Mechanisms of satiation in the nudibranch Melibe leonina

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Mechanisms of satiation in the nudibranch Melibe leonina

Abstract
In recent years, scientists have begun to study satiation as a means of understanding changes in motivational state. Satiated animals not only show a reduction in feeding behaviors, but also in locomotion, and even show changes in their responses to various stimuli. Therefore, satiation is a qualitative change in the behavioral state of an animal. Although the behavioral characteristics of satiation are well understood, as are the changes in hormone release following a meal, the neural correlates of satiation are less understood. In particular, few studies have attempted to determine how satiating signals reconfigure feeding neural networks. To begin to address this topic, I studied satiation in the nudibranch Melibe leonina, an organism that is ideally suited for studies on the neural correlates of feeding behavior. In the first chapter of my thesis I documented the time course of satiation in Melibe, and demonstrated that stomach distention from food reduces the motivation to feed in this species. Additionally, I obtained data that suggest that a small amount of stomach distention may enhance feeding, an idea that has not been previously discussed in the literature. In the second chapter I determined that the posterior nerves, which run from the buccal ganglia to the tree ganglia (a pair of ganglia that lie on the surface of the middle of the stomach), respond to stomach distention, and that posterior nerve activity reduces the motivation to feed in Melibe. I demonstrated that stomach distention changes the signaling between the brain and buccal ganglion and terminates fictive swallowing rhythms from the anterior nerve of the buccal ganglion. Additionally, I obtained preliminary evidence to suggest that the molluscan peptide SCPB enhances feeding in Melibe, although it does not appear to initiate feeding. Lastly, I demonstrated that exposure to food at night inhibits nighttime bouts of locomotion in Melibe, but consumption of a meal prior to nightfall does not appear to alter locomotion. Based on these locomotion studies, I propose a model to explain how stomach distention and circadian clocks interact to regulate behavioral state at night. These results establish an important background necessary for studies of satiation in Melibe, enabling future studies on the feeding network reconfigurations caused by stomach distention.

Keywords
behavioral state, feeding, gastropod, neuroethology, stomach distention, stretch receptor, Behavioral psychology, Biology, Neurosciences

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MECHANISMS OF SATIATION IN THE NUDIBRANCH *MELIBE LEONINA*

BY

COLIN LEE

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

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in
Zoology

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Abstract

Mechanisms of satiation in the nudibranch *Melibe leonina*

By

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*University of New Hampshire, September 2015*

In recent years, scientists have begun to study satiation as a means of understanding changes in motivational state. Satiated animals not only show a reduction in feeding behaviors, but also in locomotion, and even show changes in their responses to various stimuli. Therefore, satiation is a qualitative change in the behavioral state of an animal. Although the behavioral characteristics of satiation are well understood, as are the changes in hormone release following a meal, the neural correlates of satiation are less understood. In particular, few studies have attempted to determine how satiating signals reconfigure feeding neural networks. To begin to address this topic, I studied satiation in the nudibranch *Melibe leonina*, an organism that is ideally suited for studies on the neural correlates of feeding behavior. In the first chapter of my thesis I documented the time course of satiation in *Melibe*, and demonstrated that stomach distention from food reduces the motivation to feed in this species. Additionally, I obtained data that suggest that a small amount of stomach distention may enhance feeding, an idea that has not been previously discussed in the literature. In the second chapter I determined that the posterior nerves, which run from the buccal ganglia to the tree ganglia (a pair of ganglia that lie on the surface of the middle of the stomach), respond to stomach distention, and that posterior nerve activity reduces the motivation to feed in *Melibe*. I demonstrated that stomach distention changes the signaling between the brain and buccal ganglion and terminates fictive swallowing rhythms.
from the anterior nerve of the buccal ganglion. Additionally, I obtained preliminary evidence to suggest that the molluscan peptide SCP_B enhances feeding in *Melibe*, although it does not appear to initiate feeding. Lastly, I demonstrated that exposure to food at night inhibits nighttime bouts of locomotion in *Melibe*, but consumption of a meal prior to nightfall does not appear to alter locomotion. Based on these locomotion studies, I propose a model to explain how stomach distention and circadian clocks interact to regulate behavioral state at night. These results establish an important background necessary for studies of satiation in *Melibe*, enabling future studies on the feeding network reconfigurations caused by stomach distention.
Introduction

Over the past 40 years, gastropods have emerged as an excellent model group for studies of the neural basis of behavior (Elliott and Susswein, 2002). Unlike vertebrates, gastropods have simple nervous systems and large, individually recognizable neurons. This facilitates the characterization of neural circuits; a task that ultimately helps define the relationship between the activity of individual neurons and the expression of specific behaviors, such as the swimming behavior of the nudibranch *Melibe leonina* (Watson *et al.*, 2001) or the feeding behavior of the euopisthobranch *Aplysia californica* (Rosen *et al.*, 1991). However, much less is known about how nervous systems control changes in the motivation to perform a behavior.

Most of the research on the mechanisms underlying motivational change has focused on satiation, or a decrease in the responsiveness to food. The behavioral characteristics of satiation have been described in a number of species, and in some cases the stimuli leading to satiation have been identified (e.g. the blowfly *Phormia regina* [Bowdan and Dethier, 1986]; gastropod *Pleurobranchaea californica* [Croll *et al.*, 1987]; *Aplysia* [Kuslansky *et al.*, 1987]). However, few studies have investigated the changes that occur in feeding networks during satiation. An excellent organism in which to address this topic is the nudibranch *Melibe leonina*, which, due to its simple nervous system, lack of a buccal mass, semitransparent skin, and easily quantified feeding behavior, is well suited for studies of satiation. In this thesis I determined if *Melibe* satiates due to stomach distention, and then determined how stomach distention influences the activity of several nerves involved in feeding. Additionally, I examined the influence of feeding on locomotion, and also the influence of the peptide SCP$_B$ on feeding. These experiments take an
important first step in the study of this topic in *Melibe*, and facilitate further research in *Melibe* that will how satiating cues influence feeding networks.

**Feeding in gastropods**

Before discussing satiation in *Melibe*, it is important to first understand how the gastropod nervous system controls feeding. In most gastropods (e.g. *Aplysia* [Kupfermann, 1974]; *Lymnaea stagnalis* [Rose and Benjamin, 1981]; *Pleurobranchaea californica* [Croll and Davis, 1982]) feeding movements can be broken down into two to three phases: protraction, retraction, and swallowing. During protraction, the mouth opens and comes forward, and (depending on the food item) either the radula extends to grasp food or the mouth performs repeated bites to capture food (Elliott and Susswein, 2002). During retraction food enters the mouth, and the mouth closes and pulls back (2002). Finally, the food is swallowed and brought to the gut (2002). In some species (e.g. *Aplysia* [Hurwitz and Susswein, 1992]), food enters the mouth during protraction and is swallowed during retraction, removing the need for a third ingestive phase.

The paired buccal ganglia, which lie on top of the esophagus, control the mouth in gastropods (Cohen *et al.*, 1978; Rose and Benjamin, 1979; Rosen *et al.*, 1992), but the interneurons that command feeding are generally located in the cerebral ganglion. These interneurons project processes into the buccal ganglion, where they synapse onto the motor neurons that control mouth musculature (Chiel *et al.*, 1986; Rosen *et al.*, 1991). A different population of interneurons produces each movement phase, and these different populations cyclically inhibit each other to produce rhythmic feeding cycles (London and Gillette, 1984; Elliott and Benjamin, 1989; Rosen *et al.*, 1992).
Chemosensory stimulation of the lips, tentacles, and rhinophores elicits these feeding rhythms (Kupfermann, 1974; Davis et al., 1983). Multiple groups of neurons respond to food stimuli (Kupfermann, 1989), but foremost among these neurons is a pair of electrically coupled serotonergic neurons called the metacerebral or cerebral giant cells (Gillette and Davis, 1976; Weiss and Kupfermann, 1976; Kupfermann and Weiss, 1982; Chase and Tolloczko, 1992), which strongly excite feeding (Granzow and Kater, 1977). In some species metacerebral cell activity is necessary for feeding to occur (Granzow and Kater), whereas in others such activity simply strengthens feeding motions (Gillette and Davis, 1976; Rosen et al., 1989) or depolarizes buccal motor neurons (McCrohan and Benjamin, 1980) to make feeding more likely. Lastly, research in several species suggests the metacerebral cells promote the transition from protraction to retraction (Granzow and Kater, 1977; McCrohan and Benjamin, 1980). Thus the metacerebral cells track food-induced arousal in gastropods, and coordinate the activity of different populations of feeding interneurons and motor neurons to produce rhythmic feeding movements.

