Fall 1997

Neuroethology of the swimming behavior in the Pacific nudibranch, Melibe leonina

Kathryn Ann Lawrence

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NEUROETHOLOGY OF THE SWIMMING BEHAVIOR IN THE PACIFIC NUDIBRANCH, *Melibe leonina*

BY

**KATHRYN A. LAWRENCE**  
Bachelor of Arts, Colgate University, 1989

DISSERTATION

Submitted to the University of New Hampshire  
in Partial Fulfillment of  
the Requirements for the Degree of  

Doctor of Philosophy  

in  

Zoology  

September 1997
This dissertation has been examined and approved.

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ABSTRACT

NEUROETHOLOGY OF THE SWIMMING BEHAVIOR IN THE PACIFIC NUDIBRANCH, Melibe leonina

BY

Kathryn Lawrence
University of New Hampshire, September 1997

Melibe leonina is a subtidal nudibranch which is found in association with eelgrass and kelp. Melibe swims when disturbed, or knocked off of its substrate, by slow lateral undulations. Swimming is stereotyped in its form and execution, and can be reliably reproduced in the laboratory. Swimming consists of phases: (1) withdrawal, (2) flattening, (3) lateral flexions, (4) unfolding and swinging and (5) termination. Swimming can be reliably elicited using 1M KCl or contact with the tube feet of the predatory seastar Pycnopodia helianthoides. The duration of a single swim cycle and the amplitude of swimming flexions remain relatively constant, however, both the latency to swim, and the swimming duration, are more variable. Swimming moves the animal upward and anteriorly and the direction of travel is fairly predictable from one flexion to the next. Melibe swimming is a stereotyped fixed action pattern that is probably used, in part, to escape from predators.

The brain in Melibe is composed of four bilateral pairs of fused ganglia which surround the esophagus. The neural components for swimming make up a central pattern generator within the brain of Melibe. The nerves which innervate swimming structures arise from the pedal ganglion, and the motorneurons which drive swimming are also located within the pedal ganglion. Intracellular recordings of swimming can be reliably obtained in semi-intact and
isolated brain preparations, however only if the pedal commisure is left intact and the ambient lighting is turned down. *Melibe* swimming is amenable for further neurophysiological studies to more rigorously look at the neural basis of swimming.

Light has a profound effect on the likelihood of swimming in *Melibe*. The initiation or termination of light cannot start or stop swimming, however, animals locomote and swim significantly more in the dark. This effect was also seen in isolated brain preparations, where fictive swimming was disrupted by, even low level, light. This effect could be reversed, however, if the eyes were removed. Thus *Melibe* is a model swimming system, that of a lateral bend swimmer, whose behavior is reliably modulated by natural inputs which can be methodically tested in the laboratory.
INTRODUCTION

"Rhythmic behaviors are those in which all or part of an animal's body moves in a cyclic, repetitive way: examples are walking, swimming, scratching and breathing."

F. Delcomyn 1980

All animals exhibit rhythmic behaviors of one sort or another. Locomotion is the most universal of these behaviors and one that is easily identified in many species. Walking, crawling, swimming and flying are all behaviors which, once initiated, are accomplished "automatically" and, for the most part, without variation until the need for locomotion is past. Each form of locomotion may be simple or complex, depending on the needs and resources of the animal.

The prevailing ideas of how the nervous system produces rhythmic behavior have gone through many iterations, especially in the last 20 years. A concept which has been refined a number of times, but has remained central in this field is that of the central pattern generator (CPG). A CPG is defined as "a network of interconnected neurons that can provide rhythmic output with neither rhythmic input nor reflexes" (Arshavsky et al., 1993). This idea originated as a strict hierarchy; with a "command neuron" (Kupfermann et al., 1978) at the top of the hierarchy which directed the production of a behavior by activating subsequently "less important" interneurons and motoneurons. This concept has been modified many times and is now viewed as a more integrated system, with many neurons having the ability to participate in multiple behaviors, and the system allowing for modulation by sensory and state inputs (Getting, 1976; Getting et al., 1985b; Kristan et al., 1988; Getting, 1989; Harris-Warrick et al., 1989; Hooper et al., 1989; Wu et al., 1994; Jing et al., 1995).
In marine habitats, the primary forms of locomotion are crawling and swimming. Crawling is accomplished by either ciliary or muscular action, and is, in general, slower than swimming. Swimming is accomplished by muscular contractions which cause parts of the body to "push" against the water to effect movement. These contractions range from very simple, "all-or-none" abrupt contractions (as in the swimming anemone, *Stomphia* (Robson, 1961)) or intricately orchestrated, graded contractions of multiple muscle groups to effect a variety of swimming speeds as seen in Clione (Satterlie *et al.*, 1985a; Satterlie, 1991) as well as many aquatic vertebrates (Grillner, 1985). The complexity of the swim need not be related to the level of the animal on an evolutionary scale. Simple systems are often used to uncover the basic components of how nervous systems produce patterned behaviors.

Opisthobranchs have been used extensively for studying the neural control of rhythmic behaviors, such as swimming, because these stereotyped and easily identifiable behaviors are expressed in semi-intact preparations (Willows *et al.*, 1973b; von der Porten *et al.*, 1980) as well as isolated ganglia (Dorsett *et al.*, 1973; Getting *et al.*, 1985a; Jing *et al.*, 1995), and their "simple" nervous systems contain neuronal cell bodies which are: 1) relatively limited in number; 2) arranged at the ganglion surface; 3) typically large in diameter (20-100µ) and; 4) located in consistent positions within the ganglia of different animals. These factors facilitate the study of individual, identifiable cells in multiple preparations (Audesirk *et al.*, 1985).

Molluscan swimming behavior has been described behaviorally and neurophysiologically for a number of species, including *Hexabranchus* spp. (the Spanish Dancer) (Edmunds, 1968; Thompson, 1972), *Pleurobranchaea membranaceus* (Thompson *et al.*, 1959; Jing *et al.*, 1995), *Dendronotus giganteus* (Agersborg, 1922), *Gastropteron pacificum* (Reinhart, 1967), *Tritonia diomedea*
(Dorsett et al., 1973; Getting, 1983b), Clione limacina (Satterlie et al., 1985a; 1985b; Satterlie, 1991) and Aplysia brasiliana (McPherson et al., 1991a; 1991b). In most cases, swimming is highly stereotyped and reliably expressed in intact, semi-intact and isolated nervous system preparations (von der Porten et al., 1980). In opisthobranchs, three types of swimming have been described (Farmer, 1970): 1) parapodial or mantle flapping (for example, Gasteropteron, Hexabranchus and Aplysia); 2) dorso-ventral undulation (as in Tritonia and Pleurobranchaea) and; 3) lateral bending (as in Dendronotus). Out of the 47 species discussed, Farmer found 21 that swim by flapping either the mantle or some part of the foot, 5 that swim by dorso-ventral undulation and 18 that swim by lateral bending of the body; the latter being the most common type used by aeolidacean and dendronotacean members of the Order Nudibranchia. Lateral bend swimming in these animals does not seem to propel them in a particular direction; rather, it appears as if swimming moves animals into the water column where the current may carry them away from potential predators (Jing et al., 1995).

Thompson hypothesized that swimming in opisthobranchs evolved as a means of escape (Thompson, 1976). For most of the species studied, this seems to be a feasible explanation since it is an elective behavior that is often triggered by external, noxious stimuli, such as poking or the touch of a potential predator (Edmunds, 1968; Dorsett et al., 1973; McPherson et al., 1991a, 1991b; Page, 1993). Some animals, such as T. diomedea, apparently employ swimming solely as a means of escape (Willows et al., 1973a). While in others, like A. brasiliana, which has no known predators, swimming appears to be a means of migration (Hamilton et al., 1975).

Melibe leonina, a Pacific, subtidal dendronotacean nudibranch, is a lateral bend swimmer, unlike the other swimming dendronotaceans that have been studied (Tritonia and Pleurobranchaea), and is relatively plentiful and accessible.
for research purposes. In *Melibe* there have been conflicting hypotheses as to the function and means of execution of the swimming behavior. In some of the earliest papers on *Melibe*, Agersborg (1919; 1921) states that the position of animals during a swimming episode may vary from dorsal aspect up to ventral aspect up. He further states that swimming seemed to be correlated with copulating masses of animals, suggesting that it may be a voluntary means of locomotion for finding mates. Agersborg also refers to a method of "falling" through the water column, by completely relaxing the body musculature, which looks like "a feigned death". Hurst's 1968 paper contains a brief description of the swim, in which she states that swimming is only accomplished dorsal aspect up, and no mention is made of the ecological significance to the behavior. Most recently, Bickell-Page (1991; Page, 1993) suggested that swimming appears to have significance as an escape response, although uncertainty was expressed concerning which organisms might elicit escape swimming in the natural habitat of *Melibe*. To date, only one account of an active predator on *Melibe* has been published (Ajeska et al., 1976) which states that *Pugettia* kelp crabs prey on adult *Melibe* and do not elicit a swimming response from *Melibe* when they attack.

The relative paucity of information on *Melibe* swimming and lateral bend swimming in general, indicates a need for further investigation. This dissertation begins to look at the many behavioral and neurophysiological questions regarding swimming in *Melibe leonina*. Specifically this work tests the hypotheses that swimming in *Melibe* is a stereotyped escape behavior that is centrally generated and modulated by inputs from the animal's natural environment. Chapter 1 describes swimming behavior, its anatomical basis (e.g. musculature Fig 1.2) and whether the swim is directional. Chapter 2 describes the neural mechanisms that are responsible for the initiation, maintenance and termination of the swimming behavior. The final chapter describes the influence of ambient
light on the swimming behavior. Overall, this dissertation seeks to provide a structural and functional basis for the swimming behavior of the nudibranch, *Melibe leonina*. 
CHAPTER 1

THE SWIMMING BEHAVIOR OF MELIBE LEONINA

Introduction

Swimming, as a form of locomotion and/or defense from predation, has been studied in many marine organisms, from anemones to fishes, and marine mammals. The form of the swim, as well as its initiation and termination cues, vary widely. Gastropods mollusks have a number of swim styles (Farmer, 1970) and are amenable to neurophysiological studies (Audesirk et al., 1985). Swimming in both Tritonia diomedea and Clione limacina has been extensively studied, and the behavioral (Hume et al., 1982; Satterlie et al., 1985a) and neural (Dorsett et al., 1973; Getting, 1983b, 1985a; Satterlie, 1989; Arshavsky et al., 1993) components are now fairly well understood. These animals use dorso-ventral and parapodial flying, respectively (Farmer, 1970). A third less studied but common style, "lateral bend swimming", is used by aeolidaceans and dendronotaceans including Melibe leonina.

