we have here 3 distinct types of turns: a high-angle, high-velocity escape turn, a high-angle, low velocity dark-evoked turn, and a low angle, low velocity routine turn.
DISCUSSION

The larval zebrafish responds to many environmental cues/stimuli in order to avoid predation and remain in a safe and favorable environment. One such stimulus is the amount of light in the environment. Zebrafish will move towards a lit area if given preference between light and dark areas [Brockerhoff et al. 1995]. This could be due to the fact that zebrafish larvae require light to feed efficiently [Gahtan et al. 2005; McElligott and O’Malley 2005]. In this study we characterized behaviorally and kinematically the visuomotor response to whole-field illumination changes and showed that the zebrafish responds to a sudden increase in illumination (LES) quite differently than a sudden decrease in illumination (DET) and that both responses were highly distinct from the escape response.

In response to a sudden decrease in illumination, the larval zebrafish performed a previously unknown motor pattern that consisted of a high angle, low velocity turn. The turn was usually followed by a slow swim pattern. When the responses to long-lasting decreases in illumination or to a short dark flash (flicker) were compared, there was no significant difference. In the wild, there are many instances that the larval zebrafish may find itself in a dark environment, one such being wandering into unlit areas. The high angle, low velocity turn described here could be used to reorient the larva back towards the well lit area for better feeding. This is similar to the response seen in Xenopus tadpoles. Decrease in illumination stimulates a vertical swimming behavior in unattached Xenopus tadpoles to aid in tadpoles attaching to objects that cast shadows [Jamieson and Roberts 2000]. In lamprey, illumination in one eye will result in a contralateral turn and forward swimming motion; a behavior called negative phototaxis [Ullen et al. 1997]. This is thought to be an effective way in preemptively avoiding predators by avoiding light exposure [Ullen et al. 1995].

In response to an increase in illumination, the larval zebrafish performed a behavior that combined two previously described motor patterns: routine turn and slow swim [Budick and O’Malley 2000]. When eight kinematic variables were analyzed, the only difference found between the slow swim and the forward swimming pattern seen in LES was the bout duration. The other variables such as swim velocity, tail beat frequency, bend location and bend angle, to name a few, remained unchanged. The kinematic similarities between the LES forward swimming and the slow swim imply a shared premotor circuit. The longer latency and lack of burst swim suggests that the LES response is a part of a different neural pathway than that of the short latency escape response, which employs the burst swim
pattern. Our data shows that the LES and DET responses with the subsequent swimming behavior is not a predator avoidance behavior because the escape response C-bends are of high angular velocity (mean = 21.2±4.4° ms⁻¹ [Budick and O’Malley 2000]), short latency response and result in the larva turning away from the predator thus rapidly removing itself from the location of a possible impending strike. In both the LES and the DET turns, the larvae turn towards the stimulus (seen in our data and [Burgess and Granato 2007]) with a low angular velocity turn.

The Mauthner cell is part of the neural circuit controlling the escape response. Upon ablation of the Mauthner cell the latency of the escape response is increased [Liu and Fetcho 1999]. Since the LES and dark evoked turns had a longer latency then the escape turns, it seems that the Mauthner cell is not part of the light evoked turns. Current research showed the ablation of the Mauthner cell did not affect the dark-evoked turn response [Burgess and Granato 2007]. Further experiments will be needed to determine if the Mauthner cell plays a role in the whole-field illumination responses. However, due to the dissimilarities between the LES and escape response, we hypodissertation that ablation of the Mauthner cell will have no affect on the LES response. It seems that the latency between visual stimuli and neuronal response varies considerably between fish. Canfield [2003] showed the M-cell is stimulated from a flash of light with a latency of 15ms in adult cichlids and 25 ms in adult goldfish, which results in an C-start escape behavior. These responses were transmitted via the optic nerve and optic tectum to the Mauthner cell [Zottoli et al. 1987]. Neil and Smith [2005] in larval zebrafish show an increase in calcium response in the tectum to a moving spot within 100ms. This is similar to the latency seen in tectal cells in response to a strobe flash (20-120 ms; [Guthrie and Banks 1990]). These longer latency responses correspond well to the latency from stimulus to behavior in our study, being 234±128 ms for the increase in illumination and 379±289 ms for the light off response.

Visuomotor behaviors utilize a multisynaptic pathway. Ganglion cells in the retina show a variety of responses to full-field illumination, both increases and decreases and transient and sustained [Roska et al. 2006; Emran et al. 2007]. Melanopsin retinal ganglion cells are part of the non-image forming visual pathway for mediating activity in response to light (Panda et al 2003). ON –centre and OFF-centre ganglion cells are part of two separate parallel pathways [Schiller et al. 1986] that take information about the visual world via the optic nerve to one of the 10 afferent field in the zebrafish brain, including the optic tectum.
Both retinal bipolar and ganglion cells as well as tectal cells have been shown to respond to a moving spot as well as increase and decrease in whole-field illumination [Sajovic and Levinthal 1983; Guthrie and Banks 1990; Niell and Smith 2005; Emran et al. 2007]. Due to the different receptors and pathways for light and different bend angles, but similar angular velocity, observed in response to changes in whole-field illumination, we hypothesize differences in the neural circuits and signaling pathways may underlie the observed differences in the bend angles between the LES turns and dark-evoked turns.

The optomotor response (OMR), prey tracking, spontaneous slow swims and light evoked slow swims all share a similar motor pattern: a slow swim. We hypothesize then that there is a common spinal CPG governed by different descending neurons for the slow swim pattern. Current research shows an active inhibition of a spinal interneuron (multipolar commissural descending – MCoD) at higher swimming speeds that was initially active at slow swim speeds. At the same time, a different spinal interneuron the circumferential descending (CiD) is recruited only at the higher swimming speeds [Masino et al. 2005; McLean et al. 2007]. As with many behaviors in vertebrates, motor patterns are often combined together to make different behaviors [Stein and Daniels-McQueen 2002; Day et al. 2006]. Part of the time, the LES swim was preceded by a turn that was not significantly different from the previously described routine turn. Thus the LES response consists at times of two previously described motor patterns, a slow swim and a routine turn. Both the OMR and the prey capture utilize the slow swim motor pattern and ablation of the largest afferent field, optic tectum, does not disrupt the OMR response [Roeser and Baier 2003] but does disrupt prey capture by potentially disrupting the neural circuits necessary to orient to the prey [Gahtan et al. 2005]. This suggests that a number of visuomotor transformation systems utilize a final common pathway to activate the slow swim pattern. The locus of this pathway is uncertain but it presumably utilizes reticulospinal neurons and activates a spinal, slow-swim CPG.

Previous research has shown that the same stimuli are capable of eliciting different motor response. The optokinetic response (OKR), an eye movement behavior, and optomotor response (OMR) an undulatory swimming response, are both elicited by large-field motion. However, in zebrafish with mutations in the retina it was found that the loss of one response was accompanied by some aspect of the other response being diminished or
abolished. However, there seemed to be no correlation between the magnitude of deficit lost between the two behaviors [Muto et al. 2005]. This suggests that OKR and OMR share some underlying neural circuitry, potentially involving a shared population of retinal ganglion cells [Emran et al. 2007]. Ablation of the tectum, just slightly downstream from the ganglion cells, resulted in loss of OKR without loss of OMR [Roeser and Baier 2003] suggesting a divergence of the neural circuits prior to the tectum. However, when the visual stimulus is a difference in illumination, it is possible to lose one behavior without deficits in the other [Emran et al. 2007], which suggests a independent neural circuits. We showed that both increased and decreased illumination led to a low angular velocity turn, but of different bend angle magnitudes. It appeared that the dark-evoked turns used more of the body during bending and the zebrafish brought their head around to the tail while the routine turn and LES turns were mostly performed by the tail, with little movement from the head. It is plausible then that there are different sensory receptors for increase and decrease in illumination but a common reticulospinal or spinal circuit.