**Neuroethology of satiation**

Once animals have consumed sufficient amounts of food they become satiated and no longer feed (Kupfermann, 1974) or respond to food stimuli (1974; Lent and Dickinson; 1987; Bowdan and Dethier, 1986), and will even flee from their preferred food (Gillette et al., 2000). One of the primary causes of satiation is distention of the stomach by food (Kupfermann, 1974; Reingold and Gelperin, 1980), a relationship that becomes apparent when the volume in the stomach is artificially manipulated. *Aplysia californica* (Susswein and Kupfermann, 1975a; Kuslansky et al., 1987), and the medicinal leech *Hirudo medicinalis* (Groome et al., 1993) both
eat less when the stomach is pre-filled with an artificial bulk substance, and conversely *Hirudo* feeds four times longer than normal when food is unable to distend the stomach (Lent and Dickinson, 1987). Although *Hirudo* normally goes months without feeding, if blood is removed from its stomachs following a meal it immediately begins to eat again (Lent and Dickinson, 1987). Thus, stomach distention holds a dominant role in regulating feeding behavior. Furthermore, in invertebrates stomach distention is the only known internal cause of satiation (Gillette, 2006; Gaudry and Kristan, 2012; Gelperin, 1966); hormones, like leptin or ghrelin, influence feeding in vertebrates (Inui, 1999; Nakazato et al., 2001), have not been demonstrated to influence hunger in invertebrates, although leptin and ghrelin-like sequences have been found in the *Aplysia* genome (personal observation). Chemosensory feedback from ingested food also does not appear to play a role in satiation in invertebrates (Susswein and Kupfermann, 1975a; Lent and Dickinson, 1987).

Stomach distention causes gastric stretch receptors to fire (Paintal, 1954; Dethier and Delperin, 1967; Bowdan and Dethier, 1986), and this activity helps terminate feeding. In the blowfly *Phormia regina* the recurrent nerve of the stomach conveys stomach distention to the brain, and sectioning this nerve leads to increases in feeding (Dethier and Gelperin, 1967). *Pleurobranchaea californica* feed far more when their stomatogastric nerves, which contain processes from stretch receptors, are lesioned (Croll et al., 1987), and *Aplysia californica* show a similar effect with the lesioning of their analogous esophageal nerves (Kuslansky et al., 1987). Research in invertebrates reveals several ways that stretch receptor activity could alter feeding. In blowflies with full stomachs, tarsal chemosensory receptors are less responsive to sugar, so the flies do not feed when presented with food (Evans and Browne, 1960). Thus, in some species distention prevents feeding by inhibiting the initial response to food. Satiation can also
temporarily change the physiology of feeding interneurons. In *Aplysia*, the burst threshold of the B51 feeding interneuron neuron increases in individuals that have fed to satiation (Dickinson et al., 2014), as do the thresholds of the *Pleurobranchaea* paracerebral feeding interneurons (Davis et al., 1983). Interestingly, the *Pleurobranchaea* interneurons show a similar reduction in excitability when individuals are conditioned to avoid food (Davis et al., 1983), demonstrating that different signals can yield the same cellular changes. Changing the excitability of command neurons ensures that food does not elicit feeding, even if food stimuli activate sensory receptors. Lastly, in individuals that are not motivated to feed, the feeding network is biased towards the retraction phase. In *Lymnaea*, an identified esophageal mechanoreceptor excites retraction phase motor neurons while inhibiting protraction phase ones (Elliott and Benjamin, 1989), and in *Pleurobranchaea* that have been conditioned to avoid food, prey stimuli cause the same inhibition/excitation pattern (London and Gillette, 1984). By exciting retraction and inhibiting protraction, these sensory inputs prevent the animal from opening its mouth.

Although the studies in *Lymnaea* and *Pleurobranchaea* furnish an explanation of how sensory inputs reconfigure feeding networks, they do not explicitly demonstrate that stomach distention produces these changes. An aversive stimulus reconfigured the *Pleurobranchaea* network (London and Gillette, 1984), and in *Lymnaea* it was a proesophageal receptor, and not a gastric one, that inhibited fictive feeding (Elliott and Benjamin, 1989). The function of this receptor is unknown, and it may respond to touch and not sustained distention; in *Aplysia* radula mechanoreceptors inform animals that food has entered the mouth and other mouth proprioceptive neurons help produce retraction during rhythmic ingestive movements (Rosen et al., 2000). The *Lymnaea* stretch receptor may combine the function of these neurons, inducing
swallowing once food is detected in the mouth. Thus, it is of interest to directly determine if stomach distention produces the same network reconfigurations.

Another important element in satiation is the relationship between stomach distention and neurotransmitter release. In invertebrates a number of transmitters appear to influence the decision to feed (Elliott and Vehovsky, 2000), but the most thoroughly studied is serotonin (5-HT), which exerts a clear excitatory influence on feeding in both gastropods and leeches. In gastropods, 5-HT lowers feeding thresholds (Palovcik et al., 1982), and is used by the metacerebral cells to excite feeding (Rosen et al., 1989; Kupfermann and Weiss, 1982). Similarly, the serotonergic Retzius cells in leeches excite feeding (Lent, 1985), and application of 5-HT to leeches initiates feeding (1985). Additionally, stomach distention changes 5-HT levels. Both metacerebral cells (Hatcher et al., 2008) and Retzius cells (Gaudry and Kristan, 2012) have reduced 5-HT after a meal, and both cell types are inhibited by stomach distention (Horn et al., 2001; Lent, 1985; Lent and Dickinson, 1987). Moreover, distention prevents 5-HT levels from returning to normal in satiated leeches (Gaudry and Kristan, 2012). Thus, stomach distention appears to reduce feeding in part by reducing 5-HT transmission.

Of the other transmitters, the one that is the most interesting in the context of satiation and stomach distention, especially in Melibe, is SCP_B. Eight gastropod species, including Melibe, have been shown to have a large SCP_B cell in the buccal ganglion (Lloyd et al., 1985; Watson and Willows, 1992), and an apparent feeding rhythm can be recorded in most of them (1992). Additionally, in Limax maximus SCP_B application increases the responsiveness of the feeding motor program to chemosensory stimuli (Prior and Watson, 1988). No studies have explored the relationship between SCP_B and stomach distention, but several studies suggest that they may interact. Stomach distention is conveyed to the buccal ganglion in most species (e.g. Aplysia
[Kuslansky et al., 1987]; Limax [Reingold and Gelperin, 1980]; Pleurobranchaea [Croll et al., 1987]), so it is possible that there are stretch receptors that make synaptic connections to buccal SCP$_B$ cells. Thus, it is of interest to determine if stomach distention influences SCP$_B$ release in Melibe.

**The relationship between feeding and locomotion**

Satiation changes not only feeding behavior, but also locomotion. *Aplysia* (Kupfermann, 1974) moves less after a meal, and distention reduces locomotion in the blowfly *Phormia regina* (Browne and Evans, 1960) and in *Hirudo* (Gaudry and Kristan, 2010). Moreover, *Hirudo* will not move at all immediately after a meal (Gaudry and Kristan, 2012). Conversely, hunger enhances locomotion; in *Lymnaea* locomotor neurons show increased excitability in hungry animals (Dyakonova et al., 2015), and as *Hirudo* gradually digests a meal it becomes increasingly more active (Gaudry and Kristan, 2012). Thus, stomach distention appears to influence general arousal; animals with empty stomachs are active and responsive to food, and satiated animals are quiescent.