There have been conflicting hypotheses as to the method and function of Melibe swimming. In all cases, the swim movements are described as lateral undulations, however Agersborg (1919; 1921) describes the position of animals during a swimming episode with dorsal or ventral aspect up and suggests that swimming is correlated with copulating masses of animals proposing that it may be a voluntary means of locomotion for finding mates. Hurst's 1968 paper states that swimming occurs dorsal aspect up, no mention is made of the significance to
the behavior. Most recently, Bickell-Page (1991; Page, 1993) suggested that swimming is an escape response, although uncertainty was expressed concerning which organisms might elicit escape swimming in the natural habitat of Melibe. To date, the only account of active predation on Melibe (Ajeska et al., 1976) states that Pugettia kelp crabs prey on adult Melibe however do not elicit a swimming response when they attack.

In this chapter swimming is described and quantified, a second study reports the types of stimuli and predators that reliably elicit swimming. Finally, the motion of the animal through the water is analyzed to determine whether swimming has directional bias. These studies add a fundamental component to our knowledge of Melibe swimming behavior and should facilitate future neurophysiological and behavioral investigations.

Materials and Methods

Animals

Melibe leonina were collected from Squaw Bay, Neck Point and Blind Bay, Shaw Island, Fisherman’s Bay, Lopez Island and Grifffen Bay, San Juan Island in the San Juan Archipelago, Washington. Animals were collected by divers using SCUBA and used at the Friday Harbor Laboratories (FHL) of the University of Washington or shipped to the University of New Hampshire, Durham, NH. In Washington, the animals were maintained in flow through seawater tanks, in New Hampshire they were kept in a recirculating closed seawater system. Water temperature and salinity were held constant at 15° C and 33 ppt. Animals were provided with eelgrass (Zostera marina) to crawl upon and planktonic crustacean food from the unfiltered water supply, supplemented with Artemia nauplii two to five times weekly.
Analysis of normal swimming

The progression of a complete bout of swimming (from initiation to termination) was analyzed in 29 animals. Each was placed in a 20 l aquarium with a small amount of eelgrass. Seawater was continuously perfused through the tank, with a mean flushing time of approximately 15 min. Swimming was initiated by applying 1 ml of a 1M KCl solution in seawater to the skin of either the head or the tail using a 3 ml syringe without a needle. Measurements were taken of the: 1) latency between application of a stimulus and initiation of the swim; 2) swim duration; 3) number of complete swim cycles in each swimming bout; 4) direction (right or left) of the first and last flexions and; 5) number of occurrences, and the duration, of motionless pauses. I also calculated the average swim cycle duration for all 29 animals to determine the consistency of the swimming rhythm within the population. Finally, in one animal, using a videotape of a complete swim episode, I measured the length of each individual swim cycle, to determine the consistency of the swimming rhythm. Averaged values are presented as the mean ± SEM.

To assess lateral flexion angles through a swim, three animals were videotaped. Animals had loops of 4-0 surgical silk implanted bilaterally through the medio-lateral body wall at the point where the body pivots during swimming. After one day of recovery, animals were individually suspended by these loops, ventral aspect up, in a Plexiglas chamber. The chamber had flow-through seawater so that it remained at 10°C. A video camera was mounted above the chamber and attached to a VCR to allow continuous videotaping of swimming as viewed from the ventral side. Swimming was induced by dislodging the foot from its attachment to the surface tension of the water in the chamber. The video recordings were digitized, one frame every second, and the program NIH Image (version 1.55) was used to measure body angle throughout
the swim. The angles were calculated by marking the anterior tip of the foot, the middle of the foot in between the silk loops, and the posterior tip of the foot.

**Anatomy**

The anatomy of the body wall musculature was determined by stepwise dissection of the lateral body walls of three animals, in the same manner used for *Aplysia* by McPherson and Blankenship (1991a). Each layer of muscle was peeled away from the rest of the body wall and the relative density and direction of the fibers was assessed and drawn.

**Stimuli which elicit swimming**

A number of stimuli that might elicit swimming were applied to animals to determine their effectiveness. The stimuli tested included: 1) pinching the cerata with self-closing forceps; 2) prodding the foot with a glass rod; 3) application of a 1M KCl solution; 4) odor of an injured conspecific (attained by application of seawater from a previously dissected animal as described for other aqueous stimuli) and; 5) presence of, odor of, or contact with, potential natural predators (including *Pycnopodia*, a number of crab species native to eelgrass beds and the anemone, *Epiactus prolifera*). In each experiment the stimulus was presented at time zero, and the latency to swim, and the duration of any ensuing swim episode, were recorded. Two of the stimuli were paired (pinching the cerata and application of salt solution) to test whether previous stimulation would increase the responsiveness of the animal to a subsequent stimulus. In pairing experiments, application of the second stimulus occurred within one minute of the previous stimulus. All aqueous stimuli were introduced in the same manner as previously described for 1M KCl.
To determine if certain animals commonly found in the natural habitat (eelgrass beds) of *Melibe* were potential predators, I performed a series of predation experiments. Individuals of several crab species, seastar species and *Epiactus* (a small brooding anemone) were placed individually in 20 l aquaria with flow-through seawater, and a small amount of eelgrass; then one *Melibe* was placed in each tank and left for 24 hrs. Every 6-8 hours the *Melibe* was examined for evidence of an attack.

**Direction of swimming**

The movement of *Melibe* during a swimming episode was plotted in 3 dimensions using five animals induced to swim in an 80 l aquarium. X-y coordinates were drawn on the Plexiglas cover (top) and on one side. Each animal was placed on a blade of eelgrass which was secured to the bottom in the center of the tank. Animals were induced to swim using a brief touch with a seastar tube foot and their position was drawn on both of the aquarium grids at 5 sec intervals. These points were then converted into x, y and z coordinates and 3-D reconstructions of the swims were plotted to facilitate examination of the overall direction of travel.

The initial tracking studies led to the hypothesis that *Melibe* moved ventrally, in a direction roughly perpendicular to the plane of the foot. To test this, an additional seven animals were tracked during a swim in a manner similar to the experiment described above. The tank design was the same, except that a line representing the orientation of the foot, as well as the animal's location, was drawn on the side of the tank at 5 second intervals. These lines were then plotted in two dimensions and used to calculate the predicted position of the midpoint of the foot at successive time intervals. The "variance angle" (the angle between the actual and predicted position of the foot at the next time point)
was then calculated for each 5 second time interval, averaged, plotted using polar coordinates, and compared to the predicted path.

The instantaneous swimming velocity throughout a swim episode was calculated for each animal by measuring the distance moved at successive time intervals and dividing it by the time interval (5 sec). These data were used to determine whether there was a change in swim velocity during a normal bout of swimming.

**Results**

**Normal behavior**

Although *Melibe* are occasionally observed freely swimming (Mills, 1994), typically they are found attached to eelgrass or kelp blades (Agersborg, 1921; Hurst, 1968; Ajeska *et al.*, 1976; Behrens, 1981; Bickell-Page, 1991). When disturbed *Melibe* release from the substrate and swim. This swimming behavior is characterized by rhythmic lateral flexions 1-2 seconds in duration (2-4 sec. for a full swim cycle; Fig. 1). During each flexion the body bends laterally into a shape resembling the letter C, with the oral hood approaching the tip of the tail (Fig. 1).

The overall organization of a swim episode can be broken down into five basic components (Fig. 1). The swim begins with closure of the oral hood and detachment of the anterior foot from the substrate (1-withdrawal). Once free, the bottom (sole) of the foot is folded medially and the body is laterally compressed (2-flattening), increasing the lateral surface area of the body, which may provide better propulsion during swimming flexions. Swimming movements consist of an alternating bending, or flexion, of the body that brings the rhinophores on the oral hood close to the tail (3- flexions; Fig. 1A-C). This may continue for a few sec to over an hour. In these studies, the average swim duration was 174 ± 32 sec (n=29) for swims that were initiated by salt application and 53.7 ± 9.4 sec (n=34)
Figure 1: Phases of *Melibe* swimming. A&B. Ventral views of lateral flexions. Note the proximity of the rhinophores (indicated by the asterisk) to the tip of the tail. The folded foot is indicated (f). C is a dorsal view. D. The “unfolding and swinging” stage of the swim, as the anterior tip of the foot searches for a suitable substrate. The anterior tip of the foot (indicated by the arrow) is broad and flattened compared to the remainder of the foot. Scale bar = 1cm.

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for those initiated by the touch of a seastar. The conclusion of a swim episode is preceded by the anterior tip of the foot unfolding and beginning to "search" for a suitable substrate (4-unfolding and swinging; Fig. 1D). The swim is terminated (5-termination) when the anterior foot comes in contact with a suitable substrate, followed by attachment and settlement of the animal. Termination need not occur during the first encounter of the foot with a substrate; often the animal will make multiple contacts before ceasing to swim. Moreover, animals will occasionally stop swimming, without contact of the foot with a substrate.

The flexions involved in the swim are not equivalent along the entire length of the body. As well as the lateral flexions, there is a concurrent bending of the foot towards the dorsal aspect of the body with each flexion. This sculling motion of the foot provides a secondary propulsive force which pushes water dorsally and moves the animal ventrally through the water column. This sculling movement was originally described by Hurst (1968). The combination of lateral bending of the entire body, and the dorsal twisting of the foot, typically propels the animal upwards and ventrally through the water column.

**Anatomy of the putative swimming musculature**

The body wall musculature of *Melibe* is arranged in four layers, from an epidermally associated fine lattice layer, to a pair of oblique layers that surround the body cavity (Fig. 2). The outer layer (Fig. 2A) is difficult to dissect away from the skin and therefore was not examined as a discrete muscle layer. The second layer (Fig. 2B) is made up of a loose meshwork of apparently randomly oriented fiber bands that join the first and third layers through loose connective tissue. This second layer may be involved in lateral compression during the flattening phase of swimming. The third layer (Fig. 2C) is oriented in an anterior to posterior direction and is probably used during crawling, rather than swimming.
Muscular Layers in *Melibe* are Arranged to Facilitate Crawling and Swimming

Figure 2: The body wall musculature of *Melibe* is arranged to facilitate both swimming and crawling. It is layered from an epidermally associated layer (A) to a deep, densely interwoven sheet that surrounds the visceral cavity (D). The two layers in between (B and C) appear to assist in crawling (B) and connecting the innermost layer to the outer layers (C). The innermost layer (D) appears to be the major contributor to the lateral flattening and head-to-tail bending necessary for *Melibe* to swim. The line density in the drawings is representative of the density of the fibers in that layer. Scale bar = 5 cm.
Figure 3: Swimming in *Melibe* is driven by lateral flexions of the body (ave # of flexions is 10-30 per episode). These motions are repeated without significant variation in timing or amplitude throughout the duration of the swim episode. There is little variation in the flexion amplitude, with the exception of the initiation and termination of the episode.
The interior two layers are closely associated with, and oriented orthogonally to, one another and are aligned at oblique angles to the long axis of the body (Fig. 2D). They may responsible for generating most of the flexion and sculling movements underlying rhythmic swimming.