By understanding the motor outputs of stereotypical behaviors, researchers can begin to dissect the sensory-motor transformation circuitry that drives these behaviors. In this study, we showed that there is a conservation of the motor pattern slow swim that can be reliably elicited via visual stimuli. This behavior may be part of a different neural circuit then that of the dark-evoked behavior but probably contains some common circuitry for the turning behavior. Both the light evoked and dark evoked responses utilize neural mechanisms distinct from those that control the escape behavior.
**FIGURE 4.1**  

Examples of responses to increase in illumination. The larval zebrafish responds to increased illumination with one of two behaviors, either (A) a forward swimming behavior or (B) a turn followed by a forward swimming behavior. This first image in both examples is the first sign of movement in the larvae following the stimulus. Thus the latency of response was 150 ms (A) and 190 ms (B). (A) Images were collected at 600 frames s\(^{-1}\) and every 4\(^{th}\) frame is shown. (B) Images were collected at 400 frames s\(^{-1}\) and every 4\(^{th}\) frame is shown.
FIGURE 4.2. Bout duration comparison. The light evoked swimming pattern due to increase in whole-field illumination was significantly shorter in duration than the spontaneous slow swimming pattern (t-test, P <0.001). Mean duration of the light-evoked response was 116±28ms (N=23, mean ± S.D.) while the slow swimming pattern was 152 ±40ms. However, there was great variation in the slow swimming bout duration. The box represents the 1st to 3rd quartile, the dark line the mean and the bars the maximum and minimum durations.
FIGURE 4.3 Bar graphs showing the comparison of swimming patterns between the 3 forward swim patterns: slow swimming, light evoked by increase in illumination, and burst swim during escape response. In the 7 kinematic variables measured, there was no significant difference between light evoked and slow swimming (P>0.1). There was a significant difference between the burst swimming and both the slow swimming and light evoked swimming (P<0.001). The slow swimming and light evoked swimming are characterized by a slow velocity (A), low tailbeat frequency (B) with a small bend angle (C) that occurs more caudally along the length of the body (D). The slow swimming patterns also showed little head movement (yaw) (E). The stride length (F) and slip (G) was also significantly lower in the slow swimming and light evoked swimming compared to the escape response. The error bars represent standard errors (S.E.M.). Comparisons were done with 2-way ANOVA.
FIGURE 4.4. Histogram of response to light. The initial orientation of the larvae in relation to the light (offset angle) was binned into 45° increments. A 0° orientation represents the larvae facing directly into the light. We observed no preference between turning or slow swimming if the larvae were facing the light (0 °). However, the larvae were more likely to respond with a turning behavior towards the increase in illumination if the larvae was oriented away from the light.
FIGURE 4.5. Histogram of latency for response to increase in illumination. The latency of response time was measured from the initial onset of the stimulus, increase in illumination, to the time that movement was first seen. The average latency was 233.61 ± 127.93 ms.
FIGURE 4.6. Comparison of turning behavior in response to short sudden decrease in illumination (flicker N=37), long-lasting decrease in whole-field illumination (light off N=17), increase in whole-field illumination (light on N=21) and previously described routine turns (N=17) and C-bends in tactile escape response (escape N=12). All values are means ± s.e.m. (A-C) The routine turn and the turn in response to increase in illumination were indistinguishable in all variables measured. Both exhibited a low velocity, low angular turn that resulted a small directional change. Both the turns in response to a flicker and light off were high angle, slow velocity turns. Although the bend angle in light off and flicker were greater than that of the escape response (ANOVA, P<0.005), the resulting change in direction were similar. Also, the flicker and light off were slower velocity turns compared to the escape response (ANOVA, p<0.001). (D) A scatter analysis of the directional change and angular velocity revealed 3 different types of turns: a high angle, high velocity escape response, a high angle, low velocity dark evoked turn, and low angle, low velocity routine turn. Each of the 3 turns were significantly different from each other (ANOVA, P<0.001).
FIGURE 4.7. Example of the dark-evoked response. In response to a sudden decrease in whole-field illumination the 7 dpf larva responded with a large angle (190°) slow velocity (6.8°/ms) turn. Note the unique O-shaped turn in the 6th frame (star). This turn resulted in the larva making an orientation directional change of 142°. Images were captured at 400 frames s⁻¹ under infrared light and every 3rd frame is shown. The first frame represents the first sign of movement after the decrease in illumination (i.e. latency = 173 ms).
FIGURE 4.8. Example of a small proportion (10 out 44 trials) of the responses seen to a decreased whole-field illumination. Note the reverse propagation of the wave on the tail of the 8 dpf larva in the first 4 images that cumulates in an s-shape position (arrow). Following this motor pattern, the larva proceeded to perform a sharp turn and forward swim similar to other larva’s dark-evoked behavior. The white spot and two-toned background are artifacts from either the camera lens or video. Images were captured under infrared illumination at 400 frames s⁻¹. Images are displayed in 5 ms intervals.
FIGURE 4.9. Latency of response to decrease in illumination. Histogram of latency to either a short decrease in illumination (flicker N= 37) or to a long-lasting decrease in illumination (light off N= 17) shows a wide range of times. However, there was no significant difference in the average latency time between the light off (379±289ms) and flicker (415±314ms).
CHAPTER 5

RHEOTAXIC BEHAVIOR AND THE EFFECTS OF SWIM SPEED ON AXIAL KINEMATICS
INTRODUCTION

When a fish is presented with a water current, it will orientate itself with respect to the source of the current; a well known response called rheotaxis that is present in many species of fish, some tadpoles and lamprey [Montgomery et al. 1997; Ellerby et al. 2001; Kanter and Coombs 2003; Montgomery et al. 2003; Simmons et al. 2004]. The rheotaxis behavior could allow the fish to swim upstream traversing velocity barriers or allow it to maintain its relative position in the environment. The lateral line, a mechanosensory system that is found on the superficial surface of fish as well as in fluid filled channels along the body of the fish, has been shown to be involved in the neuronal circuits that elicit the rheotaxis response in fish [reviewed in Montgomery et al. 1997][Liao 2006]. The rheotaxic behavior is a common response that can be used to investigate the speed effects on midline kinematics [Tytell 2004], gait transitions [Hale et al. 2006] and physiological effects of chemicals on development in fish [Johnson et al. 2007].

Zebrafish are growing in popularity as a model for understanding the neural circuits that control locomotion [reviewed in: Fetcho et al. 2008]. Zebrafish, having their ecological home in the rapid mountain streams in Asia [Laale 1977] and having a lateral line system [Metcalf et al. 1985], probably use rheotaxis to maintain their position in the environment. Although the kinematics of two types of forward swimming in larval zebrafish have been previously explored [Budick and O’Malley 2000; Muller and van Leeuwen 2004], the question that remains unanswered is what motor pattern does the larval zebrafish utilize during rheotaxis.

Larval zebrafish exhibit two types of forward swimming behavior; a slow swim of low tailbeat frequency with small caudal tail bends and a burst swim of high tailbeat frequency with large more rostral tail bends. A common pattern of movement that is seen in a number of behavioral contexts of the larval zebrafish is the slow swim [shown in this thesis as well as in Day et al. 2006]. In this study we investigated whether the behavior of rheotaxis utilized the slow swimming pattern. Research indicates that the slow swim is kinematically and potentially neurological distinct from the burst swim [Budick and O’Malley 2000; Ritter et al. 2001; Masino et al. 2005; McLean et al. 2007]. If rheotaxis
utilizes the slow swimming pattern, this could provide implications for the conservation of the underlying neural circuits that control locomotion.

Vertebrates have a locomotor repertoire that allows them to utilize different gait patterns in different situations [Cabelguen et al. 2003; Marsh et al. 2006; Seay et al. 2006]. Rheotaxis has been used to elicit some of these different gait patterns in fish [Archer and Johnston 1989; Jayne and Lauder 1995a; Peake and Farrell 2004]. We used the flow channel to examine the effects of current on the patterns of behavior in the larval zebrafish. The lateral line system that stimulates locomotor behaviors during rheotaxis in zebrafish has also been shown to have synaptic connections on the Mauthner cell [reviewed in: Korn and Faber 2005]. The Mauthner cell upon reaching threshold, either by auditory or visual cues, results in a C-start escape response characterized by a high velocity, high angle C-bend and a burst swim away from the stimulus [Kimmel et al. 1974; Faber et al. 1989; Eaton et al. 2001]. We wondered if the rheotaxic behavior, that utilizes the lateral line sensory modality, could elicit a burst swim.

To understand the neural circuits that control diverse swimming behaviors in the larval zebrafish, it is necessary to describe the motor patterns that occur in response to different environmental contexts and how varying environmental stimuli affects swimming patterns and speeds. This study aims to explore the motor pattern exhibited by larval zebrafish during rheotaxis and determine whether the slow swim is the pattern of choice for situations of less urgency than the escape response.