In addition to stomach distention, circadian clocks strongly regulate behavioral state. Many animals exhibit daily patterns of locomotion (Konopka and Benzer, 1971; Silver et al., 1996), which are regulated by internal circadian clocks. The interaction between inputs from clock neurons (internal neurons whose activity oscillates on a 24 hour cycle) and stretch receptors has yet to be thoroughly explained, but is essential to our understanding of how nervous systems control behavioral state. Recent studies with *Melibe leonina* have established it as a good model system in which to study the neural basis of circadian rhythms (Newcomb et al.,
2014), so in addition to studying the neural correlates of satiation in *Melibe*, we attempted to determine how feeding influences locomotion patterns.

**Melibe leonina as a model organism for satiation studies**

*Melibe* is a nudibranch in the family Tethyidae, and is well suited for studies of satiation. All gastropods have simple nervous systems, but the *Melibe* central nervous system is simple even by the standards of the taxon. The *Melibe* central nervous system is organized into only six ganglia: the paired cerebropleural, buccal, pedal ganglia. As in all gastropods, the buccal ganglia control feeding (Trimarchi and Watson, 1992), but unlike other gastropods, *Melibe* lack a buccal mass, allowing for exceptionally simple buccal ganglia. Each ganglion has only 30-40 neurons and with only four nerves emanating from each ganglion (Trimarchi and Watson, 1992). One of these nerves, the posterior nerve, innervates the esophagus and stomach, suggesting a similar role to that of the stomatogastric nerves studied in other animals (Kuslansky et al., 1987; Croll et al., 1987; Bowdan and Dethier, 1986).

The *Melibe* morphology also makes it amenable to feeding studies; it has semi-transparent skin that allows for non-invasive visualization and imaging of organs, including the stomach. The feeding motions, termed oral hood closures, are stereotyped, easily visualized, and easily quantifiable (Watson and Trimarchi, 1992). Finally, it feeds predictably when exposed to small zooplankton, such as the brine shrimp *Artemia* (Watson and Chester, 1993). The simplicity of the nervous system, along with these morphological and behavioral properties, makes *Melibe* a highly tractable organism for studies of feeding and satiation. Moreover, the *Melibe* buccal ganglion contains a large SCPB cell (Watson and Willows, 1992), which extensively innervates the esophagus, and has branches that extend to the stomach via the
posterior nerve. Recently, we collected data suggesting that SCP_B influences locomotion in *Melibe*, so SCP_B may not only influence feeding, but also integrate several different behaviors. Thus, *Melibe* is well suited to answer questions about the relationship between SCP_B and feeding.

In the first chapter of this thesis I demonstrate that satiation occurs in *Melibe*, and that it does so due to stomach distention. In the second chapter I determine that stomach distention is communicated by the posterior nerves, reduces the activity of the cerebral buccal connective, and terminates feeding rhythms from the anterior nerve. Additionally, I demonstrate that feeding reduces circadian locomotion in *Melibe*, and that the peptide SCP_B enhances feeding.
Chapter 1: The influence of stomach distention on feeding in the nudibranch mollusk *Melibe leonina*

**Abstract**

In this study, I sought to characterize the time course of satiation in the nudibranch *Melibe leonina* and determine if satiation in this animal is caused by stomach distention. When brine shrimp (*Artemia*) were provided *Melibe* immediately commenced performing feeding motions, but despite the continued availability of food, stopped feeding after approximately five hours. The stomach filled with food as a feeding bout progressed, and as the stomach filled the feeding rate slowed. Furthermore, injecting artificial food into the stomach reduced feeding activity, and lesioning in the stomach wall to prevent it from filling prevented satiation. Taken together, these data demonstrate that stomach distention influences the motivation to feed, and set the stage for research on the influence of stomach distention on feeding circuits.

**Introduction**

In order to understand the neural mechanisms underlying changes in motivational state, a number of scientists have focused their attention on satiation: a decrease in the motivation to feed after a meal. Studies in several invertebrate taxa, notably blowflies, gastropods, and leeches, have shown that satiated animals are less responsive to food stimuli (Getting and Steinhardt, 1972; Kupfermann, 1974; Lent, 1985; Bowdan and Dethier, 1986), and will reject (Kupfermann, 1974) and even flee from their preferred food (Gillette et al., 2000). Conversely, in some species hungry individuals will respond to noxious stimuli with appetitive behaviors (Gillette et al., 2000). In addition to these changes in feeding behaviors, satiation causes reductions in locomotion (Browne and Evans, 1960; Kupfermann, 1974), whereas hunger leads to increases in activity (Strong, 1957; Green, 1964; Dyakonova et al., 2015). Thus, satiation after a meal is not merely a reduction in feeding behaviors, but a qualitative shift in the behavioral state of an animal.

These behavioral changes are caused by stomach distention (Susswein and Kupfermann, 1975a,b; Bowdan and Dethier, 1986; Lent and Dickinson, 1987); food fills the stomach and
excites gut stretch receptors, (Paintal, 1954) whose activity reduces feeding (Kuslansky et al., 1978; Croll et al., 1987). Additionally, studies in several species demonstrate that stomach distention itself can reduce locomotion. In both Phormia (Browne and Evans, 1960) and the leech Hirudo medicinalis (Gaudry and Kristan, 2010) artificial stomach distention reduces activity, and in Hirudo firing from body wall stretch receptors terminates swimming (2010). Simultaneous research, notably in the euopisthobranch Aplysia californica, has revealed much about the neural basis of feeding in gastropods (see Elliott and Susswein [2002] and Cropper et al. [2003] for a detailed review). To summarize, ingestive movements in gastropods break down into two to three phases: protraction, retraction, and in some cases swallowing (Elliott and Susswein, 2002), and a different population of interneurons produces each phase (Rose and Benjamin, 1981; London and Gillette, 1984; Rosen et al., 1992). The different interneuron populations cyclically inhibit each other, and each population controls different buccal motor neurons (Elliott and Benjamin, 1989; Rosen et al., 1992).

Despite our understanding of both the behavioral characteristics of satiation and the neural circuitry that produces feeding, few studies have attempted to determine how satiating signals (i.e. stomach distention) alter feeding circuits to produce satiation (but see London and Gillette [1984] and Elliott and Benjamin [1989]). Additionally, little focus has been given to the relationship between stomach distention and locomotion, a topic that would help reveal how satiating cues influence the overall behavioral state of an animal. One species that is well suited to fill these gaps in our understanding of satiation is the filter-feeding nudibranch Melibe leonina. Gastropod feeding circuitry has been thoroughly described in Aplysia, the Melibe central nervous system, behavior, and morphology offer several advantages over Aplysia. First, the lack of a buccal mass enables Melibe to have exceptionally small buccal ganglia, with only 30-40
neurons in each ganglion (Trimarchi and Watson, 1992). Such simplicity will allow the neural mechanisms of satiation to be described with greater precision, as the impact of satiating stimuli can potentially be determined for each feeding interneuron and motor, rather than a select subset of neurons. Second, *Aplysia* uses several different types of feeding motions to consume food (Elliot and Susswein, 2000), whereas *Melibe*, a filter feeder that does not need to cut or grasp food, only performs one type of ingestive motion. With less variability in *Melibe* feeding motions, it will be easier to determine how satiating stimuli influence feeding circuits. Third, *Melibe* has semitransparent skin that allows for non-invasive imaging of the stomach, facilitating studies of the relationship between stomach fullness and satiation. Lastly, *Melibe*, with eyes that lie directly on the brain, is uniquely suited for studies of circadian rhythms (Newcomb et al., 2014). Therefore, *Melibe* provides a highly tractable system in which to study not only the neural mechanisms that underlie satiation, but also the interaction between satiating signals and circadian clocks to produce behavioral state.

The overall goal of this study was to characterize the time course satiation in *Melibe*, and determine if satiation is caused by stomach distention. If *Melibe* satiates similarly to other animals, then *Melibe* should initially feed when exposed to prey, but terminate feeding while food is still available. Additionally, if stomach distention is the primary cause of satiation, then and the stomach should fill with food during a meal, and artificially manipulating stomach fullness should alter feeding duration. Testing these predictions will provide important data on the behavioral characteristics of satiation in *Melibe* establishing the background necessary to address the effect of stomach distention on feeding neural networks.
Methods:

Animals

Adult *Melibe leonina* were acquired from eelgrass beds near the University of Washington’s Friday Harbor Laboratories and in Monterey Bay, California. *Melibe* were then shipped to the University of New Hampshire, Durham, NH and maintained in an aquarium with recirculating seawater, at approximately 13 °C, until experimentation.