Time course and frequency of swimming

Although some aspects of Melibe swimming are quite variable, the actual rhythmic swimming flexions are very constant. In a single swim episode of 58 sec, the average duration of each swim cycle was 2.03 sec ± 0.03, with no appreciable variation during the course of the episode. In 29 different animals the average duration of a swim cycle was 2.7± 0.2 sec. The magnitude of the lateral flexions was also quite consistent throughout a swim episode once the withdrawal reaction due to dislodging the foot from the surface tension has passed (Fig. 3). Other than the first and last few flexions in a swimming episode, the contractions of the body in both directions have similar amplitudes.

In contrast to the stereotyped swimming escape response of Tritonia, where the duration of the swim and the direction of the first and last swimming flexion can be reasonably predicted (Dorsett et al., 1973), there seems to be little consistency in those parameters between one Melibe swim and the next. There was similar chance that animals would start with left or right flexion (58% of the animals tested began with a left flexion, n=29) or finish with flexion to the left or right side (53% of the animals ended with a left flexion). The variation in the duration of swim episodes was also quite large. The mean swim duration in response to a salt stimulus was 175 ± 32 sec; range 33 to 1546 sec (25.7 min).

A unique feature of Melibe swimming is the periods of motionless floating which Agersborg (1923) referred to as "feigned death". During these events animals lay in one place, dorsal aspect up, with the cerata inflated and spread.
parallel to the water surface. *Melibe* occasionally remain in this position for several minutes. During 60-90 min swimming experiments, in which the animals were not allowed to attach to any substrate, these events occurred at 10 - 20 minute intervals.

**Stimuli that trigger swimming**

To determine the most likely trigger of swimming in the natural habitat of *Melibe*, and the most reliable stimulus to use in the laboratory, I screened a number of possible noxious stimuli including: pinches with forceps, salt (KCl), and contact with several different putative predators (seastars: *Pycnopodia helianthoides*, *Henricia leviuscula*, *Pisaster* spp; four species of crabs: *Cancer magister*, *Scyra acutifrons*, *Oregonia gracilis*, *Cancer productus*; and an anemone found on eelgrass, *Epiactus prolifera*). Of these, the touch of the predatory seastar, *Pycnopodia*, gave the most consistent response (61% of the animals touched swam, n=32). In fact, contrary to previous studies (Page, 1993), a very brief (< 1 sec) touch with an individual *Pycnopodia* tube foot was sufficient to elicit a swim in many animals. Single pinches to a ceras, as well as trains of pinches, caused rapid escape crawling but swimming in only 20% of animals (n=20). Salt solution applied to the skin of the head elicited swimming in 22% (n=49) of cases. Interestingly, when a pinch preceded a less than sufficient salt stimulus (1 cc of 0.5M KCl), the percentage of animals that swam increased slightly (28%, n=20), as compared to salt alone (22%).

Of the three effective stimuli tested (pinch, salt, seastar tubefoot), seastars elicited a much more rapid escape response (average latency = 0.8 ± 0.1 sec) than either a pinch (42 ± 31.4 sec) or salt (22.3 ± 4.5 sec). However, there was no consistent relationship between the latency to respond to a particular stimulus and the duration of the subsequent swim episode. Swims in response to salt
lasted 175.0± 32.1 sec, while pinch and seastar stimuli produced swims of 91± 76.9 sec and 52.6± 45 sec, respectively. However, animals often swam multiple times in response to a single seastar contact. If these multiple swims are viewed as one long swim, then, in general, stronger stimuli caused animals to respond more quickly, and swim for a long duration; while weaker stimuli cause long latency, shorter duration swims.

The crabs and anemones tested elicited no swimming responses in Melibe. Neither animal responded to the other's presence. When contact between the crab and the nudibranch occurred, the nudibranch would often simply crawl over the carapace of the crab without incident. In contrast to the study in Monterey (Ajeska et al., 1976), none of the kelp crabs in this study ate Melibe, however no Pugettia were found in the habitats used for this study. No contact between the anemone and the nudibranch was ever observed. Furthermore, no predation was observed, even with the seastar species. Some Melibe were left with crab or seastar predators for up to 48 hrs, with no apparent interactions occurring. Finally, in a number of cases, Melibe were placed on the oral surface of potential seastar predators, including Pycnopodia, and no ingestion occurred. Perhaps Melibe's reaction to Pycnopodia relates to the juvenile stages when Pycnopodia is an active predator on Melibe (Bickell-Page, 1991).

Direction of swim

Three dimensional plots of swimming Melibe (Fig. 4) indicate that the initial movement during a swimming episode is towards the surface of the water, regardless of the initial orientation of the animal. After this initial upward thrust, the direction of movement for the remainder of a swim episode was not consistent (Fig. 4). However, swim direction was generally ventral, perpendicular to the plane of the foot, and upward through the water column.
Overall Movement During a Swim Episode is Upward Through the Water

Figure 4: Tracks of two individual *Melibe* during a swim episode. Animals were induced to swim, and their position every 5 sec was measured and plotted. Although there is a general tendency to move upward through the water column, it is difficult to predict the location of an animal at the end of a swim due to variation in initial starting position, duration of the swim episode, and the likelihood of termination swimming on substrate contact.
Swimming Directionality in *Melibe* is Perpendicular to the Long Axis of the Foot

Figure 5: Direction of travel during a *Melibe* swim. Seven animals were observed during a swim episode and their direction of movement was measured, relative to the long-axis of the foot, every 5 sec (see Methods). In order to test the hypothesis that animals moved in a ventral direction perpendicular to the long-axis of the foot, for each 5 sec interval we compared the predicted location of the animal with the actual location. The angle between these two points was calculated, averaged, and plotted. The majority of animals tested (5 of 7) moved in a direction that was within one SD (14 degrees) of the predicted angle.
Five of the seven animals used in this experiment moved, on average, in a direction which varied less than one SD (14°) from 90° from the long axis of the foot (Fig 5). This supports the hypothesis that the general direction of a Melibe's movement, from one swimming flexion to the next, can be predicted from the position of the foot. This prediction is most accurate after the first two swimming flexions, which tend to propel the animal upward. Subsequently, most movement generated by individual flexions is in a plane that is perpendicular to the orientation of the foot.

The instantaneous swimming velocity of individual Melibe was fairly constant, after the first 2-4 strong flexions of a given swim. The increased vigor of the initial flexions may be necessary to carry them into the water column and away from potential predators. The change in swimming speed after the first 2-4 cycles is small, as suggested by the consistent amplitude of each flexion throughout a swim episode (Fig. 3).

**Discussion**

This chapter describes the progression of Melibe swimming behavior from initiation, elicited by a variety of stimuli, to termination, when the animal re-attaches to a suitable substrate. Like other lateral bend swimmers (Agersborg, 1922), and swimming mollusks in its genus (Thompson *et al.*, 1984), the behavior is triggered reliably by noxious chemical or tactile stimuli, such as the predatory seastar, *Pycnopodia*, and it is stereotyped in terms of the form and timing of rhythmic flexions.

Swimming Melibe are rarely observed in the natural habitats of these animals, even though potential predators such as *Pycnopodia* are present (Ajeska *et al.*, 1976). This may be due to the low probability of encounters between predators and Melibe. Melibe may also be reluctant to swim because this behavior
may carry them away from their preferred habitat and potential mates due to strong local currents. The high density of swimming Melibe observed intermittently at considerable distances from local eelgrass beds suggests that other factors, besides predators, may also trigger swimming (Mills, 1994). For example, voluntary swimming may be a means of dispersal, which would allow mixing of rather isolated gene pools in these spatially separate populations.

Despite similarities to other well-studied molluscan swimmers, Melibe swimming is different in several ways. Perhaps the most unique feature, which has not been described in other swimming slugs, is motionless floating. These periodic floating episodes may represent an energy saving strategy that allows Melibe to rest and remain in the water column for longer periods, at relatively low energy cost. An alternate hypothesis is that floating may enable Melibe to periodically open the oral hood to sample the water column for prey. Melibe cannot feed and swim at the same time, so this sampling activity would allow them to "forage" until they encountered a high density of food, and then they could stop and feed (Trimarchi et al., 1992; Watson et al., 1992, 1993).

Experiments are currently underway to investigate more rigorously the influence of prey, as well as state of hunger, on swimming behavior.

The musculature in Melibe is similar to a basket which surrounds the internal organs (Agersborg, 1923). The organization of the muscles allows the animal to bend maximally in either direction (Fig. 1) for the force stroke of the swim. The lateral compression of the body and oral hood creates a large surface area that generates the propulsive force in the swim. However, the movement is not symmetrical, and the secondary sculling motion of the foot creates a propulsion cone that pushes the animal through the water in an anterior-ventral direction.
The most effective stimulus for eliciting *Melibe* swimming from the several tested is touch with the tube foot of *Pycnopodia*. This stimulus is probably effective due to the surfactants found on the tube feet of certain predatory seastars (*Mauzey et al.*, 1968; *Mackie*, 1970). It is curious to note that I never observed seastars attacking adult *Melibe*, however this observation was also noted by *Ajeska* (*Ajeska et al.*, 1976). *Page* (1993) suggests seastars avoid *Melibe* because they find the secretion of the repugnatorial glands, located throughout the epidermis, repulsive. These glands do not mature until 4-7 weeks after settlement, and before this time, seastars do attack and consume *Melibe* (*Page*, 1993). These secretions do not, however, have any effect on the Monterey predator, the *Pugettia* crab (*Ajeska et al.*, 1976). It is interesting then that even though their repugnatorial glands help deter potential seastar predators, mature *Melibe* retain their tendency to swim as an escape when they encounter these seastars but do not react when attacked by a crab predator.