**METHODS**

**Rheotaxis Recordings:**

All experiments were conducted on larval zebrafish between 6 and 9 days post-fertilization (dpf; N=8). The 5 dpf larval zebrafish has a lateral line system that has been shown to be responsive to water flow [Metcalfe et al. 1985; McHenry and van Netten 2007]. Larvae were placed in a flume which on one end is connected to a pump that produced constant water flow and the other end is connected to a water collection container. The flow apparatus was placed underneath a high-speed camera that was connected to a dissecting microscope (as described in chapter 2). In order to observe the larva in the flow channel in the dark, an IR LED was placed under the flow channel. When initially placed in the
chamber, the larvae were allowed to acclimate for 5-10 minutes without a current. An initial recording of swimming was obtained after acclimation to the flow chamber and was used as a baseline for the slow swimming. Then a steady current was applied to the chamber and the resulting swimming behavior was recorded for 1-5 seconds at 400 frames s\(^{-1}\). Since the previously described forward swimming bouts last less than 200 ms [Budick and O’Malley 2000], videos of 1-5 seconds were deemed appropriate to capture repeated swim bouts in response to the flow current. Three videos were recorded at a given current flow speed with the first video collected at the start of the flow, the second video 5 minutes into the experiment and last video after 10 minutes. After the final recording the flow was stopped and the larva was allowed to rest with no water flow for 10 minutes to minimize the effects due to fatigue. These steps were then repeated for all the water flow levels. The flow rate each time was randomized so as to eliminate any potential fatigue or motor learning. When a higher flow rate was tested, the flow rate was increased as quickly and steadily as possible while ensuring the larvae are not swept downstream. In order to minimize wall effects, only those videos/behaviors where the larva was in the center of the channel were retained and analyzed.

Each larval zebrafish’s escape response was also elicited and recorded so as to obtain representative burst swimming patterns from each fish. To do so, we elicited the well describe tactile response by tapping on the head of the zebrafish with a small probe during a period of relative quiescence. The behavior was elicited 2 times from each of the fish, once from tapping on the right and once from the left. These behaviors were elicited outside the flow channel, in a small petri dish.

We were able to collect the most data at 3 fixed flow velocity speeds and thus used this data for our analysis. Since our flow pump did not specify water current in a usable form, the current flow was directly measured. This was done by placing drops of methyl blue dye at the beginning of the flow channel and recording the speed of the front of the dye. This procedure was repeated 5 times at each flow speed and the average of the 5 was taken. Visual inspection of the video revealed dye flow was unidirectional, spatially homogenous in plain view and a uniform velocity over the 5 videos. The water current was measured to have three reliable speeds: slow water flow of 1.88 mm/s (level 3), a moderate water flow of 4.22 mm/s (level 5) and high water flow of 6.55 mm/s (level 7). In the results we will refer to these water flow speeds a slow, moderate and fast.
Behavioral and Statistical Analysis

As described in the general methods, each of the saved behaviors’ axial kinematics were analyzed with the use of MATLAB. In short, 16 points were manually selected evenly spaced down the midline of the larva and were smoothed with a cubic smoothing spline [Johnston et al. 1995]. From the spline, 9 variables from 8 fish under 5 swimming conditions (slow swim, three rheotaxic flow speeds, burst swim) per fish were analyzed. This resulted in 19 trials for the slow swims, 42 trials for slow current flow, 43 trials from moderate flow, 52 trials from the fast flow and 10 from the burst swim. The variations in the number of trials were due to exclusion of videos for reasons such as the fish swimming against the wall or the fish swimming out of field of view. Variables analyzed included tailbeat frequency (TBF), bend angle, bend location, yaw, and swim velocity, bout duration, rest time between bouts, stride length and slip. All velocity variables analyzed were relative velocities obtained by adding the measured absolute velocity to the water flow velocity. Measurements in distance were normalized to body length (BL).

In addition to basic calculations of statistics for the kinematic variables mentioned above, several analyses of variance (ANOVA) and regression models were performed using SPSS to test the effect of swimming speed and flow speed on these kinematic variables. First, comparisons of the variables were performed using a two-way mixed model ANOVA with swim level as a fixed factor and individual as a random factor. For those ANOVA’s that showed significant difference among the levels, Tukey post hoc tests were performed to determine out which levels differed. Also regression analysis was used to measure the effect of swimming speed on the kinematic variables. The average of the velocity and the average of the kinematic variable of interest for each fish at each speed were used for the regression analysis. Linear regression analyses were not intended to produce equation, but rather to describe trends in behavior. Separate from the above analysis, other two-way mixed model ANOVA’s and regression analysis were used to compare the differences between the rheotaxis behavior, slow swimming and burst swimming. P values were subjected to Bonferroni correction to maintain alpha=0.05 due to use of multiple comparisons on the data set. Results are presented as mean±S.D.
RESULTS

Larval zebrafish (N=8) swimming in different flow conditions were compared and the same data was analyzed as a function of swim velocity to distinguish channel flow and larval swim speed effects. These behaviors were then compared to slow and burst swimming data collected in the same larvae. The larva ranged between 6-9 days post-fertilization and 3.71 – 4.88 mm in length. Each larva’s swimming behavior was recorded, at low water flow (1.88 mm/s), a moderate water flows (4.22 mm/s) and high water flow (6.55 mm/s). At speeds greater than 6.55 mm/s, the larvae were swept back with the current and the larval zebrafish could not perform coordinated swim patterns in a timely fashion to swim against the flow, so data collected at these speeds could not be analyzed. However, the motor patterns seen did not exhibit burst swimming patterns. The larvae’s behavior was also recorded with no water flow, used as a slow swim, and in response to a head tap, used as a burst swim. The means of the kinematic variables measure are presented in table 5.1 and a summary of the regression analysis performed for swim velocity on the kinematic variables are presented in table 5.2.

Rheotaxis is a behavior in which fish orient themselves and swim into oncoming current. Upon increasing current flow, fish will generally increase swim velocity. To determine whether larval zebrafish exhibit a rheotaxis response, individual larval zebrafish (N=8) were placed in a flow channel with 3 varying water speeds, previously mentioned. As the flow velocity increased, there was a linear increase in swim velocity (Figure 5.1; r²=0.907; p<0.001). When the swim velocity due to the slow water current was compared to the slow swimming velocity, with no water current, there was a significant increase in swimming velocities from 2.38±0.67 BL/s to 4.16±0.81 BL/s (P<0.001). There was a large increase in swim velocity during the channel swimming from an average of 4.16±0.81 BL/s at the slow flow to 9.06±0.77 BL/s at the fast flow. However, the fastest swimming velocity obtained by an individual larva in the flow channel (10.52 BL/s) was quite different then the velocities recorded during a burst swim (mean 29.79±2.19 BL/s, P<0.001 ANOVA).

To determine whether the behaviors we captured were influenced by visual cues, a larval zebrafish (N=1) was placed in the flow channel at the three different speeds in the dark. With the use of an IR LED placed under the flow channel, we observed that the kinematics of the larva appeared the same as that exhibited during whole-field illumination (data not shown).
Tail beat frequency (TBF) increased in the flow channel (figure 5.2) from 25±2.9 Hz during the slow flow to the 27±2 Hz at the moderate flow (P<0.001), but did not continue to increase significantly at the faster current speed (28.6±1.8, P=0.054). The slow swim TBF (25.2±2 Hz) was similar to the TBF in the slow current (P=0.702). Since the swim velocity increased with the water current, we decided to see what the effect of swim velocity was on TBF. Regression analysis showed a strong correlation between swim velocity and TBF (R²=0.923) when burst swimming was added to the regression model (Table 5.2, figure 5.2B). The higher 4 velocities in figure 5.2B represent the mean velocities and mean TBF of 4 individual fish that occurred during the burst swim. The gap between the fastest speed obtained in the flow channel (10.52 BL/s) and the slowest burst swim velocity (27.5 BL/s) correspond to the velocities we were unable to elicit in the flow channel due to the larval being swept downstream by the current.

The distance traveled forward during each tail beat (stride length) was also significantly different over the increase in current flow (figure 5.3; P<0.001). The stride length showed an increase at each level from the slow swimming (0.09±0.02 BL/tail beat) all the way to burst swimming (0.52±0.02 BL/tail beat). A strong correlation of stride length to swim speed was seen when the average stride length for each fish was plotted (figure 5.3B). In the lower swim velocities (<11 BL/s) there is a strong linear correlation to stride length (R²=0.965; Table 5.2). However, as swim velocity increased above 11 BL/s, there is a smaller rise in stride length. Although data was not obtained for the intermediate swim speeds, a cubic regression equation (SL=−0.017+0.050U−0.002U²+0.00002U³; U=swim velocity) best explained the relationship between stride length and swimming velocity (R²=0.985).

There was no significant difference in bend location (figure 5.5; P=0.682) or maximum bend angle (figure 5.6; P=0.303) with the increasing speed in the flow channel. When the bend location during swimming in the flow channel was compared to slow swimming, there visually appeared to be a slight decrease from 0.83±0.04 BL in slow swimming to 0.81±0.02 BL in the fast flow current. However, this was not a significant change (P=0.257). The bend location in burst swimming in the same larva occurred more rostrally (0.66±0.00BL) compared to the flow channel and slow swimming (P<0.001). The bend angle varied a little with the increase flow speed (slow=52±8° fast=55±6°; P=0.303), although this increase was not found to be significant. The slow swim bend angle (51±8°)
was the same as the 3 flow speeds bend angle, which was different from the burst swim (78±4° P<0.001). Both bend location and bend angle showed no significant correlation to swimming velocity (Table 5.2, figures 5.4 & 5.5).