Feeding experiments

In order to determine if *Melibe* satiates and if satiation is caused by stomach distention, several different experiments were performed in which individual *Melibe* were given food and their feeding rates were recorded. In each experiment subjects received a different manipulation (described in the following sections) prior to feeding, but experiments proceeded in the same way otherwise. Prior to each individual trial, subjects were placed in circular buckets (diameter of 30 cm) located within a larger tank of aerated seawater. Small mesh “windows” in the buckets allowed water to flow through them, but prevented food from escaping. The tanks were kept in a 13 °C cold room that was on a 24 hour light/dark cycle, with 10 to 14 hours of light per day, depending on the season.

Subjects adjusted to the buckets for 24 hours, and then newly hatched *Artemia spp.* (brine shrimp) were added to the bucket to yield a density of approximately 3,000 *Artemia*/L. All trials began between 10 and 11 AM to ensure that the time of day did not affect the motivation to feed, and *Melibe* fed ad libitum for approximately 24 hours. A black and white camera suspended directly above the buckets captured feeding activity, and recorded from approximately one hour before *Artemia* addition to 24 hours post-addition. Camera outputs were digitized, time-stamped,
and recorded on a computer using the video capture software Gawker, which took one picture every second and streamed the images together at a rate of ten frames per second. In the subsequent video analysis, the number of feeding motions (oral hood closures) per minute was recorded until the animal returned to its baseline rate of OHCs, which was taken from the hour before *Artemia* addition. During pilot studies we observed that animals would routinely perform incomplete feeding movements, performing the oral hood closing phase of feeding (the first phase, in which the hood comes forward and closes, drawing in water), but not the tilt and squeeze swallowing phase (in which the closed hood is tilted back; see Watson and Trimarchi, 1992, for a complete description of these phases). During these aborted feeding motions *Melibe* likely does not swallow the prey it captures (Trimarchi and Watson, 1992), and thus food does not enter the stomach. Complete feeding sequences were recorded as ingestive motions, and incomplete ones as food searching or casting motions, and the two were analyzed separately.

*Changes in feeding rate over time*

To determine if *Melibe* satiates, nine individuals, who had not received any manipulations, were fed. Animals were weighed prior to testing so that we could determine if size influenced feeding duration. Additionally, three individuals were fed on multiple days to assess the impact of a recent meal on the motivation to feed. These *Melibe* were fed three separate times: once at the beginning of the trial, a second time 24 hours later, and lastly 72 hours after the first feeding.

*Changes in stomach fullness over time*

In order to quantify how stomach volume changed over time, the feeding activity of six *Melibe* was recorded and pictures of their stomachs were taken at intervals as they ate. Prior to feeding, individuals were removed from their buckets and a picture of the stomach was taken
using a dissecting microscope. Thirty minutes after food addition, the subject was removed and another picture of the stomach was taken. This process continued until the subject went at least thirty minutes without feeding, at which point one final picture was taken. In each picture, the surface area of the stomach was calculated with the software ImageJ (Fig. 1), and used to approximate stomach fullness.

Figure 1: The *Melibe* stomach. A) The stomach, filled with *Artemia*, within the whole animal. B) Sample ImageJ surface area measurement of the stomach.

The stomachs were removed at the end of each trial and the *Artemia* in each of them were counted to determine if the surface area measurements correlated with stomach fullness. The number of *Artemia* in the stomach was also subtracted from the original number in the tank to determine if the density of *Artemia* in the tanks had changed during the feeding trial. Finally, as described previously, subjects were housed, fed, and videotaped in the same manner as in the previous experiments.
The influence of artificial stomach distension on feeding

To determine if stomach distention reduces the motivation to feed, five *Melibe* were fed after having their stomachs filled with a non-nutritive bulk substance of gelatin and methylcellulose. To prepare the substance, one gram of gelatin was heated and dissolved in 40 mL of seawater, after which one gram of methylcellulose was added to create additional bulk. The mixture was then drawn into a 1 mL syringe, and a thin strip of tubing was inserted over the needle of the syringe to act as a cannula. The solution was maintained at 12°C until needed. For the injections, animals were first pinned out ventrally in a sylgard dish, with a single pin in the foot and two through the oral hood. The cannula was inserted through the mouth and gently guided into the stomach, and the bulk substance was gradually added until the stomach was visibly distended. After the injection, subjects were returned to their arenas, and tested several hours later. Lastly, individuals were first tested with a sham injection to serve as a control, and then were tested a week later with a real injection. For the sham injection *Melibe* were pinned out as above, but did not have the substance added to their stomach.

The influence of stomach lesions on feeding

To see how feeding was affected when the stomach was not filled, four *Melibe* were fed after their stomachs were cut open, allowing food to escape, and thereby preventing stomach distention. For the lesions, subjects were pinned out dorsally, a single 0.5 cm incision was made in the skin directly above the stomach, and the stomach was cut open with scissors. The skin incision was then sewn up with a sterile suture, and the animal was given several days to recover before testing.
Statistics

All statistical tests were performed with the software JMP Pro 11. Changes in feeding rate over time were determined with repeated measures ANOVAs with multiple comparisons tests. In the bulk injection experiment, feeding rates between the two treatments were compared through paired t-tests. Lastly, we used linear regressions to compare feeding rate and the change in surface area to the total number of OHCs performed.

Results:

Changes in feeding rate over time

The feeding rates (oral hood closures [OHC]/minute) of nine Melibe were recorded before and after addition of Artemia at a concentration of 3,000/L (Fig. 2). As seen in previous studies (Watson and Trimarchi, 1992; Watson and Chester, 1993), subjects showed an immediate response to the addition of Artemia, increasing their feeding activity significantly from 0.01 ± 0.008 OHC/min at baseline to 1.66 ± 0.43 after twenty minutes (P = 0.005). Feeding generally peaked within an hour of food addition, and plateaued for the next two hours. After this point feeding started to return to baseline, reaching a rate after 5 hours (0.87 ± 0.28 OHC/min) that was significantly less than that at the peak (2.2 ± 0.42 OHC/min; P = 0.025) and no longer significantly different from baseline (P = 0.14).

In order to determine if recent consumption of a meal influences feeding, three Melibe were given Artemia three times in four days (fed on days 1, 2, and 4). On the second day, when they had fed 24 hours prior, Melibe performed significantly fewer OHC in the first hour of feeding (62.0 ± 35.8 OHC) compared to the first day (188.0 ± 9.2 OHC; P = 0.048), although feeding was similar for the rest of the bout (Fig. 3). On the fourth day, with 48 hours between
meals, feeding returned to normal, and the number of OHCs in the first hour (178.0 ± 11.9 OHC) was not different from on the first day (P = 0.18).

![Graph showing changes in Melibe feeding rate over time after Artemia addition. Feeding rate (OHC/min ± SEM) was calculated for each ten-minute bin for the first hour, and for every hour after. The rate of OHCs increased significantly shortly after food was added (bar indicates times in which the rate was significantly elevated), but returned to baseline after several hours. Feeding probably did not stop because the individuals had consumed all the food. On average, Melibe (n = 6) consumed 1357.5 ± 554.20 Artemia in 4 hours; subtracting this number from the original number of Artemia in the tank reveals that the feeding rate returned to baseline when the density of Artemia was 90.1 ± 5.2% of the starting amount, or approximately 2700 Artemia/L. Melibe performs OHCs at a similar rate for densities of 2700 and 3000 Artemia/L (Watson and Trimarchi, 1992), so the approximately 10% decrease in Artemia should not have influenced feeding.](image-url)
Figure 3: Feeding rate when subjects were fed on consecutive days. Feeding rate (OHC/min ± SEM) was calculated for each ten-minute bin for the first hour, and for every hour after. On the second day subjects fed much less for the first hour after Artemia addition.