The direction *Melibe* travel during a swim appears random however, certain features of the path taken during a swim episode are fairly predictable. First, observations here indicate there is an overall movement towards the surface. This initial upward movement may be caused by the initial release of the anterior part of the foot at swimming onset. In its natural habitat, *Melibe* are most often positioned on eelgrass with their anterior end toward the water surface (unpublished observations). This tendency may put the animal in a position that favors an initial turn towards the surface. In animals that are facing the bottom, when they release the anterior part of the foot, their head rises toward the surface so they are oriented correctly before they make their first few thrusts. This initial orientation of the foot toward the surface may account for the discrepancies in previous studies about the orientation of the animal during swimming. It may be that, although there is an initial positioning of the foot
towards the surface, interaction with the strong currents and the mixing layers of
the water tumble the animal during a swim episode. A second consistent feature
is the strength of the first few flexions of the swim. This is a form of "pushing
off" when the behavior is initiated which may be similar to the robust first flexion
seen in both *Tritonia* (Willows *et al.*, 1973a; Hume *et al.*, 1982) and *Pleurobranchaea*
(Jing *et al.*, 1995). Finally, the combination of lateral flexions and twisting of the
body to create a sculling motion reliably propels *Melibe* along a plane that is
perpendicular to the foot. Thus, although in their natural habitat current
probably has a major influence on their direction of travel, they do have some
control over their direction of swimming. This raises the question of whether
*Melibe* seeks out preferred eelgrass habitats when displaced.

Three factors are useful in neuroethological studies: reliability, robustness
and stereotypy (Audesirk *et al.*, 1985) and each applies to the swimming behavior
of *Melibe leonina*. It can be reliably initiated in the laboratory with natural stimuli,
or a simple salt solution. The robustness and stereotypy are clearly illustrated in
Fig. 3, which shows that over the time course of a swim, the flexion amplitude
and frequency do not change significantly. *Melibe* is also amenable to
electrophysiological analysis for the same reasons that other opisthobranch
species have been used; large and identifiable neurons. Moreover, because
almost all the motor neurons which bend the body in one direction are located in
the ipsilateral pedal ganglion, and the antagonistic motor neurons are in the
contralateral pedal ganglion, intracellular recordings are easily obtained in
isolated ganglia, as well as semi-intact animals (Chapter Two; Lawrence and
Watson, *in preparation*). Additionally, there are relatively few higher order
interneurons associated with each half-center of the swim central pattern
generator (Thompson, *pers. com.*), which permits detailed analysis of the neural
basis of the behavior at the level of the central pattern generator and command
neurons. Finally, during development the swimming behavior "crystallizes" in the CNS as discreet units (Page, 1993), thereby allowing the development of the behavior to be followed both behaviorally and neurophysiologically from its inception to its full expression. This piece by piece development of a behavior has not been seen in other sea slugs (Kempf et al., 1977), and offers a chance to look at the neural components of the behavior as they develop and make proper connections in the CNS. The data presented here will provide the behavioral foundation necessary for such future neurophysiological studies.
CHAPTER 2

NEURAL CORRELATES OF MELIBE SWIMMING BEHAVIOR

Introduction

Rhythmic behaviors are common and have been studied in a wide variety of animals (Delcomyn, 1980; Pearson, 1993). Invertebrate systems are particularly good subjects for studies of the neural mechanisms underlying specific behaviors (Getting, 1985; Harris-Warrick et al., 1989; Morton et al., 1994) due to the relatively simple organization and large, identifiable neurons (Getting, 1989; Harris-Warrick et al., 1989). One of the common features of rhythmic behaviors is a neural organization designed around a central pattern generator (CPG). These systems may exhibit a variety of forms, but all will drive a behavior with little or no sensory input. For example, the locomotor systems of the leech (Kristan et al., 1983), Clione limacina (Satterlie et al., 1985a, 1985b), and Tritonia diomedea (Dorsett et al., 1973; Willows et al., 1973b; Getting, 1975, 1976, 1983a) as well as the rhythmicity of the leech heart (Calabrese et al., 1989) have demonstrated underlying mechanisms like reciprocal inhibition and post-inhibitory rebound, that are conserved between phylogenetically different groups of animals. Additionally, studies of feeding systems (Lent et al., 1988; Prior et al., 1989; Trimarchi et al., 1992; Norekian et al., 1993) have shown that behavior can be modulated by either sensory input (Hooper et al., 1989, 1990; Harris-Warwick et al., 1991) or the "state" of the animal (e.g. starved, satiated, laying eggs).
The gastropod nervous system has been mapped in a number of species (Croll et al., 1985; Willows, 1985; Dorsett, 1986). This research has been facilitated by the relative accessibility of re-identifiable inter- and motor neurons which generate specific behaviors, and the consistency of relative cell position within and between preparations (Audesirk et al., 1985). The CPG’s that have been studied within the gastropods have, for the most part been those responsible for producing the rhythmic movements involved in feeding and locomotion.

This work focuses on the neural basis of swimming in the nudibranch, *Melibe leonina*. This animal’s behavior and physiology have been studied over a long period of time (Agersborg, 1921; Hurst, 1968; Bickell, 1984; Watson et al., 1992). Swimming was first described in 1919 (Agersborg, 1919) and then examined in more detail by Hurst in 1968 and Page in 1993. They described its development through the veliger stage (Page, 1992b, 1992a) and the morphology of the brain in adults (Hurst, 1968), however the neural basis of swimming was not fully addressed in any earlier work.

*Melibe* swims to escape from predators (Chapter One), and possibly, to locate mates and suitable habitat. Based on comparisons with other gastropods and the preliminary findings of Hurst (1968) and Thompson (pers. comm.), it is likely that *Melibe* swimming is produced by a CPG in the CNS. The goal of this study was to test the hypothesis that escape swimming is a centrally generated behavior.

First, a map of the adult brain was developed to provide an anatomical framework for subsequent studies. Secondly, the foot was denervated to assess the effect of lack of sensory input from that area. Third, putative swim motor neurons were identified using retrograde fills and recordings were obtained from those cells in semi-intact swimming animals. Finally, recordings were obtained
from the same putative swim motor neurons in isolated brains to demonstrate
that the swim CPG may be expressed in the absence of sensory feedback.

As a result of these studies the neural components that control swimming
in *Melibe* are more fully known, and this will aid future studies to further
understand the neural basis of locomotion.

**Materials and Methods**

**Animals**

*Melibe leonina* were collected from several bays (Squaw Bay, Neck Point
and Blind Bay, Shaw Island, Fisherman's Bay, Lopez Island and Griffen Bay) near
San Juan Island in the San Juan Archipelago, Washington. Animals were
collected by divers using SCUBA and used at the Friday Harbor Laboratories
(FHL) of the University of Washington or shipped to the University of New
Hampshire, Durham, NH. At FHL, the animals were maintained in flow through
sea water tanks, in New Hampshire they were kept in a recirculating closed
seawater system. Water temperature and salinity were held constant at 15°C
and 33 ppt. Animals were provided with eelgrass (*Zostera marina*) to crawl upon
and they fed on planktonic crustacea from the unfiltered water supply,
supplemented with *Artemia* nauplii two to five times weekly.

**Anatomy**

The gross anatomy of the brain and its nerves was revealed using CoCl₂
and 0.5% Neurobiotin backfills. Brains were removed and the nerve of interest
was left 15-20 mm long and connective tissue was removed. The brains were
placed in small two-chamber petroleum jelly wells with sea water. The nerve
was placed across a barrier between the chambers and sealed off from the well
containing the brain with additional petroleum jelly. The seawater in the nerve
chamber was replaced with distilled water and after 1 min the water was replaced with either CoCl₂ or Neurobiotin and the brains were incubated overnight at 4°C. Cobalt was precipitated with ammonium sulfide, and fixed in Carnoy’s fluid (Pitman et al., 1971; Croll, 1986). Neurobiotin was precipitated with Vectastain ABC and DAB and fixed 4% formaldehyde (Moroz, pers. comm.). The preparations were then cleared through graded alcohols and mounted with Permount. Drawings were made with a drawing tube attachment for a Nikon or a Zeiss dissecting microscope. Anatomical data was compiled to form a comprehensive picture of the CNS of Melibe. Maps of the ganglia and the relative positions of cell bodies which contribute axons to each of the nerve roots (Figure 2.1 and 2.2) were constructed. Some animals were used for extensive dissections of the paths of the nerves exiting the pedal ganglia. Nerves were traced to the most distal branch point visible at 500x (50x objective and 10x oculars) on a Wild dissecting microscope.

Denervation of the Foot

Innervation of the anterior region of the foot was severed to eliminate putative inhibition of swimming from sensory input provided by foot contact. Nine animals received either unilateral or bilateral lesions of nerve Pd4, and in some cases Pd5, approximately 2-3 mm from the ganglion. These nerves innervate the anterior and middle foot (Table 2.1). Animals were anesthetized with isotonic MgCl₂ in sea water, the nerves were severed through a 1-2 cm incision in the dorsal body wall and the incision was closed with surgical 4.0 silk using minuten pins as needles. They were allowed to recover for 24 hours in flow-through seawater cages.

Individual Melibe were induced to swim in a large tank and kept swimming for 90 minutes. The amount of time spent swimming was recorded
and compared to the amount of time spent inactive. Control animals were expected to swim less than lesioned animals because the swim CPG in isolated brains continuously exhibits neural output associated with swimming (see below), thus without inhibitory input from the foot, lesioned animals were expected to swim continuously. The results were compared to control animals (n = 4) and sham operated controls (n = 4) to test this hypothesis.

**Electrophysiology**

Animals were prepared for intracellular recording from semi-intact animals using the preparation of Willows (1973b). The brain was exposed through a small incision in the dorsal body wall and muscle layers and connective tissue were cut away so that the brain was clear for impalement with microelectrodes. The brain was then immobilized on a wax coated platform by pinning through the remaining connective tissue with minuten pins. Strings with pins for hooks, were used to hold the incision above the brain open, and help support animals in a 1 liter flow-through chamber. Animals prepared in this way are capable of many normal behaviors including crawling and swimming.

Putative swim neurons were identified by simultaneously recording intracellular bursts of action potentials while observing and recording lateral flexions of the body. Movements were monitored using a Fotonic Sensor by MTI Instruments which consisted of a light sensor within a narrow pipe that was aimed at the animal’s tail. Each time the tail passed by the sensor, the chart recorder pen was deflected. Recordings were obtained in this way for up to 24 hours. Intracellular recordings were obtained using the same methods described below.
For isolated nervous system experiments, the cerebral, pleural and pedal ganglia were dissected from the animal, with nerve roots 8-10 mm long (Dorsett et al., 1973), and the commissure between the pedal ganglia intact. Connective tissue was removed by cutting the sheath at the midline and carefully pulling it away from the ganglia. The buccal ganglia were often lost in this preparation. The brain was pinned out, dorsal aspect up, in a small (approx. 5 ml) recording dish that was continuously perfused with cold seawater (10 - 15 °C). The recording dish was additionally kept cool by recirculating polyethylene glycol through a water jacket that surrounded the dish. Isolated brains provided useful recordings for up to 24 hours.