The duration of each swim bout and the rest time between the bouts was measured at the 3 swim levels (figure 5.6). The rest time was inversely proportionate to the water flow speed with a significant decrease in rest time occurring between the slow flow speed (566±236 ms) and the moderate flow speed (427±178ms; P<0.01). Although the rest time decreased further at the fast flow speeds (372±162 ms), this was not shown to be significant (P=0.198). The bout duration remained fairly constant over the 3 flow speeds (F=3.143, P=0.08). Although no correlation was found between swim velocity and bout duration, there was a significant but weak correlation between swim velocity and rest period (R²=0.220 table 2). By decreasing the rest period between swim bouts, the larval zebrafish was able to maintain a relatively constant placement in the environment.

We also found no significant difference in yaw over the flow speeds measured (F=1.619, P=0.215; figure 5.7). When yaw in the flow channel was compared to slow swimming, there was no significant difference. When yaw was plotted against swim velocity, we found a weak correlation (R²=0.220), but the slope was not significantly different from 0 (Table 2). This suggests that yaw does not change with increasing slow velocity in the flow channel. However, there was almost a 8-fold increase in yaw when the burst swim bouts were compared to rheotaxis (table 1; P<0.001).

There was a steady, almost even, increase in slip over the course of slow swimming, flow channel swimming and burst swimming (F=108.28, P<0.001; figure 5.8). When slip was compared to swim velocity in the flow channel only, there was a strong linear correlation (Table 2; R²=0.885). This correlation was strengthened when burst swimming, not performed in the flow channel, was added to the model (R²=0.928 for cubic regression). These regressions show a steady increase in slip with the increase swim velocity measured up to 11 BL/s. After this swim velocity, slip seems to plateau at a mean of 0.52±0.02. Reynolds numbers ranged from 38 to 273 (Table 5.1; mean: 130±51) during the rheotaxis behavior. This is a range in which conditions change from viscous forces dominating to an intermediate regime.

When propulsive wavelength was measure for slow swim, rheotaxis and burst swim, we found no significant difference between the different flow speeds (F(2,12) 0.545, P=0.592)
or between rheotaxis and slow swim (figure 5.9). Also, there was no significant relationship between propulsive wavelength and swim speed (Table 5.2). However, there was a significant increase in wavelength from the fastest flow speed (0.83±0.13 BL) to that analyzed in burst swimming (1.06±0.16 BL; Table 5.1).
DISCUSSION

Animals are thought to change gaits to minimize energy cost or in response to ecological demands [Drucker and Jensen 1996; Alexander 2003]. When encountering an increase flow speed, fish will turn into the oncoming water current and swim to maintain a relatively constant position in the flow channel or environment [Montgomery et al. 1997; Donley and Dickson 2000]. Superficial neuromasts of the lateral line system respond to current flow, resulting in the rheotaxis behavior [Baker and Montgomery 1999; Voigt et al. 2000]. Ablation or chemical blocking of superficial neuromasts in three different species of fish resulted in an increase of the behavioral threshold for rheotaxis [Montgomery et al. 1997; Baker and Montgomery 1999]. In juvenile sole [Champalbert and Marchand 1994] and adult rainbow trout [Liao 2006], vision does not seem to play a role in the rheotaxis response. We briefly examined the role that vision plays on rheotaxis. Based on visual inspection, we saw no difference in the swimming pattern between rheotaxis performed in the light or dark. This indicates that the behavioral changes observed in our study have to do with water flow and not optical stimuli, which is in keeping with previous research [Montgomery et al. 2000; Liao 2006]. We consider below the linkage between water flow and larval zebrafish behaviors.

The effects of swim velocity on axial kinematics

One goal of this experiment was to look at how the larval zebrafish would respond to an increased flow speed and whether the increase in water current could elicit a burst swim response. The larval zebrafish were able to maintain a relative position in the flow channel over a range of flow speeds. As the flow speed increased, the most notable a change was in the frequency of swim bouts; the rest period between bouts decreased while the length of the swim bout remained relatively constant.

Although swimming speed elicited in the flow channel did not have a significant effect on some of the kinematic variables, we did find a significant change in tailbeat frequency (TBF), stride length and slip. Tail beat frequency is known to vary with speed in undulatory swimming animals [Hudson 1972; Webb 1975; Gillis 1997], including larval zebrafish, shown in our study. This increase in frequency has been linked to an increase in firing from the midbrain in goldfish [Fetcho and Svoboda 1993], from the nucMLF in carp [Uematsu et al. 2007] and descending reticulospinal neurons in eel [Brocard and Dubuc
2003]. It is therefore likely that a similar increase in firing rate of the descending motor control system of larval zebrafish is responsible for the increase in TBF and potentially the decrease in rest duration. While TBF did increase with speed the increase was modest and remained far below TBFs associated with burst swims which can approach 80-100 Hz [Budick and O’Malley 2000; Muller and van Leeuwen 2004].

As the swim velocity increased, we found an increase in slip which coincided with an increase in stride length (Figure 5.3, 5.8). A study in salamanders showed a similar increase in slip with swim velocity, but a decrease in slip at the highest swim velocity [Gillis 1997]. This was also previously seen when larval spontaneous slow swims were compared to burst swims [Muller and van Leeuwen 2004]. Since swim velocity increases out proportion to TBF, the fish is moving further forward for every tail beat, a stride length increase. It is assumed that a longer stride length is associated with a greater efficiency [Videler 1993], which corresponds to the increase in slip. However, this increase in stride length is not seen in other adult fish [Jayne and Lauder 1995b; Coughlin 2000] or eels [D’AoUt and Aerts 1999; Ellerby et al. 2001]. A possible explanation for this discrepancy could be due the change in fluid regime that larvae must overcome. Most undulatory swimmers swim in an inertial regime. However, the larva zebrafish swim in a viscous regime during slow swimming and combination during faster velocities (Table 5.1).

Other research shows the capability of different fish in a flow channel to increase speeds with the increasing water velocity until a different motor pattern was elicited. In smallmouth bass this pattern change was from a slow steady undulatory swim characteristic of red muscle to a unsteady burst and coast gait with the use of white muscle fibers [Jayne and Lauder 1996; Peake and Farrell 2004]. We were unable to elicit a transition in swimming in our flow regime. Although slow swim TBFs in larval zebrafish have been recorded up to 40Hz in both a fictive preparation [McLean et al. 2007] and in vivo [Budick and O’Malley 2000], neither of these experiments were performed in a flow channel. It is possible that the water current was too much for the larval zebrafish. Data from older larvae might be necessary to establish kinematic data on any intermediate tail beat frequency and swim pattern.

**Implications for neural control**

Many fishes swim via rhythmic contractions of axial muscles. These contractions are thought to be controlled by motoneurons and CPG’s located in the spinal cord [Grillner et
al. 1998]. To initiate swimming, a descending signal is sent from the brain, which synapses on the spinal interneurons, which are potentially part of the CPG [McClellan and Grillner 1984; Downes and Granato 2006; Dubuc et al. 2008; Grillner et al. 2008]. The frequency of firing of the descending neurons was found to be directly proportional to the rhythmic motor output [Rossignol et al. 2006; Uematsu et al. 2007; Gabriel et al. 2008].

Research suggests that spinal motor systems are organized into small number of fundamental units [Stein and Daniels-McQueen 2004] with spinal interneurons that preferentially located based on activity at different swimming frequencies [McLean et al. 2007]. Different spinal units are specialized for the production of particular components of motor patterns. To produce complex behaviors, a combination of the units are recruited [Cheng et al. 1998]. Perhaps then the larval zebrafish has a spinal unit for slow swim and one for burst swim. Not to say that these modules would be fully exclusive of one another in terms of number of elements contributing, but rather they are controlled and stimulated differently than one another. The slow red muscle fibers are active during slow swimming, and are controlled by small motorneurons; while the escape response, which contains the burst swim, utilizes fast white muscle fibers controlled by large motorneurons [Liu and Westerfield 1988; Fetcho 1992]. On a spinal level it was shown that interneurons known as the MCoD’s were active during slow swims [Ritter et al. 2001] but actively inhibited during the burst swim [Masino et al. 2005; McLean et al. 2007]. The interneurons and motorneurons are located in the spinal cord in a topographical map, with those neurons active in slow swimming being more ventrally located and the dorsally located neurons active at higher frequencies. This evidence suggests that the two forward swimming patterns are controlled differently.