Changes in stomach fullness over time

In order to examine the relationship between the number of OHCs and stomach fullness, and to determine how stomach fullness changes during feeding, the stomach surface area of five Melibe was recorded over the course of a meal. As feeding progressed the stomach became increasingly full (Fig. 4), and the change in fullness correlated significantly with time (P < 0.0002; R^2 = 0.69; Fig. 5). Surface area increased the most initially, while subjects were feeding quickly, and then increased more slowly as the rate of OHCs declined and the Melibe became satiated (Fig. 4). Additionally, feeding rate inversely correlated with stomach surface area (Fig. 6); as the stomachs filled, feeding rate decreased (P < 0.0001; R^2 = 0.50).
Figure 4: Changes in *Melibe* stomach surface area over time during a feeding bout. A) Images of the stomach at 0, 60 and 90 minutes after food addition. Initially, the stomach was empty and small, but as the *Melibe* consumed *Artemia*, the stomach became progressively more full. B) Percent changes in stomach surface area and cumulative number of OHCs taken per 30 min for one *Melibe* during a 4 hour feeding trial.
Figure 5: Changes in stomach fullness over time for four different *Melibe* during a feeding bout. Different symbols denote different individuals. Stomach surface area correlated significantly with size, and for each individual the final surface area measurement was the largest.
Figure 6: Feeding rate versus stomach surface area for five different *Melibe*. Each symbol denotes a different individual. As the stomach became more full, feeding rate decreased.

*The Influence of artificial distention of the stomach on feeding*

The aforementioned results suggest that the progressive increase in stomach fullness leads to satiation. To test this hypothesis, a second feeding experiment was conducted to determine if adding artificial bulk to *Melibe*’s stomach prior to prey exposure reduces feeding. Four *Melibe* were tested first with a sham bulk addition, and then a week later with a true addition of a bulk substance to their stomachs. Initially, feeding was similar between the conditions (Fig. 7), but after one hour *Melibe* fed significantly slower with the bulk in their stomach than without (P = 0.007). Additionally, in the bulk condition feeding significantly decreased from the peak rate (3.48 ± 0.457 OHC/min) after four hours (1.52 ± 0.608 OHC/min; P = 0.02), whereas in the sham condition it took six hours for the rate to significantly decrease from the peak (2.08 ± 0.400 OHC/min vs. 3.58 ± 0.256 OHC/min; P = 0.015).
Figure 7: The influence of stomach distension via non-nutritive bulk on *Melibe* feeding rate. Feeding rate (OHC/min ± SEM) was calculated for each ten-minute bin for the first hour, and for each hour after; stars indicate time points at which feeding rates were significantly different. In both conditions subjects ate at a similar rate for the first hour after food addition, but after one hour they slowed down more in the bulk condition compared to the sham condition.

*The impact of stomach lesions on feeding*

To complement the previous experiment, an additional one was performed in which *Melibe* were fed after their stomachs had been lesioned, thereby preventing food from distending the stomach. This treatment had two effects. First, lesioned individuals performed fewer OHCs than the unmanipulated ones (Fig 8). Second, their feeding rate did not decrease over time.
Figure 8: A comparison of the feeding rates of control *Melibe* and those with stomach lesions. Feeding rate (OHC/min ± SEM) was calculated for each ten-minute bin for the first hour, and for each hour thereafter; stars indicate time points at which feeding rates were significantly different. Individuals with lesioned stomachs did not eat as quickly as controls at the beginning of their feeding bouts, and their feeding rates did not slow as the bout progressed.

**Discussion**

*Melibe satiates*

The present results show that *Melibe*, when fed *ad libitum*, consumes prey until it becomes satiated. After food addition, subjects quickly increased their rate of OHCs, indicating that they were motivated to feed, and after several hours they began to slow down and returned to their baseline feeding rate by five hours (Fig. 2). The decrease in feeding was not caused by a change in food availability, as the majority of the *Artemia* originally added to the tank (90%) were still present at the end of the feeding bouts. Additionally, the results suggest that the decrease in feeding was not caused by muscular or neural fatigue, or sensory adaptation, because
several manipulations caused subjects to feed longer than 5 hours (Figs. 6, 7). In this regard, *Melibe* appears to be somewhat unique. Leeches with cannulated stomachs (i.e. preparations in which food passed through the stomach without distention) terminated feeding after two hours (Lent and Dickinson, 1987), and *Aplysia* shows decreased biting responses after an hour of repeated lip stimulation (Horn *et al.*, 2001) suggesting that within a short period of time neuromuscular fatigue terminates feeding in these species. Neuromuscular fatigue may still be possible in *Melibe*, but our results suggest that it takes more than nine hours for fatigue to influence behavior. Therefore, the observed decreased rate of OHCs was most likely due to a decrease in motivation.

As in *Aplysia* (Kupfermann, 1974), recent consumption of a meal decreased the initial motivation to feed. When individuals had fed only 24 hours prior, they performed significantly fewer OHCs (Fig. 3) in the first hour after food exposure compared to when they had gone at least 48 hours between meals. This change was not caused by sensory fatigue, as *Melibe* still performed spontaneous OHCs even when they had fed 24 hours prior (Fig. 3). Interestingly, after one hour *Melibe* fed similarly regardless of how long they gone between meals, demonstrating that although the initial motivation to feed was influenced by recent feeding, the duration of feeding bouts was not affected.

*Stomach distention influences the motivation to feed*

As expected, the volume of each individual’s stomach increased over the course of a feeding bout (Fig. 4), and stomach fullness correlated significantly with the cumulative number of OHCs (Fig. 5). Moreover, as the stomach filled during the meal, feeding rate decreased (Fig. 6). Thus, stomach distention in *Melibe*, as in *Aplysia* (Susswein and Kupfermann, 1975;
Kuslansky et al., 1987), leeches (Lent and Dickinson, 1987; Groome et al., 1993), and blowflies (Dethier and Gelperin, 1967; Bowdan and Dethier, 1986), is one of the key causes of satiation. The responses of the Melibe in the bulk addition and stomach lesion experiments demonstrate that stomach distention reduces the motivation to feed. After feeding for one hour, individuals injected with a bulk substance fed noticeably less than they did following a sham injection (Figure 7). Conversely, stomach lesioned individuals fed robustly long after the controls had satiated (Figure 8), presumably because food leaked out of their stomach and did not activate putative stretch receptors that respond to distension.

It is important to note that the feeding rates for Melibe in both sham and bulk conditions were greater than for control Melibe, but this is likely an artifact of the injection procedure. When a cannula was inserted into the stomach it likely stimulated buccal motor neurons (see Gelperin et al. [1978] and Rosen et al. [2000]), thereby lowering the threshold for feeding; the subjects consistently performed OHCs even before food addition (Fig. 6), which supports this idea.

**Feeding when the stomach has a small amount of distention**

Certain data from this study suggest that a small amount of stomach distention actually excites feeding. For the first hour after prey exposure, bulk-injected Melibe fed as robustly as when they were sham-injected (Fig. 7). The bulk material may have provided positive feedback to these Melibe, signaling that the OHCs successfully captured prey. Meanwhile, stomach-lesioned individuals actually fed less than controls initially (Fig. 8), and it was not until five hours, when the controls had satiated, that the lesioned Melibe had a faster feeding rate. If a small amount of stomach distention can promote feeding, then these individuals would have lacked this excitation, and therefore never been fully stimulate to feed.
To our knowledge no studies have demonstrated that a small amount of stomach distention excites feeding, but this lack of evidence could be due to the methods used. Most studies of satiation (e.g. Susswein and Kupfermann, 1975a; Croll et al., 1987) have used total food consumption to measure the motivation to feed rather than feeding rate, and thus could not detect short term effects. Additionally, although no studies have directly supported this idea, several reported data consistent with the hypothesis. For example, six hours after consuming a satiating meal (i.e., when the stomach is partially distended), Aplysia responds more quickly to chemosensory stimulation of the lips (Horn et al., 2001), suggesting that partial distention reduces the threshold to feed. In Pleurobranchaea, low intensity firing from the stomatogastric nerve (which conveys stomach distention [Croll et al., 1987]) elicits ingestive motor programs, whereas high intensity firing elicits a mix of egestive and ingestive programs (Croll and Davis, 1982), suggesting that there is a qualitative difference in the signaling caused by low and high stomach distention. In both of these studies, however, there were other uncontrolled variables that could have accounted for the data, and thus it is premature to say if this phenomenon indeed occurs.