Conventional intracellular recording methods were used for both semi-intact and isolated brain preparations. Glass micropipettes with a resistance of 10-30 MΩ were made using a DKI model 700 vertical pipette puller and back-filled with either 3M KCl or 4M KAc. Signals were displayed on an Astro-Med DASH 4 chart recorder. Four cells were further characterized by injecting 4% Lucifer Yellow in LiCl (Stewart, 1978). Lucifer Yellow was introduced into the cells by iontophoretic injection using a 50% duty cycle with 10 NA hyperpolarizing current. The brains were then cleared and mounted in glycerol for viewing on a Nikon fluorescence microscope with a blue B2 filter cube. Cells were designated as motor neurons if they sent projections out of the central ganglia through one of the nerve roots. Interneurons were identified as cells which only had projections within the central ganglia.
Table 2.1: Target tissues of the major nerve trunks in *Melibe*

<table>
<thead>
<tr>
<th>NERVE</th>
<th>TARGET TISSUE/INNERVATION FIELD</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>oral tube and lips*</td>
</tr>
<tr>
<td>C2</td>
<td>distal quarter of the hood margin*</td>
</tr>
<tr>
<td>C3</td>
<td>branches to join C1*</td>
</tr>
<tr>
<td></td>
<td>proximal quarter of the hood margin*</td>
</tr>
<tr>
<td>C4</td>
<td>rhinophore and rhinophore process*</td>
</tr>
<tr>
<td>Pd1</td>
<td>target tissues not known</td>
</tr>
<tr>
<td>Pd2</td>
<td>gut and ipsilateral mid-body wall</td>
</tr>
<tr>
<td>Pd3</td>
<td>anterior branch- lower ipsilateral body wall</td>
</tr>
<tr>
<td></td>
<td>posterior branch- mid and dorsal body wall</td>
</tr>
<tr>
<td>Pd4</td>
<td>anterior branch- anterior foot</td>
</tr>
<tr>
<td></td>
<td>posterior branch- mid-foot and ovotestis</td>
</tr>
<tr>
<td>Pd5</td>
<td>anterior branch- front of neck</td>
</tr>
<tr>
<td></td>
<td>posterior branch- most anterior ~1cm of the foot</td>
</tr>
<tr>
<td>Pd6</td>
<td>anterior branch- lower margin of hood</td>
</tr>
<tr>
<td></td>
<td>posterior branch- front and sides of neck</td>
</tr>
</tbody>
</table>

from Hurst (1968)


Results

Anatomy

The arrangement of the larval ganglia in *Melibe* has been described in detail by Page (Page, 1992b, 1992a), and the morphology of the adult brain is similar to *Tritonia* and *Phestilla* (Willows et al., 1973b; Willows, 1985; Dorsett, 1986). The CNS in *Melibe* is composed of bilaterally paired cerebral, pleural, pedal and buccal ganglia (Fig 2.1). The ganglia are arranged in a ring around the esophagus and extend nerves to the periphery. The cerebral and pleural ganglia are fused into a cerebro-pleural complex with the pedal ganglia extending laterally via short, stout connectives. There is an accessory neural peduncle on the cerebro-pleural complex, the tentacular lobe, which is unique to *Melibe*. The pedal ganglia are connected to one another by the pedal and parapedal commisures (PC and PPC) which wrap around the esophagus, and the buccals are joined to the cerebals by the cerebro-buccal connective and to each other by the buccal commisure (CBC and BC, respectively). The paths and target tissues of the nerves projecting from the brain were traced and recorded. Hurst (1968) published the target tissues of the nerves of the cerebral ganglion, while the nerves of the pedal ganglia are reported here. The innervation fields from both studies are listed in Table 2.1.

Most of the backfill information is from CoCl₂ due to technical difficulties with Neurobiotin. These cells whose axons make up the pedal nerves are located primarily in the pedal ganglia (Fig 2.2). Pd3 and 4 backfills also stained large cells on the posterior margin of the pleural ganglia, while Pd5 stained a small cluster of cerebral cells near the anterior midline. As in other opisthobranchs (Dorsett, 1986), the effects of torsion are not apparent in the CNS of *Melibe*. The similarities to other opisthobranchs allows preliminary assumptions to be made.
Schematic Map of *Melibe's* Brain

Figure 2.1: Brain map of *Melibe*. Note that the buccal ganglia (BG) have been drawn lateral to the cerebral ganglia. *In situ* the buccals are below the main ganglia ventral to the esophagus, and connected to one another by the BC. CG = cerebral ganglion, PLG = pleural ganglion, PdG = pedal ganglion, tl = tentacular lobes, PC = pedal connective, PPC = parapedal connective, CBC = cerebrobuccal connective. Nerves are numbered as they are referred to in text. Cells drawn on this map represent large marker cells, not all of which have been fully characterized. (Original drawing by Dr. Win Watson)

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Positions of Cell Bodies from Backfills of Pedal Nerves

Figure 2.2: Drawings of the cell bodies that were backfilled from the pedal nerves. The filled nerve is darkened in each of the figures. Grey areas were darkened with precipitate from the filling process, however individual cell bodies were not discernible. A. Pd3. B. Pd 4. C. Pd5. D. Pd6. Each drawing represents the combination of at least four separate backfills.
in beginning electrophysiological studies such as the pedal ganglia being responsible for locomotor function.

**Denervation of the Foot**

Pedal nerves 4 and 5 provide the nerve branches to the anterior and middle foot and were identified as potentially affecting initiation and termination of swimming. Similar nerves in *Tritonia* (PdN 2-4) are responsible for innervating the foot, and have been shown to control its withdrawal movements (Willows et al., 1973b). Previous observations (Lawrence et al., 1997) indicated that sensory input from the foot may inhibit an ongoing swimming motor pattern, it was therefore hypothesized that lack of input from the foot would increase swimming. However, lesioning Pd 4 and 5 in *Melibe* did not affect swimming as expected. Attachment to substrate was affected during the first 18 - 24 hours post-surgery during which time lesioned animals swam nearly continuously while normal and sham operated controls crawled normally. However in long-term swimming experiments lesioned animals swam less and floated more than normal or sham operated animals (Fig 2.3).

**Electrophysiology**

Semi-intact *Melibe* swam vigorously when their foot was detached from the substrate. During these swimming episodes cells in either pedal ganglia fired volleys of action potentials either in phase, or 180° out of phase with lateral swimming flexions (Fig 2.4). In general, swim motor neurons caused a lateral flexion toward the same side. However, there were some cells that fired while the body flexed in the opposite direction. The role of this small population of cells was not determined. When individual putative motor neurons were either depolarized or hyperpolarized there was little or no visible change in the timing
Foot Denervation in *Melibe* Decreases the Amount of Time Spent Actively Swimming

Figure 2.3: After innervation to the foot is severed animals swim less per five minute interval than in normal animals. Denervated animals spend substantially more time floating dormant in the water column (see Chapter 1) than their non-lesioned counterparts.
Intracellular Recording From a Motor Neuron With Simultaneous Movement Records

A.

B.

Figure 2.4: Recordings from a semi-intact preparation with swimming movement records. A. Movement of the tail measured using a photo cell transducer. The downward deflections of the motion sensor represent left flexions. B. Intracellular recording from a motor neuron in the left pedal ganglion. There is a slight delay between the onset of the motor neuron burst and the flexion to that side. Note that when the firing of the motor neuron ceases, the flexions also cease.
of the swim cycle. The interburst interval (IBI) in the motor neurons was 2.5 sec ± 0.1 and contralateral bursts occasionally overlapped one another. Behaviorally, when the animal stopped swimming, the motor neuron burst also ceased. If a motor neuron was driven beyond the normal termination of its burst, the animal remained tonically flexed to the ipsilateral side. And if the animal remained flexed to one side, the motor neurons on that side fired tonically until the flexion was released. Motor neurons in each half of the brain fired synergistically, however they did not appear to be electrically coupled.

The isolated brain of *Melibe* spontaneously produced a rhythmic output which very closely resembled the pattern recorded from putative swim motor neurons during swimming in semi-intact animals. Bursting in left and right pedal neurons was out of phase (Fig 2.5) and the burst duration and frequency were very similar (IBI = 2.7 ± 0.1) to the semi-intact (IBI = 2.5 ± 0.1) animal. Two conditions had to be met for the isolated brain to spontaneously swim. First, the pedal-pedal connectives had to be intact. Apparently, the CPG interneurons coordinate swimming using contralateral projections through this connective. Second, the dissecting light illuminating the preparation must be turned off. Light appears to inhibit the swim CPG, this issue will be discussed in more detail in Chapter 3.

Based on the data obtained using backfills, the cells innervating swimming structures are clustered primarily on the dorsal side of each pedal ganglion. The majority of cells identified by intracellular recording as swim cells were motor neurons located near the pleuropedal commissure, and along the posterior margin of the pedal ganglion (Fig 2.6). These motor neurons are not considered a part of the CPG because manipulation of the burst pattern does not change the timing of the behavior. This pool of cells does not appear to extend to either the pleural or cerebral ganglia.
Neuronal Firing Pattern for Swimming Recorded from Pedal Motor Neurons of *Melibe* Isolated Brain

Figure 2.5: Normal swimming in the isolated *Melibe* brain is characterized by out of phase oscillation of bursts in motor neurons in opposite ganglia. The bursts are robust and no input is necessary to start the rhythm.
Approximate Location of the Motor Neuron Pool

Figure 2.6: Map of the locations of putative swimming motor neurons involved in swimming in *Melibe*. The motor neurons (shown as clear circles) are clustered in the pedal ganglia based on successful recordings from cells that exhibit a swimming motor program in both semi-intact and isolated brain preparations. Key to symbols in Fig 2.1.
Discussion

*Melibe* is the first of the lateral bend swimming nudibranchs whose swimming behavior has been studied neurophysiologically. The anatomy of *Melibe*’s brain and the peripheral innervation patterns of the nerve roots are similar to other nudibranchs whose brains have been mapped (Willows *et al.*, 1973b; Willows, 1985; Dorsett, 1986; Jing *et al.*, 1995). The nervous system in *Melibe* is centralized in a circumesophageal ring consisting of four pairs of ganglia. *Melibe* also has a bilaterally paired tentacular lobe on the anterior cerebral ganglia. The motile eyes are mounted prominently on the cerebral ganglia by short stalks. Similar to *Dendronotus*, the nerves which provide input to swimming structures (the foot and lateral body wall) arise from the pedal ganglion and the motor neurons that control swimming are located primarily in the pedal ganglia.