The increase in firing rate of the descending neurons in the hindbrain could also account for the increase in stride length and efficiency. Increased firing in the superficial neuromasts due to increased water current might directly or indirectly result in increased firing of descending motor control neurons in the brainstem. This increased firing would result in a sequential recruitment of more dorsally located interneuron and/or motorneurons, substantiated by the decrease in resistance of the dorsally located neurons [McLean et al. 2007]. A recruitment of either more red fibers or a start of recruitment of white fibers could explain the increase in stride length. The increase in fiber activation would allow for a more powerful tailbeat and thus increase the distance covered in one tailbeat (i.e. stride length).
Jayne and Lauder [1994] showed that adult bluegill sunfish had an intermediate swim speed in which both red and white muscle fibers were active. At even faster swim speeds, white fibers were recruited more and red fibers were deactivated. This decrease in recruitment was also seen in 3 day post-fertilization larval zebrafish [Buss and Drapeau 2002].

**Conservation of slow swim motor pattern**

To further understand the motor pattern of the larval zebrafish, the swimming pattern in the flow channel at increased current speed was compared to the previously described slow swim and burst swim patterns [Budick and O’Malley 2000; Muller and van Leeuwen 2004]. The kinematic data in our study is consistent with previous literature. Budick and O’Malley reported slow swims with a bend angle of $46.3\pm8.1^\circ$, a bend location of $0.80\pm0.06$ body lengths and minimal yaw of $1.4\pm0.8^\circ$, compared with values from this study of $51\pm8^\circ$, $0.83\pm0.04$ and $2.5\pm0.3^\circ$, respectively. During slow swimming, we observe the pectoral fins used in alternation to assist in the forward swimming in agreement with Thorsen et al. [2004]. This behavior is sharply contrasted to the burst swim which involves rapid, large and more rostral bends of the trunk with the pectoral fins adducted resulting in a high velocity swim with large amounts of yaw [Budick and O’Malley 2000; Thorsen et al. 2004]. Again, our data on burst swim was consistent with these findings.

We then used these variables to compare the slow swim and burst swim pattern to the swimming of the rheotaxis behavior. With increase in swim velocity in the flow channel, there was no significant change in bend angle, bend location or yaw. The larval zebrafish used a mild caudal bend with relatively little yaw during all swim velocities in the flow channel. These kinematics were the same as those analyzed in the slow swim of the same larva but significantly and largely different to that of the burst swim pattern. Although the tailbeat frequency did increase with the increase in swim velocity, a high tailbeat frequency of that of the burst swim was not obtained in the flow channel. At the highest speeds in the flow channel, the larvae did elicit a slightly larger bend angle and a small shift of bend location more rostral location. While these changes were not found to be significant, some mechanical parameter must account for the greater velocity and stride length. Potentially with an older larva, a higher tailbeat frequency might be obtainable and a transition swim between slow and burst swim might be seen.
As noted above, the larval zebrafish during slow swimming used the pectoral fins in alternation, while during burst swim the fins were adducted [Thorsen et al. 2004]. It is quite rare to see alternating fin movement without slow swim axial movements [McLean et al. 2007]. During all speeds in the flow channel, we observed the alternation of the pectoral fins. In the same larva, we noted the adduction of the fins during elicited burst swims. This evidence along with our research, suggests that burst swimming where the body has more lateral undulation, is a different locomotor pattern than the rheotaxis behavior, which is kinematically similar to the slow swim locomotor gait.

Our results in this study add to the growing body of evidence that the larval zebrafish have two distinct forward swimming patterns [Hudson 1972; Budick and O’Malley 2000; Buss and Drapeau 2001; Thorsen et al. 2004; McLean et al. 2007]. Due to the complexity of the neural networks, it is theoretically possible that these swimming patterns are conserved and utilized via different sensorimotor transformations. The fact that the same motor pattern of slow swimming is seen in the previously described OMR and light-evoked as well as the current study, suggests a preference in gait pattern for navigational behaviors.
Figure 5.1. Bar graph showing the mean swim velocity of zebrafish larvae (N=8) during spontaneous slow swim, 3 speeds of flow channel swimming and burst swim elicited by a tactile escape response. A slow (1.88 mm/s); moderate (4.22 mm/s) and fast (6.55 mm/s) current flow was used to elicit a swimming behavior. There was a significant increase in swim velocity at each of the different levels measured. **P<0.001. Error bars represent standard errors (S.E.M.).
Figure 5.2. Graphs showing the affect of swim speed on tailbeat frequency (TBF). (A) TBF of the slow swim pattern was similar to the TBF seen in the slow velocity swim channel. There was a significant difference between the TBF of burst swimming and the fastest swimming attained in the flow channel. (B) Markers represent the mean TBF and mean swim velocity for each individual fish at the 5 different levels tested in A. As swimming velocity increased, there was a concurrent increase in TBF. $R^2 = 0.921; P<0.001$ **$P<0.001$. Error bars represent standard errors (S.E.M.).
Figure 5.3 Stride length, the distance traveled (expressed in body lengths) in one tailbeat, was compared over the different motor patterns (A) and over swimming velocity (B). (A) There was an increase in stride length from slow swimming to flow channel swimming, over the varying speeds in the flow channel and from flow channel swimming to burst swimming. **P<0.001. Error bars represent standard errors (S.E.M.). (B) Markers represent the mean stride length and mean swim velocity for each individual fish at the 5 different levels tested in A. The was a strong correlation between stride length and swim velocity. (R² = 0.985)
**Figure 5.4** Maximum bend location as a fraction of body length. (A) Bend location was consistent from slow swimming to the different speeds in the flow channel. However, there was a significant change to a more rostral bend location during the burst swim. **P<0.001. Error bars represent standard errors (S.E.M.).** (B) Markers represent the mean bend location and mean swim velocity for each individual fish at the 5 different levels tested in A. The was no affect of swim speed on bend location (R² = 0.102)
**Figure 5.5** Bend Angle. (A) There was no significant difference of bend angle between the different flow speeds or between the flow speeds and slow swimming. There was a significant difference between the fastest flow speed and the burst swim. **P<0.001.** Error bars represent standard errors (S.E.M.). (B) Markers represent the mean bend angle and mean swim velocity for each individual fish at the 5 different levels tested in A. With the increasing swim velocity during the slow swim or flow channel swimming, we saw no correlation between swim speed and bend angle.
Figure 5.6 Bout duration and rest time. (A) The mean length of time for the bout and rest period between bouts seen during slow swimming (water flow speed = 0) and flow channel swimming. In order to maintain position in the faster water current, the larval zebrafish decreased the rest time between bouts. There was no significant difference in bout duration time over the changing water flow speeds. Water flow speeds 1.88 mm/s = slow flow; 4.22 mm/s = moderate flow and 6.55 mm/s = fast flow. (B) Markers represent the mean bout duration (open circles) or mean rest time (filled circles) and mean swim velocity for each individual fish at the 5 different levels tested in A. When time was plotted over swim speed, the bout duration remained constant (open circles; $R^2 = 0; P=0.956$) while the rest time between bouts decreased (filled circles $R^2 = 0.220; P<0.05$).
Figure 5.7 Yaw. (A) There was no significant difference in yaw between slow swimming and the swimming exhibited in the flow channel. However, there was a large increase in yaw when rheotaxic behavior was compared to burst swimming of an escape response. **P<0.001. Error bars represent standard errors (S.E.M.). (B) Markers represent the mean yaw and mean swim velocity for each individual fish at the 5 different levels tested in A. When plotted over swimming velocity, we see no correlation. (R² = 0.164) between yaw and swim velocity.
**FIGURE 5.8**

Figure 5.8 Slip is the ratio of swimming velocity to propagating body wave velocity. (A) We found an increase in slip from slow swimming to flow channel swimming and from flow channel swimming to burst swimming. **P<0.001. Error bars represent standard errors (S.E.M.).** (B) Markers represent the mean slip and mean swim velocity for each individual fish at the 5 different levels tested in A. With the increasing swim velocity up to 11 BL/s there was an increase in slip. At higher velocities measured there was a more consistent slip.
Figure 5.9 Wave length is the length the propulsive wave of contraction travels down the body during steady swimming. There was no significant change in wave length during the increase in velocity in flow channel swimming. However, the wave length of burst swimming was significantly different than that of slow swimming and of flow channel swimming. (A)**P<0.001. Error bars represent standard errors (S.E.M.). (B) Markers represent the mean wave length and mean swim velocity for each individual fish at the 5 different levels tested in A.
## Table 1