Small amounts of stomach distention may provide a feedback loop to guide feeding behavior. Melibe consumes food that is both ephemeral and patchy, and occasionally performs food-capturing motions even in the absence of food. Although it has been demonstrated that both tactile stimulation and chemical cues can elicit feeding motions in Melibe (Chester and Watson, 1993), the additional confirmation of successful ingestion, from stretch receptors in the esophagus and stomach, might be necessary to maintain feeding activity. Studies in Aplysia support this idea, as information from the esophageal nerves is necessary for Aplysia to learn that food is inedible (Schwartz and Susswein, 1986). Stomach distention may also inhibit behaviors
in *Melibe* that compete with feeding. In *Hirudo*, a highly opportunistic feeder, stomach distention inhibits swimming (Groome *et al*., 1993; Gaudry and Kristan, 2010), preventing the animal from prematurely terminating a meal.

**Conclusions**

As has been seen in other gastropods, *Melibe* satiates. The reduction in feeding at the end of a meal is caused by stomach distention, but distention may have a distinct, second behavioral effect. A small amount of distention appears to provide a post-ingestive signal that feeding motions were successful, maintaining and enhancing feeding. Conversely, a large amount of distention signals that the stomach is full and terminates feeding behaviors. These conclusions enable future studies on how information about stomach distension is communicated to the CNS and, ultimately, the neural mechanisms involved in modulating the feeding circuits.
Chapter 2: Neural correlates of satiation in *Melibe leonina*

Abstract

Research in gastropod mollusks has revealed that stomach distention causes satiation, but the impact of stomach distention on feeding neural networks remains poorly understood. To explore this topic, we determined the pathway by which stomach distention is communicated to the brain in the nudibranch *Melibe leonina*, and examined the influence of stomach distention on the buccal ganglion. Distention is communicated by the posterior nerves, which connect the buccal ganglion with the tree ganglion on the stomach, but the posterior nerve does not appear to contain processes from stretch receptors themselves. Additionally, stomach distention reduces signaling from the buccal ganglion to the brain via the cerebral buccal connective, and terminates fictive swallowing in the anterior nerve. These results demonstrate that stomach distention alters the rhythmic output from feeding circuits.

Introduction

In the past several decades satiation (a decrease in the motivation to feed after a meal) has emerged as an ideal model for research on the neural basis of motivational change. The results from a number of studies, particularly on invertebrates, have revealed that stomach distention reduces the motivation to feed (Susswein and Kupfermann, 1975a,b; Bowdan and Dethier, 1986; Kuslansky *et al.*, 1987; Lent and Dickinson, 1987). Mechanosensory stretch receptors on the gut transduce stomach distention (Paintal, 1954; Gelperin, 1967; Kuslansky *et al.*, 1978), and loss of communication from these receptors leads to increases in feeding (Dethier and Gelperin, 1967; Belzer, 1978; Croll *et al.*, 1987).

Although it is clear that signaling from gastric stretch receptors reduces the motivation to feeding, the manner in which such activity reduces feeding is less clear. In the gastropod *Pleurobranchaea californica* an aversive stimulus inhibits specific feeding interneurons to bias the feeding central pattern generator towards the retraction phase of feeding (London and Gillette, 1984), and firing from a proespophageal mechanoreceptor produces a similar effect in the pulmonate *Lymnaea stagnalis* (Elliot and Benjamin, 1989). However, it remains to be seen if this network reconfiguration occurs following stomach distention, and also if the aforementioned
changes inhibit feeding in intact animals. In *Aplysia* radula mechanoreceptors inform animals that food has entered the mouth and help produce retraction during rhythmic ingestive movements (Rosen *et al*., 2000), so the *Lymnaea* proesophageal receptor may actually serve a role in food consumption.

One species that is well suited for research on the impact of stomach distention on feeding circuits is the nudibranch *Melibe leonina*. Although feeding circuits have been best characterized in *Aplysia* (Cropper *et al*., 2003), aspects of the *Melibe* nervous system, behavior, and morphology make *Melibe* perhaps more suitable for satiation studies. First, the *Melibe* buccal ganglion, which controls the mouth and esophagus, contains only 30-40 neurons (Trimarchi and Watson, 1992), far fewer than in *Aplysia*. As such, the neural mechanisms of satiation can be described with greater precision, as the impact of satiating stimuli can potentially be determined for each feeding interneuron and motor, rather than a select subset of neurons. Second, *Aplysia* uses several different types of feeding motions to consume food (Elliot and Susswein, 2000), with corresponding differences in feeding interneuron activity, whereas *Melibe* only performs one type of ingestive motion. With only one possible feeding motion, it will be easier to determine how satiating stimuli influence feeding circuits in *Melibe*. Third, *Melibe* has semitransparent skin that allows for non-invasive imaging of the stomach, thereby facilitating studies of the relationship between stomach fullness and satiation. Lastly, *Melibe*, with eyes that lie directly on the brain, is uniquely suited for studies of circadian rhythms (Newcomb *et al*., 2014). Therefore, studying satiation in *Melibe* will provide not only an understanding of the influence of stomach distention on feeding circuits, but also of how stomach distention and clock neurons interact to influence behavioral state. In the first chapter of my thesis I demonstrated that stomach distention causes satiation in *Melibe*, establishing a framework for further inquiry into
Stomach distention in *Melibe* is most likely conveyed by the posterior nerves, which run from the esophagus to the stomach and connect the buccal ganglion to the tree ganglion, which is located at the junction of the esophagus and the stomach (Trimarchi and Watson, 1992). The buccal ganglion controls swallowing in *Melibe* via the anterior nerve (Trimarchi and Watson, 1992), and also connects to the brain via a third nerve, the cerebral buccal connective. The buccal ganglion thus serves an important role in feeding in *Melibe*, and also likely serves as a relay center for signaling between the brain and stomach. Moreover, the direct connection between the buccal ganglion and the stomach via the posterior nerve makes the buccal ganglion the likely target of putative stomach stretch receptors that might inhibit feeding.

The goal of the study summarized in this chapter was to determine how stomach distention alters nervous system activity in *Melibe*. Specifically, I sought to determine: 1) if stomach fullness is communicated to the CNS by the posterior nerves and 2) if stomach distention alters the output of the buccal ganglion. If the posterior nerves convey information to the CNS about stomach fullness, then distending the stomach should cause changes in posterior nerve activity. Moreover, posterior nerve lesions should remove feedback about stomach fullness and thus alter feeding activity. Finally, if stomach distention alters feeding activity, then artificially inflating the stomach should change the activity of both the anterior nerve of the buccal ganglion, which is involved in swallowing, and signaling from the buccal ganglion to the brain via the cerebral buccal connective.
Methods

Animals

Adult *Melibe leonina* were acquired from eelgrass beds near the University of Washington’s Friday Harbor Laboratories in the Puget Sound, WA and in Monterey Bay, California. *Melibe* were then shipped to the University of New Hampshire, Durham, NH and maintained in an aquarium with recirculating seawater, at approximately 13 °C, until experimentation.

Identification of putative gastric mechanoreceptors

In order to characterize the putative mechanoreceptors in the gut that respond to stomach distention, cobalt chloride fills of the posterior nerve were performed. The nerve was cut close to the buccal ganglion, and the nerve and the tree ganglion were separated from the stomach. The anterior end of the nerve was then immersed in cobalt chloride for approximately 24 hours. Lastly, the preparation was developed with ammonium sulfide, fixed, cleared, and mounted according to the methods described in Watson *et al.* (2002).