In the semi-intact I showed that the swimming movements are coordinated with the firing of a number of motor neurons in the pedal ganglion. When these cells fire, the animal flexes to the ipsilateral side, as in swimming. When there is an ipsilateral flexion, the putative swimming motor neurons fire bursts of action potentials that are temporally in phase with the flexion. Most importantly, when the putative swimming motor neurons are driven by injected current, the animal flexes to the ipsilateral side and if the motor neuron is inhibited, the animal remains flexed to the contralateral side. When flexions cease in the semi-intact animal, the bursts also cease in the motor neurons. This provides strong evidence that the neuronal firing patterns that I am recording are the neural correlates for swimming in the brain. These firing patterns are also expressed in the isolated brain, which indicates that peripheral input is not
necessary for generating swimming. It is likely, however, that input from sensory structures is used to modulate the expression of swimming.

The neural components of the swimming system in *Melibe leonina* are also comparable to those of other swimming nudibranchs that have been studied (Getting et al., 1985a; Jing et al., 1995) however differences in the locations of individual components of the CPG are striking. Swimming in all of these animals is an escape response which is highly stereotyped and repeatable. In species such as *Tritonia, Pleurobranchaea,* and *Clione* swimming is coordinated by a small number of interneurons and executed by a larger pool of motorneurons. In these systems, the interneurons set the timing and make the "decision" as to whether or not the behavior will occur. In *Tritonia,* the cerebral C2 neuron is responsible for providing the excitatory input to the dorsal and ventral swim interneuron pools which, in turn, drive the swimming motor neurons (Getting, 1976). The dorsal and ventral swim interneurons are mutually inhibitory and fire out of phase with one another, but in phase with either dorsal or ventral flexion (respectively) (Getting, 1983b). The interneurons in *Tritonia* are located in the cerebral and pleural ganglia, while in *Clione,* whose swim is accomplished by lateral parapodia, the interneurons are located in the cerebral and pedal ganglia (Satterlie, 1991). The swim CPG in *Clione* is based on reciprocal inhibitory synapses between the dorsal and ventral phase interneurons (Satterlie, 1989). *Melibe* is different from *Tritonia* in that it is a lateral bend swimmer with the swim CPG is divided into lateral halves and the interneurons located in the right and left pedal and pleural ganglia. The number of swim cycles that are involved in a swim episode in *Melibe* is lower than in *Clione,* which swims continuously, but often higher than in *Tritonia* whose number of swim flexions is the same (8 - 10) from swim to swim (Lennard et al., 1980). While the components of the swim are highly conserved throughout, and between swim episodes in *Melibe,* the length
of the episode varies between swims. Also, there does not appear to be a homologue to the C2 neuron found in dorso-ventral swimmers (Willows et al., 1973a; Getting, 1977; Jing et al., 1995) and, in fact, no cells were found in the cerebral ganglia that fired in a coordinated fashion with a swim episode. The timing of swimming flexions, however, does not seem to be substantially different from that of Tritonia (Lennard et al., 1980) (5.6 sec -vs- 6-8 sec) even though the neural mechanisms which generate swimming appear to be different. These differences and similarities make Melibe an excellent model for comparing mechanisms used by diverse animals to produce rhythmic swimming.

This work has concentrated primarily on the motor neurons which appear to represent the last step in the neural hierarchy controlling swimming. These motor neurons are not considered part of the central pattern generator because they are not capable of resetting the timing of the behavior when their firing patterns are artificially manipulated. This is in contrast to the leech swimming CPG (Kristan et al., 1983) and the stomatogastric ganglion in decapods (Hooper et al., 1989) where some proportion of the CPG is made up of the motor neurons which drive the action. The CPG in Melibe is divided into lateral half-centers, each of which controls the flexions to the ipsilateral side. In Dendronotus, another lateral bend swimmer, the CPG appears to be confined to the pedal ganglia (unpublished observations; Gillette and Brown, pers. comm.) and there is apparently no input from either the cerebral or the pleural ganglia. In Melibe, the motor neurons are output only, and the CPG is controlled by interneurons. Thompson (pers. comm.) and Watson (pers. comm.) have found two bilateral pairs of interneurons which appear to be necessary and sufficient to produce the swimming motor pattern in Melibe. These cells are located in the pedal and pleural ganglia, are electrically coupled and appear to be the only cells which are capable of producing swimming in a quiescent preparation. These cells provide
a rich opportunity for investigation and comparison to other swimming systems such as *Tritonia, Clione* and *Hirudo*.

*Melibe* appears to be unique in that the CPG is distributed between the pedal and pleural ganglia, with each ganglion exerting approximately equal control over whether the behavior will be executed. This poses an interesting question as to whether *Melibe* may actually be an intermediate of some sort between dorso-ventral and lateral bend swimming, or whether the larval reorganization of the ganglia (Page, 1992b) has moved a previously cerebral cell (as in dorso-ventral swimmers) or a previously pedal cell (as in *Dendronotus*) into the pleural ganglion. In either case, *Melibe* is the first lateral bend swimmer that has its swimming CPG distributed outside of the pedal ganglion. Now that the behavioral and neural groundwork of swimming in *Melibe* is known, more extensive studies of the synaptic connections, neurotransmitters and environmental and chemical modulators can be undertaken. This study provides a starting point for a deeper look into the swimming neural circuit of *Melibe leonina*. 
CHAPTER 3

THE EFFECT OF LIGHT ON MELIBE LOCOMOTION

Introduction

Light is known to influence locomotion and feeding in a number of opisthobranchs, *Aplysia* (Lickey et al., 1977), *Bursatella* (Block et al., 1981), *Bulla* (Block et al., 1982) and *Hermisenda* (Alkon, 1983), and other gastropods (for example, *Limax* (Beiswanger et al., 1981)). No consistent pattern is exhibited across the group. Some animals are nocturnal, others diurnal, some exhibit phototaxes, some of the circadian patterns are controlled by the eye, others by extraretinal photoreceptors and still others by complex interactions between the two (Page, 1982). Both *Aplysia* (Lickey et al., 1977) and *Bursatella* (Block et al., 1981) exhibit diurnal locomotor patterns while *Bulla* (Block et al., 1982) and *Limax* (Beiswanger et al., 1981) are nocturnal, and *Hermisenda* exhibits a phototaxis in its natural habitat.

All species except *Bursatella* exhibit circadian locomotion rhythms which, after entrainment, continue in constant darkness. The roles of the eyes in the different species range from being the center of the circadian oscillator in *Bulla* (Block et al., 1982) to non-essential for locomotor rhythms in *Bursatella* (Block et al., 1981). In *Hermisenda* the eyes react to light differently during the day and night, causing a change in phototaxis from positive to negative. Also the presence or absence of extraretinal photoreceptors varies from one animal to the
next, and has not been fully explored in many species. In Aplysia (Lickey et al., 1977), Hermissenda (Getting, 1985), and Bulla (Block et al., 1982) extraretinal photoreception has been demonstrated, however in Bursatella (Block et al., 1981) and Limax (Beiswanger et al., 1981) no definitive evidence has been found. Locomotion patterns in each of these gastropods have been entrained, with varying degrees of success. The actual eyes of each of these animals are located in different places. Hermissenda’s eyes are fixed on the brain, lateral to the cerebral ganglia (Alkon, 1983), while in Aplysia the eyes are located in slight depressions on the head of the animal, lateral to the rhinophores (Kandel, 1979). Thus, although each is utilizing light cues, they do not all employ the same neural or sensory pathways.

Light also appears to have an influence on locomotion in Melibe. Hurst (1968) observed that juvenile Melibe swam to a light that was placed in the water at night and Hurd et al. (1993a) noted a negative phototaxis in adult animals when given the choice between light and dark. Melibe are only found in well lit, shallow waters in association with seagrass or kelp which raises the question of whether light is playing a role in modulating behavior and influencing habitat selection.

First, I report on how a light gradient influences the vertical distribution of Melibe in cylindrical experimental chambers. Secondly, I describe the effects of light on Melibe’s response to swim inducing stimuli and whether changes in light were able to initiate or terminate swimming. Lastly, videotapes of freely behaving animals provide evidence for the effect of three light regimes ("natural" light cycle, constant dark and constant light) on locomotion and swimming. The results of these experiments suggest that light is an important natural factor influencing locomotion in Melibe.
Materials and Methods

Vertical Distribution in a Light Gradient

Animals were placed in a 2 m tall, cylindrical Plexiglas tank located outside at FHL. The tank was fully covered with 4 mil black plastic to block ambient light. Light inside the tank was provided by a 100 watt incandescent lamp, mounted at the top of the tank, which established a light gradient within the tank that was similar to the natural habitat as measured with a HOBO Light Intensity data logger (Onset Computers, Pocasset MA) (Fig 3.3). The seawater was continuously changed during the experiment and an eelgrass habitat was provided in the bottom 1 m of the tank. Ambient light was altered on a 24 hour light/24 hour dark schedule. Lighting was changed after the last observation of the previous treatment (i.e. after the 10 PM observation on a lighted day, the lamp was turned off. The next observation was made the following morning.). The height of nine animal relative to the bottom of the tank was recorded at 8 and 11 AM, 1, 4, and 10 PM. The results from 10 d of experimentation (five light and five dark with nine animals per experiment; total n = 45 each for light and dark) were combined and percentages plotted. An ANOVA analyzed the differences in distribution between the light and dark treatments.