<table>
<thead>
<tr>
<th></th>
<th>Slow Swim</th>
<th>Slow Flow</th>
<th>Moderate Flow</th>
<th>Fast Flow</th>
<th>Burst Swim</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBF (Hz)</td>
<td>25.9 ± 1.9</td>
<td>24.6 ± 3.4</td>
<td>27.2 ± 2.4</td>
<td>28.1 ± 2.3</td>
<td>57.8 ± 12.4</td>
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<tr>
<td>Bend Angle (degrees)</td>
<td>54.8 ± 11.5</td>
<td>52.3 ± 13.2</td>
<td>53.3 ± 14.1</td>
<td>56.2 ± 10.2</td>
<td>78.0 ± 10.6</td>
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<tr>
<td>Bend Location (BL)</td>
<td>0.84 ± 0.05</td>
<td>0.83 ± 0.03</td>
<td>0.82 ± 0.04</td>
<td>0.82 ± 0.03</td>
<td>0.66 ± 0.01</td>
</tr>
<tr>
<td>Yaw (degrees)</td>
<td>2.5 ± 1.2</td>
<td>2.6 ± 1.4</td>
<td>3.7 ± 2.1</td>
<td>3.0 ± 2.1</td>
<td>24.8 ± 4.7</td>
</tr>
<tr>
<td>Wave length (BL)</td>
<td>0.84 ± 0.05</td>
<td>0.83 ± 0.11</td>
<td>0.82 ± 0.18</td>
<td>0.83 ± 0.13</td>
<td>1.06 ± 0.16</td>
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<tr>
<td>Stride Length (BL)</td>
<td>0.10 ± 0.03</td>
<td>0.17 ± 0.04</td>
<td>0.27 ± 0.06</td>
<td>0.32 ± 0.04</td>
<td>0.53 ± 0.07</td>
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<tr>
<td>SLIP</td>
<td>0.12 ± 0.02</td>
<td>0.20 ± 0.05</td>
<td>0.32 ± 0.09</td>
<td>0.40 ± 0.08</td>
<td>0.52 ± 0.03</td>
</tr>
<tr>
<td>Duration (ms)</td>
<td>174.3 ± 27.7</td>
<td>174.9 ± 57.1</td>
<td>186.3 ± 88.7</td>
<td>155.5 ± 41.0</td>
<td>82.8 ± 20.6</td>
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<tr>
<td>Rest (ms)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>548.6 ± 296.0</td>
<td>396.5 ± 200.5</td>
<td>320.2 ± 185.1</td>
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<td></td>
</tr>
<tr>
<td>Reynolds Number</td>
<td>54 ± 18</td>
<td>81 ± 27</td>
<td>127 ± 37</td>
<td>172 ± 36</td>
<td>591 ± 59</td>
</tr>
</tbody>
</table>

Table of means +/- standard deviation of the kinematic variables for each of the behaviors tested; spontaneous slow swim, 3 different water current levels and burst swim during a tactile escape response.
# Table 2

<table>
<thead>
<tr>
<th>Variable</th>
<th>Constant</th>
<th>Slope</th>
<th>R²</th>
<th>F_{(1,22)}</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBF</td>
<td>23.3±1.0</td>
<td>0.57±0.15</td>
<td>0.363</td>
<td>14.828</td>
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<tr>
<td>Bend Angle</td>
<td>45.7±3.4</td>
<td>1.11±0.53\textsuperscript{a}</td>
<td>0.146</td>
<td>4.444</td>
<td>0.055</td>
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<tr>
<td>Bend Location</td>
<td>0.82±0.01</td>
<td>-0.004±0.002\textsuperscript{a}</td>
<td>0.102</td>
<td>2.944</td>
<td>0.098</td>
</tr>
<tr>
<td>Yaw</td>
<td>2.1±0.5</td>
<td>0.17±0.07\textsuperscript{a}</td>
<td>0.164</td>
<td>5.119</td>
<td>0.032</td>
</tr>
<tr>
<td>Bout Duration</td>
<td>180±27</td>
<td>0.24±4.21\textsuperscript{a}</td>
<td>0.000</td>
<td>0.003</td>
<td>0.956</td>
</tr>
<tr>
<td>Rest</td>
<td>669±97</td>
<td>-34.63±14.23</td>
<td>0.220</td>
<td>5.923</td>
<td>0.024</td>
</tr>
<tr>
<td>Stride Length</td>
<td>0.02±0.01</td>
<td>0.03±0.001</td>
<td>0.965</td>
<td>713.608</td>
<td>**</td>
</tr>
<tr>
<td>Slip</td>
<td>0.03±0.02</td>
<td>0.04±0.003</td>
<td>0.885</td>
<td>199.226</td>
<td>**</td>
</tr>
<tr>
<td>Wave Length</td>
<td>0.80±0.04</td>
<td>0.01±0.01\textsuperscript{a}</td>
<td>0.042</td>
<td>1.147</td>
<td>0.294</td>
</tr>
</tbody>
</table>

Table of regressions against swim velocity for data collected from slow swims and flow channel. Burst swimming was not included in the above calculations. Graphs are shown in the figures on this paper. F and P values are for the effect of swim speed

** P<0.001

\textsuperscript{a} not significantly different from 0 (P<0.05)
CHAPTER 6

GENERAL DISCUSSION:
CONSERVATION OF MOTOR PATTERNS AND
MUSCLE SYNERGIES: HYPODISSertation AND
FUTURE DIRECTIONS
Much of the data provided here has been derived by kinematic analysis of the larval zebrafish in specific sensory contexts and with regard to the underlying neural controls. This approach and analysis has both general and specific relevance to systems neuroscience in that it specifies the motor output that an animal’s nervous system must produce and it constrains the range of possible solutions to behavioral tasks by showing the actual movement patterns used in different circumstances. While these two general attributes of kinematic analysis are central to the understanding of neural systems, further insights are often gleaned by virtue of the specific kinematics seen during motor pattern generation and sensorimotor integration. For example, an early attempt to categorize the variety of larval zebrafish motor patterns [Budick and O’Malley 2000] led to the proposal of independently controlled spinal central pattern generators. This kinematically-motivated insight has been increasingly supported by a range of different neuronal, muscular and behavioral analyses [Buss and Drapeau 2002; Thorsen et al. 2004; Masino et al. 2005; Day et al. 2006; Bhatt et al. 2007; McLean et al. 2007]. The findings of the present dissertation touch upon this issue and more substantially extends into issues of muscle synergies and sensorimotor integration.

Although this dissertation found many interesting outcomes, the unifying theme that emerges from these kinematic results is that one of the building blocks upon which diverse motor patterns are built is the slow swimming pattern; a forward swimming pattern quite distinct from the burst swimming pattern. The subsequent sections attempt to construct the simplest possible model that will account for the breadth of sensorimotor and kinematic observations of this dissertation. It will be argued that by defining the precision and details of motor patterns, in relation to the sensory input, that one can learn many details that define the functional organization of the larval locomotor neural networks.

**Muscle Synergies or Modules**

With an infinite number of neural circuits capable of being formed in the central nervous system, it is amazing to see an animal perform behaviors effortlessly and efficiently. Recent research has put forth the hypodissertation that the nervous system is composed of discrete building blocks that can be utilized in different combinations and strengths to perform a variety of different behaviors [Grillner 1985; Stein et al. 1997; Bizzi et al. 2008]. This research has been performed in spinalized frogs [Giszter et al. 1993], intact freely moving frogs [d'Avella et al. 2003], rats [Tresch and Kiehn 1999], turtles [Stein and
Daniels-McQueen 2004], cats [Ting and Macpherson 2005] and humans [Torres-Oviedo and Ting 2007]. The concept is referred to as a module or a muscle synergy; a functional unit with a distinct motor output. These modules could be combined together to perform a variety of motor patterns and behaviors in the same way letters in the alphabet can be combined together to make a variety of words and sentences, respectively. By changing the amplitude, duration and sequence of these motor patterns a variety of behaviors can be performed to allow for animals to detect and avoid prey, find and secure food, and move about in their environment. It is possible that these modules are composed of smaller subunits and it is possible that the interneurons contained within the modules are mutually exclusive. Nonetheless, individually stimulating these independent modules, by supraspinal input or artificial means, results in the same motor output via activation of a specific pattern of muscle contraction. It is thought to be the job of the descending motor control system, such as the reticulospinal network in zebrafish, to coordinate the modules to perform behaviors. With modification from sensory feedback, these modules can be adapted to external environment [Bizzi et al. 2008; Grillner et al. 2008].