Feeding assays

To determine if posterior nerve signaling influences the motivation to feed in *Melibe*, feeding experiments were performed that compared feeding rates between individuals with posterior nerve lesions and control individuals. Individual *Melibe* were placed in circular buckets (diameter of 30 cm) located within a larger tank of aerated seawater. The buckets had small mesh “windows” that allowed water to flow through them, but prevented food from escaping. The tanks were kept in a 13 °C cold room on a 24 hour light/dark cycle, with 10-14 hours of light per day, depending on the season. After subjects adjusted to the buckets for 24 hours, newly hatched *Artemia spp.* (brine shrimp) were added to the bucket to yield a density of approximately 3,000
Artemia/L. All trials began between 10 and 11 AM and Melibe were allowed to feed ad libitum for approximately 24 hours. A black and white camera suspended directly above the buckets captured feeding activity, and recorded from approximately one hour before Artemia addition to 24 hours post-addition. Camera outputs were digitized, time-stamped, and recorded on a computer using the video capture software Gawker, which took one picture every second and streamed the images together at a rate of ten frames per second.

In the subsequent video analysis, the number of feeding motions performed per minute was counted for the entire experiment. Melibe feeds using rhythmic movements termed oral hood closures (OHCs), which consists of an oral hood closing phase (in which the hood comes forward and closes, drawing in water) and a tilt and squeeze swallowing phase (in which the closed hood is tilted back; see Watson and Trimarchi, 1992, for a complete description of these phases). During pilot studies we observed that individuals would routinely produce incomplete feeding movements, performing only the oral hood closing phase. Individuals likely not do not swallow captured prey with incomplete oral hood movements (Trimarchi and Watson, 1992), and therefore these incomplete motions do not cause food to enter the stomach. Consequently, in the video analysis incomplete motions were recorded as food searching or casting motions whereas complete motions were recorded as ingestive, and only the ingestive motions were considered in the data analysis.

The influence of posterior nerve lesions on feeding

To determine if posterior nerve signaling reduces the motivation to feed, six Melibe were fed after their posterior nerves had been lesioned. For the lesions, Melibe were pinned out dorsally on a sylgard-coated dish with a single pin through the foot and two through the oral hood, and viewed under a dissecting microscope. A single incision was made in the skin directly
above the brain, exposing the brain, buccal ganglia and the posterior nerves. The posterior nerves were then either cut with scissors or torn with tweezers. Incisions were sewn up with sterile sutures, and the subjects were given several days to recover. After this recovery period lesioned animals were fed as described above, and their feeding activity was compared to that of control animals.

Additionally, the number of *Artemia* consumed was recorded for six control and six posterior nerve-lesioned individuals. After a feeding session subjects were removed from the testing arenas, pictures of their stomachs were taken, and the stomachs then were removed and the *Artemia* were counted. The surface area of the stomachs in the pictures was then calculated using the software ImageJ.

**Electrophysiology**

Extracellular electrophysiological recordings were obtained from several buccal ganglion nerves (the posterior nerve, the anterior nerve, and the cerebral buccal connective) while the stomach was distended. For each preparation, the combined mouth, esophagus, stomach, and intestine were dissected out of a *Melibe*, pinned out in a dish, and continuously perfused with 10.7 °C seawater. A cannula was inserted through the esophagus into the stomach, and then both the esophagus and the intestine were tied off with thread (Fig. 1) to make them watertight. To artificially distend the stomach, seawater was injected in through the cannula, and was expanded to one of four different levels of fullness (1/4, 1/2, 3/4, full; Fig. 1).

For the posterior nerve recordings (n = 6), the nerve was cut near the connection to the buccal ganglion and then sucked up into a suction electrode, so that the information traveling to the buccal ganglion was recorded, whereas for the anterior nerve (n = 2) and cerebral buccal connective (n = 4) recordings, the nerve was cut as far from the buccal ganglion as possible and
drawn up into a suction electrode, so that the output of the ganglion was recorded. Signals were amplified and filtered with an AM Systems Microelectrode AC Amplifier, digitized with an AD Instruments Powerlab 4/30, and displayed with Labchart software. Changes in firing rate over time were determined by counting number of spikes/min using Labchart software.

Figure 1: Different levels of stomach inflation for neurophysiological recordings from the posterior nerve. A) uninflated, B) \( \frac{1}{4} \) inflated, C) \( \frac{1}{2} \) inflated, D) \( \frac{3}{4} \) inflated, E) fully inflated.

Statistics

All statistical tests were performed with the software JMP Pro 11. For the feeding
duration experiment, a repeated measures ANOVA was used to determine if feeding rate changed significantly over time within the two treatments. Differences in the number of *Artemia* consumed between lesioned and control *Melibe*, and in firing activity for the posterior nerve electrophysiology experiment were assessed with paired t-tests.

**Results:**

*Anatomy of the posterior nerve*

The posterior nerve runs between the buccal ganglion and the tree ganglion, with processes that emanate from it to innervate the esophagus (Fig. 2; Trimarchi and Watson, 1992). The nerve may also contain axons that communicate information from the tree ganglion and stomach to the buccal ganglion and brain. To identify these neurons, the posterior nerve was backfilled toward the tree ganglion with cobalt chloride. These fills revealed approximately ten different cell bodies in the tree ganglion (Fig. 3).
Figure 2: Anatomy of the posterior nerve. A) Isolated stomach showing the posterior nerve (PN) running between one of the buccal ganglia (BG) and the tree ganglion (TG). There are at least three points at which processes (P) branch off of the posterior nerve and innervate the stomach; within these side processes branching is extensive, covering much of the surface of the esophagus. B) Drawing of the posterior nerves and buccal ganglia (modified from Trimarchi and Watson, 1992).
The influence of posterior nerve lesions on feeding

To determine if posterior nerve signaling affects feeding, I compared the feeding rates between six *Melibe* with posterior nerve lesions and nine control animals (Fig. 4). For the first four hours after the addition of *Artemia* feeding was similar between the two groups (Fig. 4); both groups immediately increased their rate of OHCs, and both reached a similar peak rate at three hours ($P = 0.51$). However, after five hours of feeding the control *Melibe* began to satiate ($P = 0.14$), while lesioned individuals continued to feed at a significantly elevated rate for seven hours ($P = 0.04$). Additionally, after six hours the feeding rate of the controls was significantly slower than that of the lesioned *Melibe* ($P = 0.04$).
Figure 4: Feeding rate over time of *Melibe* with posterior nerve lesions and control *Melibe*. Feeding rate (OHC/min ± SEM) was calculated in ten-minute bins for the first hour, and for every hour thereafter. Stars indicate the times when the rate was significantly different between the two groups. The number of OHCs performed was similar between control and lesioned *Melibe* for the first 4 hours, but after four hours the control *Melibe* returned to baseline, while lesioned animals continued to feed at an elevated rate.

To verify that the lesioned *Melibe* consumed more prey, the stomachs of six lesioned and six control *Melibe* were removed after they had ceased feeding (8.9 hours for lesioned individuals, 4.1 hours for controls), and the number of *Artemia* in the stomach was counted (Fig. 5). Lesioned *Melibe* consumed significantly more *Artemia* (P = 0.04).
The influence of stomach distention on posterior nerve activity

To determine if information about stomach fullness travels from the tree ganglion to the buccal ganglia and CNS via the posterior nerves, extracellular recordings were obtained from six posterior nerve preparations while the stomach was artificially inflated with seawater. The posterior nerve fired even when the stomach was empty (0.43 ± 0.11 Hz), but distention immediately caused a significant (P = 0.04) increase in firing (Fig. 6). After ten seconds the rate decreased slightly from the initial peak, but firing persisted at approximately 1.2 Hz. In most cases a unit that had been silent began to fire, and in several instances a unit that was tonically active prior to distension stopped firing and a different one became active. In all cases, when the stomach was deflated activity in the posterior nerve immediately stopped (Fig. 7).
Figure 6: Response of the *Melibe* posterior nerve to stomach distention. A) Average firing rate over time for the posterior nerve when the stomach was distended; zero seconds represents the point at which the stomach was distended. B) Representative neurophysiological recording, inset portrays experimental procedure. When the stomach was distended with water, signaling from the tree ganglion to the buccal ganglion via the posterior nerve increased.
Figure 7: Deflating the *Melibe* stomach (arrow) caused posterior nerve firing to cease entirely.