Influence of Light on Swimming

The most effective of the stimuli tested for eliciting swimming in *Melibe* is the touch of an individual tubefoot from the seastar *Pycnopodia* (Chapter 1). In this experiment the hypothesis that light altered the likelihood of swimming in response to a noxious stimulus was tested. Individual *Melibe* were placed into 8 l plastic aquaria with flow through seawater and allowed to acclimate for 12 hours. In natural light cycles, sixty-four animals were tested for their likelihood to swim in response to a touch with a single seastar tubefoot during the day (10
AM PST) and at night (10 PM PST). The latency to swim and swim duration were recorded for all animals. A consistent difference was noted between the light and dark treatments. Therefore the experiment was repeated under constant conditions to determine whether animals possessed an endogenous rhythm of responsiveness. The constant condition experiments were conducted in a small room with the windows covered with aluminum foil and 4 mil black plastic. Animals (n = 15) were acclimated as above and white light was provided by one 60 watt white light bulb for the constant light condition. The spectral composition of light generated by this bulb was measured using a spectroradiometer to assess the composition of light reaching the animal (emission spectrum Fig 3.1 A). A 40 watt red ceramic coated bulb (emission spectrum Fig 3.1 B; visual light intensity = 5.5 lumens/m²) illuminated the tanks at all times to facilitate observations in the dark.

An additional 18 animals were used to test the immediate impact of light on swimming. Animals were acclimated as above in a darkened room. At 10 AM and 10 PM the animals were induced to swim using a Pycnopodia tubefoot. While the animals were swimming, a white bulb was illuminated and the number of animals that ceased swimming within 15 seconds was recorded. The room was then left lighted for 12 hours, and the animals were tested to see if sudden darkness elicited swimming. For this experiment, after making sure that each animal was attached to a substrate, the light was turned off for 30 sec. The number of animals that commenced swimming within 15 sec was recorded.

**Video Analysis of Locomotion**

Experiments were performed at UNH and at FHL. Each experiment was video taped to determine the amount of locomotion (combined swimming and crawling) undertaken by animals per day. At UNH, the water temperature in a

50
Spectral Analysis of the Lighting in Experimental Chambers

White Bulb Spectrum

Red Bulb Spectrum

Figure 3.1: Wavelength profiles of the light sources used to analyze locomotion in *Melibe*. A. The wavelength profile for a GE 60 watt white bulb which provided the "day" light for locomotion experiments. B. Wavelength profile of a ceramic coated 40 watt bulb used for "night" conditions. This bulb did not alter the swimming motor pattern in isolated brains (*pers. obs.*). The absolute vales for irradiance are values x10^12, and were not standardized and therefore not directly comparable.
20 l aquarium was maintained at 8°C with a recirculating water jacket. The tank was continuously aerated, and water was changed following each experiment. Light regimes were set at 12:12 (L:D) with a household electrical timer attached to two lamps. One lamp contained a 60 watt white light bulb which provided the subjective day, and the other a 40 watt ceramic coated red light bulb for subjective night. The light intensities in the experimental tanks were recorded with a HOBO. The chamber was covered with 4 mil black plastic to eliminate external light effects. The tank at FHL was exposed to natural light for the first set of experiments, and was subsequently covered with black plastic for constant condition experiments.

At both sites, the experiments ran for 40-72 hours and monitored by a low-light video camera. Digitized frames were collected every 10 minutes using a Macintosh® Quadra 660AV computer. These frames were reviewed and the position of the animal (X-Y coordinates of the mouth of the animal) were measured using NIH Image 1.60. These coordinates were used to calculate movement per hour for the duration of the experiment. Movement distances were also pooled into "day" (7AM - 5PM in the winter; 5AM - 7PM summer) and "night" (5PM - 7AM winter; 7PM - 5AM summer) to assess the gross effect of light on movement. Results are presented as averages ± SEM.

Influence of Light on Spontaneous Swimming

These experiments were carried out as described above but the data collection and analysis was different. In these studies, the 40 hour experiments were video taped using a Panasonic AG-RT600P time-lapse VCR. The tapes were subsequently analyzed to determine the time and duration of each swim episode. These data were used to determine the influence of light on the temporal patterns of spontaneous swim episodes.
The Influence of Light on Fictive Swimming

As described in Chapter 2, the isolated Melibe brain spontaneously expresses the swimming motor program and in preliminary experiments light appeared to inhibit the swimming motor program. To test this, intracellular recordings were made in putative swim motor neurons exhibiting fictive swimming (methods and criteria are outlined in Chapter 2) and the effect of light changes was recorded. In the first series of experiments 26 brains that were spontaneously expressing the swim motor program were exposed to bright light from a fiber optic illuminator to determine if light inhibited swimming. In the second series of experiments six animals were used to determine the light threshold necessary to inhibit swimming. The room lights were turned off after impaling a pair of antagonistic motor neurons and the preparation was left in total darkness for 30 min. The preparation was then exposed to varying light intensities using a rheostat controlled fiber optic illuminator located 6 ft from the preparation. The amount of light actually impinging on the brain was measured with a HOBO light meter located next to the recording chamber. The brains were exposed to a measured light intensity for approximately 30 sec and the intensities were changed every 5 min. Threshold was determined to be the least amount of light able to inhibit fictive swimming. Finally those same brains were threshold tested again after excising both eyes. The electrodes were removed from putative swim motor neurons during surgery and then cells were re-penetrated. Threshold tests were repeated as described above.

Results

Vertical Distribution in a Light Gradient

In artificially illuminated water columns with a light gradient ranging
from 2.7 log Lumens/m² at the surface to 1.5 log Lum/m² at the bottom (as measured with a Licor LI-185 submersible light meter), animals were found primarily in the uppermost 20 cm (Fig 3.2). The lighting gradient in their natural habitat is also small, with daytime values ranging from 2.8 just above the eelgrass canopy to 1.6 log Lum/m² at 4 m depth (Fig 3.3). There was no preference for the eelgrass substrate in the lower 1 m. Animals were found alone and in pairs near the surface in constant darkness and constant light and at all times of day. Animals did not appear to move within the tank during the day, as the relative number of animals at each depth did not change throughout the day. There was, however, some redistribution of animals over the course of the experiment all of which occurred during the night. The animals in this experiment were used for both light and dark experiments, thereby repeating measures for each animal in light and dark. A two tailed t-test was performed to look for differences between the distribution of animals in light and dark. The two-tailed p value was 0.9992, showing no significant difference between the lighting regimes. An ANOVA was also performed to compare each of the depth categories. No difference was found between light and dark, however the concentration of animals at the 20 cm and surface depth was significantly higher than at other depths (p<0.001 at both depths) suggesting that this distribution was not related to light.

Influence of Light on Swimming

Under natural lighting conditions, 42% of 64 animals tested swam in response to a Pycnopodia tubefoot during the day while 64% swam at night (p<0.01). In constant darkness, animals were more likely to swim than during natural light/dark cycles (67% during the subjective day vs. 80% during subjective night). However the only statistically significant difference was between natural day and subjective night (p<0.05). In constant light, animals
*Melibe* Cluster Primarily at the Surface in Both Lighted and Darkened Tanks

Figure 3.2: In an artificial water column, *Melibe* were found primarily in the upper 20 cm of the tank. This was the case in both light and dark tanks and at all times of day. This suggests that light is not influencing the vertical distribution of *Melibe*.
Light intensity in *Melibe*'s natural habitat as compared with the experimental tank.

Figure 3.3: Light intensity in the natural habitat as compared to the experimental tanks. The peak of the natural light cycle is approximately equal to the maximum light intensity in the habitat, and the dark lighting treatment is approximately equal to very early morning. Note: most of the dark light regime and nighttime baseline light intensity recorded here is due to the red bulb which is >600 nm and has been shown in isolated brain experiments not to disrupt the swimming motor program.
swam about the same amount during subjective day as in normal daylight (47% vs. 42%) but less during the subjective night than normal nighttime (27% vs. 64%). The constant dark results, however, reinforce the hypothesis that light has an inhibitory role in the swimming motor system of Melibe. The results of each experiment suggests that light reduces the probability that Melibe will swim in response to a noxious stimulus.

The results from the immediate impact of light were very convincing. When swimming was induced in the dark, and the lights were turned on, no animals stopped swimming (n=18). In the opposite experiment, where animals were acclimated to light and the lights were turned off, no animals began to swim (n=18). This indicates that, on a short time scale, changes in light intensity neither induce nor stop, swimming behavior in an intact animal.

**Video Analysis of Locomotion**

When exposed to natural light/dark cycles Melibe locomotion is highest at night, in the dark, and lowest during the day (fig. 3.4). At night animals (n = 12) moved an average of 27.8 ± 43.9 cm per hour, while during the day animals moved only 11.5 ± 16.3 cm per hour (Fig. 3.4). In the constant dark experiments, the animals (n = 8) moved more per unit time (43 ± 28.1 cm per hour) during subjective night than during subjective day (24.3 ± 20.2 cm per hour), and movement was recorded at all time points (Fig 3.5). Constant light was not assessed, yet those experiments must be completed in order to make a definitive statement on the presence or absence of a natural locomotor rhythm. However, it appears that the overall locomotion is greater in darkened conditions regardless of the total light input.

In an attempt to determine if there was a free running rhythm of locomotion in Melibe, animals were subjected to extended periods without light.
Animals in Natural Light Cycles Exhibit
Diurnal Cycling of Locomotion

Figure 3.4: Individual and population locomotion profiles in natural light. A. Representative locomotion pattern of one individual over a 40 hour experiment. The grey areas represent nighttime hours. B. The average distance moved for pooled night and day hours by 12 animals. High variability was seen between individuals yet there was significantly less movement (p<0.001) for daytime hours than at night. C. Population averages for locomotion over the 40 hour experiment. Note that locomotion is depressed in daylight hours.
Figure 3.5: Individual and population locomotion profiles in constant darkness. Boxed areas in A and C represent subjective nighttime. A. Locomotion pattern of one representative individual. B. The average locomotion distances for night and day. There is a weak, nonsignificant (p = 0.4) difference between subjective day and night. C. Population averages for locomotion (n = 8).
*Melibe* Does Not Exhibit a Freerunning Circadian Rhythm of Locomotion

Figure 3.6: In constant darkness, one *Melibe* previously entrained to a 12:12 light cycle did not exhibit a freerunning circadian locomotion pattern. The grey bars represent the subjective day and night from the previous entrainment light cycle. However, locomotion patterns in some animals break down to seven hour cycles, not related to the previous light cycle.
In these cases none of the individuals \((n = 11)\) gave a strong rhythmic response similar to light entrained animals (Fig 3.6). Four animals showed a weak depression in locomotion during the subjective day of these experiments, however these animals also tended to be only sporadically active. A weak cycling with a period of 7 to 8 hours was seen in 6 animals. The significance of this rhythm is not known.