**Conservation of Slow Swim Motor Pattern**

Larvae exhibit a large number of locomotor behaviors, but a smaller number of motor patterns. The conserved use of slow swim patterns and turning pattern, discussed later, is substantial case in point. This is not to say that there are no variations on these themes or transitional behaviors, because both such deviations from ideal behaviors do occur, as will be discussed. Rather, the point is that larval spinal cord has the necessary infrastructure to generate slow and fast forward swimming, and the larval brain can use its control of said spinal circuits to generate a diversity of locomotor behaviors using conserved building blocks.

We propose that there is a common module (or synergy) for slow swimming. We find that a slow swim pattern, such as that used spontaneously [Budick and O’Malley 2000] and during prey tracking [McElligott and O’Malley 2005] is also used during OMR, light-evoked swimming and the rheotaxis behaviors. The slow swimming pattern shown in this dissertation all share some common kinematics; caudal bend location (approximately 81% of the body length), low bend amplitude (approximately 54°) and minimal yaw (<5°). That
the pectoral fins are used in alternation during these behaviors coincides with the definition of this pattern as a slow swim gait by Thorsen et al. [2004]. Thus we conclude the slow swim is a conserved motor pattern that different sensory inputs (retina, lateral line) are able to modulate. However, at no time could we elicit or did we see a motor pattern similar to the escape response burst swimming, unless we elicited an escape response with a tactile stimulus. Nor did we see characteristics of both burst and slow swim at the same time such as high tailbeat frequency with small caudal bends. When other researchers used calcium imaging to look at the activity of 2 spinal interneurons during forward swimming in the partial restrained larvae, the MCoD cell showed increased activity during slow swimming, but not during burst swimming where the CiD cell showed increased activity in burst swimming but not in slow swimming [Ritter et al. 2001; Bhatt et al. 2007; McLean et al. 2007]. Therefore, we speculate that there is a preference to stimulate slow swim circuits over burst swim circuits during navigational behaviors.

This fits well with the idea that the 5 to 10 day old larva principally uses two distinct forward swimming patterns: slow and burst. The slow swim pattern has been associated with the use of red muscle [Buss and Drapeau 2002] and is observed in the present work during navigational behaviors wherein zebrafish larvae attempt to maintain their position within the environment. Given that small larvae may be exposed to overwhelmingly large currents [Laale 1977; Spence et al. 2008], it is reasonable to expect them to cautiously expend energy to maintain position as OMR and rheotaxic behaviors will tend to do. Burst swimming being expensive [Blake 2004], it seems likely that continuous usage of the high-performance burst swim pattern might quickly exhaust larvae, leaving them unable to hunt or escape predators. In this context, it makes sense that zebrafish larvae do not regularly employ burst swimming, even when optic-flow or water-flow sensory input indicate that they are being displaced from a particular location. Our data are also consistent with the idea that zebrafish larvae prefer (at least within a laboratory setting) to maintain position within a lit consistent environment since they swim toward an area that is broadly illuminated, or in response to stimuli that represent their being swept from their current environment (current flow, optic flow). Interestingly, changing the illumination conditions from light-to-dark produces a directly opposite behavior, evoking a dark-evoked turn (DET) or dark response (original data provided here; also see [Burgess and Granato 2007]). This dark response is clearly not an escape behavior, either by kinematic definition or by consequence. Larval
escape behaviors are categorized by high angular velocities [Liu and Fetcho 1999; Budick and O’Malley 2000] and an accompanying burst swim that removes the larva from harm’s way. The dark-evoked turn is of a very low angular velocity – within the parameters of larval routine turns—and results in the larva turning toward where the light was extinguished [Burgess and Granato 2007], somewhat of a disadvantage if in the path of a charging predator. The simplest interpretation of this data is that the sudden decrease in illumination is indicative to the larva of movement into a darkened environment. The proper response in this model is re-orientation into the previously lit environment. Figure 3.10 shows that OMR responses are observed primarily in response to forward motion of the optic flow (indicating rearwards motion of the larva). This stimulus indicates displacement from the current environment. Based on the simple idea that larvae should orient into the optic flow (an idea consistent with all of our forward moving stimuli), one would predict that rearwards optic flow would produce the very largest OMR turns. Instead, larvae are virtually unresponsive to directly rearwards optic flow, under our experimental conditions. Further experimentation combining hydrodynamic and optic flow would help to further delineate the circumstances in which adaptive navigational behaviors are employed, but all of the evidence obtained from these first kinematic analyses of OMR, light, dark and rheotaxic behaviors is consistent with a simple preference of zebrafish larvae for a lit environment.

It is not the case that these data rule out the presence of discrete, independent motor control systems for each of these different behaviors, but rather that the simplest model is one with a slow-swim generating CPG that is driven from brainstem and that in turns uses the more economical red muscle system. This is also our preferred model given the relatively small number of neuronal cell types in spinal cord (Hale et al., 2001)—it does not seem that spinal cord has enough cells or cell types to provide independent spinal generation mechanisms for all the larval behaviors. Hudson [1972] suggested that the separation of red and white muscles into two distinct systems must occur at the spinal cord either by different level of excitability of motoneuron pool or different neuronal pathways. This proposed model contrasts that seen in lamprey models in which there is a unified network of spinal interneurons that are controlled by varying the descending supraspinal inputs [Grillner et al. 1991; Soffe 1993; Zelenin et al. 2001; Dubuc et al. 2008]. However, anatomical, physiological and behavioral evidence presented throughout this dissertation suggests that
there are differences between the spinal networks used in larval zebrafish for forward locomotion.

The proposed slow swim module may have some neurons that overlap with other modules [McLean et al. 2007] and/or it may vary slightly due to different stimulation or neuromodulation from the brainstem. For instance in *Xenopus*, some spinal interneurons are activated during fictive swimming and struggling behaviors [Soffe 1993]. This type of sharing was also found through the use of electrophysiology in larval zebrafish, where commissural, bifurcating, longitudinal interneurons (CoBL) were active in a wide range of swimming frequencies and in struggling behavior [Liao et al. 2006]. Since both slow swimming and burst swimming are produced by the same muscles, but different fibers, it stands to reason that there must be some overlap between the two neural circuits. Also, with the relatively little number of spinal interneurons in zebrafish, it is reasonable to think that interneurons would be used in more than one module.

Most vertebrate movements can vary in speed and duration. The gradation in movement can be explained by both the increased firing of active motor neurons and the recruitment of new motor neurons and motor units [Henneman 1957]. In our study, we saw variation in tailbeat frequency within the OMR and rheotaxis slow swimming behaviors. In *Clione*, the two modes of swimming are neuronally different in configuration and activity of individual neurons of the CPG network [Satterlie 1991]. In lamprey, the production of larger movements is thought to be due to increase firing of reticulospinal neurons as well as a recruitment of inactive reticulospinal neurons by the mesencephalic locomotor region [Deliagina et al. 2000; Brocard and Dubuc 2003; Zelenin et al. 2003; Dubuc et al. 2008]. In larval zebrafish, the recruitment of neurons with increased swim speed occurred in the spinal cord, with the more ventral excitatory neurons active at the lowest frequency and the more dorsal neurons recruited for faster frequencies [McLean et al. 2007]. However, there was active inhibition of some neurons (MCoD) at the higher frequencies associated with burst swimming [Masino et al. 2005]. During burst swimming there was little recruitment of the inactive spinal interneuron [Bhatt et al. 2007]. Instead there was an increase in firing of the already active CiD neurons that accounted for the varying strengths and velocity of escapes. Thus, the variations we saw in tail beat frequencies could be due to either increase firing of active spinal neurons or recruitment of new interneurons. The increase in tailbeat frequency
partially accounts for the increase in swimming velocity seen in the OMR and rheotaxis behaviors.

A future experiment that could elucidate this theory of a separate slow swim module would be ablation of selective cells. The MCoD spinal interneuron, through technically demanding ablations, have been shown to be active at slow swimming and inhibited during burst swimming. The ablations resulted in loss of slow swimming, determined by loss of axial movements without loss of alternating pectoral fin movements. However, the same fish were able to perform axial movements necessary for a burst swim [McLean et al. 2007]. It would be interesting to see what deficits would arise when the larval zebrafish with ablated MCoD’s is subjected to rheotaxis, OMR, and then light evoked swimming. If the slow swimming module is conserved, then one would expect to find deficits in all the behaviors that utilize the slow swimming circuit without a disruption in burst swimming. If ablation of these cells in the rostral spinal cord does not disrupt all the behaviors, this does not disprove the theory of a common slow swimming module. An alternative hypodissertation would be that there are small subunits within the slow swimming module that are employed for different behaviors. While the details of these motor control systems remain to be elucidated, this proposal of an elemental slow-behavior generating system is both parsimonious and well suited to the red/white muscle control systems described in larval zebrafish spinal cord [Westerfield et al. 1986].

Conservation of Turning Behaviors

A similar conservation of behavioral elements occurs in the turning domain, wherein routine turns which appear spontaneously are also used in the OMR and accompanied by forward swimming—appropriate with the need to maintain position in the environment. This is quite distinct from the other types of turns in the larval zebrafish’s repertoire; namely the J-turn and C-bend. Zebrafish larvae perform a prey-tracking behavior that utilizes a sequence of motor patterns including slow swims and a unique locomotor maneuver called a J-turn. First described by McElligott and O’Malley [2005], the J-turn is distinct from all other larval motor patterns, in terms of both function and neural basis. The J-turn consists of extremely far-caudal bending of the trunk in a rhythmic yet highly asymmetric fashion. This
pattern is distinct from the whole-larval axial bending patterns of escape turns, dark-evoked turns and routine turns. It is also distinct in terms of sensorimotor integration in that its use during prey tracking results in precise orientation of the larvae with the prey item, a paramecium in the published studies.

In contrast with the J-turn, larval OMR turns are a low-precision locomotor maneuver: they only coarsely align larvae with the stripe-motion axis, and sometimes move the larval orientation slightly away from the stripe motion axis. One possible interpretation is that OMR relies on a relatively coarse visuomotor transformation system. The zebrafish retina projects to 10 different afferent fields [Easter and Nicola 1996] of which the optic tectum is by far the largest. The tectum is thought to be involved in behaviors of orientation and predator avoidance, which is evident by the disruption of prey tracking upon ablation of the tectum [Gahtan et al. 2005], while OMR [Roeser and Baier 2003] and dorsal light response [Ullen et al. 1997] can survive tectal ablation and so may be subserved by one of the smaller afferent fields, which may not have the precise visuomotor transformation capabilities evident in the optic tectum’s control of prey tracking. The pretectal area was shown as a visuomotor relay for the dorsal light response [Ullen et al. 1997]. OMR is severely disrupted or completely absent in zebrafish mutants that have a pretectal areas (AF4 and AF9) absent, reduced of overinnervated [Muto et al. 2005]. It is possible that the OMR evolved as a turning behavior and so the circuits that produce OMR have integral to them a turning component—one that serves to generally orient larvae towards the stripe motion axis. Since fine-alignment does not seem necessary for good OMR performance and station holding, utilizing a more precise neural circuit, like that used in prey capture, might not have any selective advantage.

We suggest that spontaneous routine turns, OMR turns, light-evoked (LES) turns and the distinct dark-evoked turns (DET), may use conserved turn-control circuits. The routine-turn module uses comparatively low angular velocities (mean=5.1±0.9 °/ms), whereas as escape turns have much larger values (mean=20.6±5.0°/ms). The slow velocity turn module can be turned on or off and can be altered to allow for different degrees of bend angle as determined by the stimulus. The transition from the largest OMR turns (in response to slightly forwards stimuli) to no OMR response (when the stripe motion axis is shifted to a slightly rearwards direction) is a good example. This suggests that OMR is mediated by a directional control system tuned to forward optic flow. When our stimulus (optic flow axis)
transitions from angles of less than 90 degrees to angles greater than 90 degrees (with respect to larval orientation), the directional cells being activated transition from forward directional selective cells to rearwards directional selective cells. Since OMR is not a learned behavior, this means that evolutionary processes have resulted in developmental programs that have been hard-wired for forward directional selective pathways to a motor-pattern generating system that is capable of producing scaled turn angles in conjunction with the slow-swim motor pattern. In contrast, we would predict that rearward-tuned directional selective cells will either produce no motor response and/or may inhibit the elicitation of the OMR motor program.

It is possible that these 2 building blocks of low velocity and high velocity might have a subunit of a neural circuit for the generation of sharp turns. Thus the dark evoked turns would require the stimulation of a sharp turn module and the low-velocity module, and the C-bend escape response would combine a sharp turn and a high-velocity module. The supraspinal input would recruit, coordinate or modulate these different spinal circuits. In lamprey, the activation of different reticulospinal neurons results in different directional changes, either turn, pitch or roll, via indirect activation of a subset of motorneurons [Zelenin et al. 2001]. When the M-cell array (M-cell, MiD2cm, MiD3cm) in goldfish was ablated, the angular velocity of the C-bend was increased, due to an increase in duration of the C-bend while still maintaining a high bend angle. However, when just the M-cell was ablated there was no change in angular velocity of the initial C-bend [Liu and Fetcho 1999]. This suggests that the two M-cell homologues (MiD2cm and MiD3cm) might help contribute to the angular velocity, perhaps by recruiting or modulating different spinal interneurons. Recent research showed that after ablation of a small number of neurons in the larval zebrafish’s hindbrain, the turning component of the OMR response could be abolished, while not affecting the forward swim component or turns to the opposite side [Orger et al. 2008]. All of this evidence lends credence to the idea that the spinal cord is made of building blocks (slow swim and slow velocity turn in this case) that the descending motor system puts together to perform necessary behaviors.
**Combination of modules and sensorimotor integration**

We are not saying that larvae cannot combine different motor patterns or make complex, sensory guided transitions. In fact, this is exactly how muscle synergies are thought to work. The larval capture swim (Borla et al., 2002) already documented that larvae can dynamically modulate bend location, bend amplitude and tail-beat frequency on a half-cycle basis, that results in a transition from a slow-swim TBF to an instantaneous fast-swim range TBF at the moment of capture. Their interpretation is that the capture swim begins precisely as a slow swim pattern and is then altered by descending controls that tap into certain features of the burst swim spinal circuits to produce the burst-swim caliber TBFs seen at the moment of the strike (see Fig. 4 in [Borla et al. 2002]). Genetic targeting of Aequorin to the primary motor neurons might eventually be able to reveal such a transition in free swimming fish [Naumann et al. 2007].

While it is parsimonious to characterize the different slow turns as having a common neural basis, the sensory generation of different slow turning and swimming behaviors indicates diverse and specialized sensorimotor integration processes. Here we consider the neural transformations that must exist in order for such behaviors to occur. An instance of sensorimotor integration processes distinguishing related optical stimuli is in the production of LES vs. DETs. It is tempting to speculate a brainstem locus wherein the ON vs. OFF visual pathways are differentially hardwired into specific motor pattern generating circuitry. In this instance, ON-pathways would elicit a forward swimming pattern, whereas OFF pathways would elicit the largest of the routine turns, the dark-evoked turn. While both the LES and the DET are slow motor patterns, they could not be more different within the context of this motor program space. The LES stimulus feeds into a relatively simple "slow swim" command center, which we expect would also be used during other slow-swim generating behaviors such as OMR and perhaps prey tracking. In contrast, the DET stimulus must produce highly asymmetric activation of descending control fibers, and also produce stronger activation then does the OMR or spontaneous routine turn generating sensory stimuli.

One possible way to tease out the different neural circuits involved in the visuomotor transformation would be with the use of the mutant zebrafish discussed in Muto et al. [2005]. They showed a weak dissociation between the OMR and OKR neural circuits, suggesting a sharing of 2 underlying neural circuits. To understand the sensorimotor
integration, an experiment could look at the different mutants’ response to the different stimuli that elicit slow swimming. For instance, the mutant *missing link* was found to have the pretectal areas AF4 and AF9 absent or reduced. In these mutants, both the OMR and OKR response were absent, but slow swimming activity and visually mediated background adaptation (VBA) were normal. It would be interesting to see how these larvae respond to an increase in whole-field illumination. Would other mutants with photoreceptor deficits/depletions, like *lime light*, be unable to perform a LES or DETs? These mutants do demonstrate normal slow swimming activity and a normal VBA response, which was shown not to be mediated by the pineal gland but perhaps by other receptors in the retina. These experiments on the different mutants could give insight into the circuits of visuomotor transformation. Further exploration by neuronal cell ablation, electrophysiology and calcium imaging would deepen our understanding of the neuronal pathways as well as provided insight into the slow swimming and turning modules of the spinal cord.

**Summary**

The larval zebrafish brain possesses a world of unknowns, in terms of the loci of sensorimotor transformations, the computational mechanisms, and the locus of the motor pattern generating systems for the diverse turning and swimming behaviors observed in this dissertation. The favorable features of the larval zebrafish are that these systems are vastly simpler than in mammalian species and that they are optically accessible. But most importantly is that the neurons are individually identifiable. As such, there is hope that these systems can be investigated to expand upon our understanding of the neural basis of vertebrate behaviors. While kinematic analyses are often viewed as simple studies of behavior, the details emerging from this work define a variety of important sensorimotor transformations and motor patter generation mechanism: we now know what to look for in examining the functional organization of the larval brainstem and spinal cord.
REFERENCES