**Influence of Light on Spontaneous Swimming**

Swimming occurs spontaneously in freely behaving animals. In addition to the influence of light on overall locomotion, the frequency and duration of spontaneous swimming episodes was also modulated by ambient lighting conditions (Figs 3.7 and 3.8). In natural light cycles, the frequency of swimming is based on the rise and fall of light intensity in the experimental chamber. Those oscillations are not seen in animals experiencing constant darkness or constant light. Under natural light cycles, the average frequency of swimming was less than 1 event during the "daylight" hours (7AM to 5PM) as compared to 34 during the "nighttime" hours (5PM to 7AM) (Fig 3.8; \(p<0.001; n = 12\)). In constant darkness, the overall swimming frequency was lower than in natural light conditions, but the median level was higher (10 swims per hour vs. 30 in darkness and 0.6 in light for natural light cycles). There was a difference between the average number of swim events during subjective day (3 events) and subjective night (17 events) \((n = 4)\) which suggested that a weak circadian component was present, but the difference was not statistically significant \((p = 0.4)\). Thus, in whole animals, the frequency of spontaneous swimming is modulated by the presence or absence of light.

The total amount of time spent swimming and the duration of individual episodes are also affected by the light conditions. Total time spent swimming is
Melibe's Swimming Pattern is Directed by Light

A. Natural Light Cycle (10:14)

B. Constant Darkness

C. Constant Light

Figure 3.7: Melibe swimming behavior is affected by light. A. Average swim episode frequency of 12 animals in natural light cycle (10:14 winter cycle) indicates that they are nocturnal swimmers. B. In constant darkness, animals (n = 8) swim less than in natural light, but the hourly distribution is more even. C. In constant light, animals (n = 8) rarely swim, and the swim events are randomly distributed over time.
Swim Events During "Day" and "Night" for Different Lighting Regimes

Figure 3.8: The frequency of spontaneous swimming is affected by light. A. The average frequency of swim events in the "day" and "night" hours are significantly different. B. The total number of swim events in "daylight hours" and "nighttime hours" ("Day" = 7AM-5PM; "Night" = 5PM-7AM) for each of the three light regimes is represented here with statistical significance where indicated.
Figure 3.9: Swimming duration is effected by ambient lighting conditions. A. Total time shows that during the "day" substantial amounts of swimming occur in the darkened tank (p<0.05 compared to natural light). At "night" there is more time spent swimming in all light regimes compared to day values (p<0.01). B. The average duration of swim events is related to the light regime causing longer swim events in the constant condition tanks than in natural light cycles.
lower in the light than in the dark (Fig 3.9 A). This difference in swim time reinforces what was found with swims per hour, that animals are more likely to swim in the dark. There are more swim events in the dark, and these tend to be longer individual episodes. The average swim duration is significantly higher in dark conditions (Fig 3.9 B) which also reinforces the indication that light inhibits swimming probability and duration. These data are supported by field observations (all during the day) which suggest little or no movement during the day unless the animal is physically disturbed.

The Influence of Light on Fictive Swimming

Isolated brains exhibit continuous firing patterns which represents the swimming swimming motor program (Chapter 2). This "fictive" swimming is sensitive to light which disrupts the swimming motor pattern. Although the whole animal is not immediately effected by light or dark, cells in the brain react quite quickly. In isolated brains, spontaneous swimming was rapidly inhibited when lights were illuminated and resumed when the light was shut off (Fig 3.10). This effect was repeatable over multiple preparations (n = 18). The lag time between illumination of the light and a noticeable effect on the firing pattern was approximately 10-15 sec in preparations that were not dark adapted, and 4-8 sec for preparations that were dark adapted. Ablation of the eyes (n = 6) was sufficient to eliminate this effect (Fig 3.11). None of the light intensities that previously altered the swimming pattern had an effect on the eyeless preparation.

The minimum threshold for inhibiting the swimming motor pattern was determined to be -0.14 log Lum/m^2 ± 0.16. This level of light was sufficient to completely disrupt the swim motor program in 6 animals. However, the same
Figure 3.10: Light has a disorganizing effect on the generation of fictive swimming in isolated brains. In some cells the effect is relatively complete inhibition of the cell, while in others, as above, the swim pattern is altered to something which although not swimming may be another rhythmic behavior.
Melibe Without Eyes Do Not Respond To Light

Figure 3.11: *Melibe's* eyes mediate the response to light. A. Intracellular recordings from a pair of contralateral motor neurons showing a degradation of the swimming motor pattern with the onset of light (first hatch mark), and a return of the pattern when the lights were turned off (second hatch mark). B. Intracellular recordings from a brain without eyes showing that light on (first hatch mark) and off (second hatch mark) have no effect on the swimming motor pattern.

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and stronger light intensities did not affect animals whose eyes had been removed (Fig 3.11).

**Discussion**

Swimming in *Melibe* has previously been shown to be a robust and repeatable behavior that is amenable to neurophysiological study. However, there are a number of environmental factors which influence *Melibe* swimming and locomotion. One of the most influential of these factors is light. In this chapter, I have shown that light can decrease locomotion and swimming in intact animals and disrupt the fictive swimming motor pattern in an isolated brain.

In response to swim-inducing stimuli, *Melibe* swims more readily and for longer durations in darkened conditions. In constant darkness, the likelihood of a swim was much higher than in "natural" light supporting the hypothesis that light is inhibitory to the swim CPG. In constant light, the percentage of animals swimming during the subjective day was unexpected however Agersborg (1921) found that *Melibe* fed primarily at night, which may have influenced swimming in constant light. If the animals had no darkness in which to feed, they may have opted for swimming in the daylight in order to find a darker location in which to feed. It may also be that upon prolonged exposure to white light, the eyes and/or extraretinal photoreceptors become adapted, and therefore the animal no longer recognizes the lighted condition (Page, 1982). Alkon (1983) showed in *Hermissenda* that the phototaxis of the animal changes between day and night, which represents another way that nudibranchs may be dealing with light input. These data suggest that in responding to stimuli, *Melibe* appears to be modulated mainly to the photic input from the eyes.
Although light has a profound effect in the brain on the likelihood of swimming in *Melibe* it does not have an immediate effect in whole animals. Brains responded to changes in light more reliably and rapidly if they were dark adapted before the experiment. This may also be due to the lack of light filtering in isolated brains. In intact animals, there is a skin layer and connective tissue between the eyes, which are mounted on the brain, and the light source, that is lacking in the isolated nervous system preparation. This may influence the threshold values for disruption of swimming in the isolated brain. The values recorded here represent not much more light than, perhaps, a moonlit evening. It may be that in whole animals, the light intensity needs to be higher to disrupt swimming. In *Bulla* light has a profound effect on the locomotion patterns of the animal and entrainment happens relatively quickly after light shift (Block *et al.*, 1982). Light does not appear to turn swimming on or off with short latency. Quiescent animals do not begin swimming upon the extinguishing of a light source, nor do swimming animals cease swimming upon the illumination of a light source. Changes in ongoing behaviors due to changes in light take up to several minutes, suggesting that light may not be the only input affecting the swimming CPG. Other possible inputs may be coming from the foot or the stomach, as contact or hunger signals, which may modulate swimming. With the isolated brain preparation, it is possible to examine the relative influence of different variables on the probability of swimming. It is clear that foot contact with an appropriate substrate will override the swimming circuit and swimming will cease, however it is still unclear what other inputs will alter the swimming circuit.

A vertical preference in the water column was expected from field and laboratory observations (Hurst, 1968; Ajeska *et al.*, 1976). In both vertical and horizontal (unpublished data) preference experiments, there was skewed
distribution of animals, however the exact cause was not determined. Animals in both experiments clustered together regardless of the light regime. There was movement during the nighttime hours in these experiments, however the extent of the movements was not sufficient to change the overall distribution of animals. Light, however, did not seem to be the factor which caused the animals to remain at the top of the tank, as the distribution did not change between the light and dark tanks. Water flow may have introduced zooplankton that concentrated near the surface, thereby providing better feeding opportunities near the surface or the water inflow may not have been strong enough to mix to the bottom of the tank, causing a more anoxic, less hospitable environment in the bottom of the tank. Hurd (1993) stated that animals sought out the darkened portion of a tank when given the choice, but this was not borne out by these studies. The tendency for the animals to move in the dark and stop in the light might be sufficient to explain the distribution that Hurd observed.

When all locomotion is assessed, regardless of the means by which it occurs, Melibe is nocturnal. The lack of a clear circadian rhythm is reiterated in the locomotion and swimming experiments. There is strong diurnal cycling of locomotion in natural light, and a weak rhythm unrelated to the previous light cycle is demonstrated in constant conditions. Under constant darkness locomotion is consistently high over the course of an experiment (40 hours) however the weak rhythmicity of locomotion (7-8 hour cycle) that cannot be explained without some form of endogenous oscillator or perhaps it is an artifact of a dark kinesis which appears to be an endogenous rhythm.

Melibe that are entrained to a given light cycle, and subsequently placed in a constant darkness, do not show a free running rhythm of locomotion. This is in contrast to Aplysia and Bursatella both of whom, are diurnal. Of these two, Aplysia will exhibit a clear freerunning pattern when placed in a dark tank.
(Lickey et al., 1977) but Bursatella will not cycle without light input (Block et al., 1981). Bulla and Melibe are nocturnal, however Bulla is circadian when placed in constant darkness (Block et al., 1982). Each of these animals uses a different mode for entrainment to light cycles, however all of them exhibit strong responses to light. Thus, continued work with Melibe may help to elucidate the ways that animals perceive and react to their "visual" environment.

Lastly, swimming, like locomotion, shows diurnal cycling in natural light, but does not continue to cycle strongly in constant conditions. In their natural habitat, Melibe is exposed to light intensities ranging from 0.01 lum/m² or less at night, to 150 - 315 lum/m² during the day. Eelgrass, the preferred habitat, grows only in sheltered, muddy bays which are fairly shallow. Individual populations are widely separated by fast running currents and wide channels. Light may, therefore, be playing a role as a depth cue or behavioral modifier for Melibe. It has been hypothesized that swimming may be a voluntary or accidental means of dispersal and mixing of isolated gene pools for this animal (Block et al., 1982; Mills, 1994). If this is true, chances for survival during dispersal may be enhanced if light intensity could be used as a cue for depth. Melibe swims preferentially at night however, when there would be little or no light gradient in the environment. Also, the fact that swimming moves the animal upward through the water column allows the animal to maintain a height in the water where habitat and mates are likely thereby negating the need for a depth signal from light. Lastly, avoidance of visual predators may not be the primary reason for nighttime swimming since the only visual predators for Melibe are crabs, that rely on contact as well as visual cues. Thus, it may be that swimming at night lowers the probability of encounter with a predator, and keeps the animal in a depth where suitable habitat is likely to be found.
LITERATURE CITED


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