In addition to responding to immediate changes in stomach fullness, the posterior nerve also showed lasting changes in activity in response to sustained distention (applied for at least 30 minutes). Multiple units burst rhythmically regardless of the level of fullness, and the overall firing rate increased when sustained distention was applied. Partial stomach distention caused the firing rate to increase slightly from baseline, but not by a statistically significant amount (Fig. 8). However, as the amount of distension increased, so did the activity in the posterior nerve, and when the stomach was fully distended the firing rate was significantly greater than that for all other levels of distention. Therefore, the posterior nerve communicated the level of stomach fullness to the CNS. Lastly, to determine if the activity recorded in the posterior nerve was from stretch receptors, electrophysiological recordings were performed while the nerve was in a high Mg$^{2+}$/low Ca$^{2+}$ solution. Activity largely stopped while the nerve was in the high Mg$^{2+}$/low Ca$^{2+}$ solution (Fig. 9), suggesting that the spikes recorded from the posterior nerve are produced by interneurons.
Figure 8: The relationship between the posterior nerve firing rate and different levels of stomach fullness. The firing rate (percent change in rate ± SEM) increased as the stomach was distended.

Figure 9: Changes in firing when the posterior nerve was bathed in a high Mg$^{2+}$/low Ca$^{2+}$ solution (arrow). Spiking attenuated when the nerve was bathed in the solution, and returned when regular seawater was washed back in.

The influence of stomach distention on buccal ganglion output

To determine if stomach distention alters buccal ganglion activity, and therefore affects one of the neural circuits involved in feeding, we recorded from the cerebral buccal connective
(CBC) and the anterior root while inflating the stomach. Distention caused several changes in CBC activity. When the stomach was partially distended a small unit began to burst rhythmically (Fig. 10), and at full distention these bursts lasted longer, bust also occurred less frequently. In three of five preparations, a larger unit (Fig. 10) spiked tonically while the stomach was empty, but became silent when the stomach was fully distended.

The anterior nerve of the buccal ganglion causes rhythmic contractions of the esophagus (Trimarchi and Watson, 1992), and produces phasic bursts in isolated preparations. Consequently, to assess the influence of stomach distention on putative feeding rhythms, we inflated the stomach while the anterior nerve was bursting rhythmically. This bursting immediately stopped when the stomach was distended (n = 2; Fig. 11).

Figure 10: Representative activity recorded from the cerebral buccal connective (CBC) before and after stomach distension. Signaling from the buccal ganglion traveling toward the cerebral ganglion was recorded (inset); arrow indicates the point at which the stomach was distended. A large unit spiked tonically while the stomach was empty, but became silent after distention, and a smaller unit began to burst rhythmically once the stomach was distended.
Figure 11: Representative activity from the anterior nerve of the buccal ganglion in response to stomach distention. The nerve burst spontaneously when the stomach was empty, but bursting ceased as soon as the stomach was distended.

**Discussion:**

*The posterior nerve contains units that communicate stomach distention, but they are not the processes of the actual stretch receptors*

Electrophysiological recordings demonstrated that the *Melibe* posterior nerve contains axons from neurons that communicate information about stomach distention to the buccal ganglia and CNS. When the stomach was artificially inflated with water, neurons that had been silent immediately began to fire (Fig. 6), and conversely when a full stomach was deflated posterior nerve firing immediately ceased (Fig. 8). During periods of sustained distention the firing rate was significantly greater than when the stomach was empty (Fig. 8), and in these long term recordings the posterior nerve showed a graded response to stomach distention; as the stomach was incrementally distended the firing rate increased proportionally. The posterior nerve, then, communicates that the stomach is full, the degree to which the stomach is filled, and
also changes in fullness. Interestingly, the spikes we recorded from the posterior nerve do not appear to be from stretch receptors themselves, or from motor neurons. When preparations were bathed in a high Mg\(^{2+}\)/low Ca\(^{2+}\) solution designed to inhibit polysynaptic pathways, activity stopped (Fig. 4). If stretch receptors with cell bodies in the tree ganglion project processes into the posterior nerve (i.e. make a monosynaptic connection with the buccal ganglion), then activity should have continued even in the presence of a high divalent cation solution. Instead, the loss of activity high Mg\(^{2+}\)/low Ca\(^{2+}\) solution suggests that gastric stretch receptors excite interneurons in the tree ganglia, which in turn signal to the buccal ganglion. Regarding motor neurons, *Melibe* with posterior nerve lesions were still fully capable of swallowing food and holding it in their stomach, demonstrating that the posterior nerve does not control esophagus movements. Our current hypothesis is that stretch receptors activated neurons in the tree ganglia and these produced the action potentials that we recorded extracellularly in the posterior nerve. Behavioral experiments demonstrated that the recorded activity helps terminate feeding. Posterior nerve lesioned *Melibe* fed longer (Fig. 5), and consumed more *Artemia* (Fig. 6) than did controls, demonstrating that without such signaling, *Melibe* required more food to satiate. Initial feeding rates did not differ between the control and lesioned *Melibe* (Fig. 5), which suggests that the differences in feeding were caused by a change in the ability to sense stomach fullness rather than a change in the baseline motivational state; lesioned *Melibe* were not more responsive to food initially, but rather remained motivated to feed for a longer duration. This result is consistent with the idea that the posterior nerve communicates stomach distention to the buccal ganglia and CNS. Thus, the posterior nerve appears to serve a role in *Melibe* analogous to that of the stomatogastric nerve in *Pleurobranchaea* (Croll et al., 1987) and the esophageal nerve in *Aplysia* (Kuslansky et al., 1987). However, unlike the esophageal nerve, the posterior nerve
does not appear to be necessary for motor control of the stomach; in *Aplysia*, the esophageal nerve also controls the esophageal sphincter, so lesioning the nerve causes a loss of muscle tone (Kuslansky *et al*., 1987). Lesioned *Melibe* were fully capable of swallowing and retaining *Artemia*, so therefore the posterior nerve does not control mouth, esophagus, or stomach movements.

Lastly, the feeding duration results (Fig. 5) support the idea raised in chapter 1 that post-ingestive cues from the stomach initially serve to enhance feeding. For the first several hours after *Artemia* addition lesioned individuals actually fed at a slightly slower rate than controls, suggesting that the ability to sense stomach fullness is necessary for *Melibe* to be maximally excited by food.

*Stomach distention influences buccal ganglion output*

Based on the CBC and anterior root recordings, stomach distention also influenced the activity of the buccal ganglia. One cerebral buccal neuron, which spiked tonically when the stomach was empty, ceased firing when the stomach was full (Fig. 11), while a second neuron began to burst slowly when the stomach was full. The CBC likely coordinates the movements of the oral hood and mouth in *Melibe*, and in other species contains processes from feeding neurons (Rosen *et al*., 1991). Furthermore, although stomach distention is communicated by the posterior nerve, the ‘decision’ to feed in *Melibe* is made in the brain (Trimarchi and Watson, 1992), and thus reductions in the signaling between the brain and buccal ganglion likely represent changes in feeding patterns.

Distention also altered the bursting activity we recorded from the anterior nerve of the buccal ganglion. When the stomach was inflated while the nerve was bursting rhythmically, bursting immediately ceased. The anterior nerve innervates the anterior region of the esophagus
and posterior portions of the mouth and causes rhythmic contractions of these areas (Trimarchi and Watson, 1992). Thus, rhythmic bursting of units in the anterior nerve is most likely fictive swallowing and stomach distention appears to terminate these swallowing rhythms in *Melibe*. Our working hypothesis is that stomach distension, via the posterior nerve, modulates both swallowing and feeding neural circuits.

In *Lymnaea* (Elliot and Benjamin, 1989) and *Pleurobranchaea* (London and Gillette, 1984), esophageal stretch receptors excite the motor neurons that cause retraction (the closing of the mouth after a bite), while inhibiting those that cause protraction (the extension of the mouth to bite food), a reconfiguration of the feeding network that presumably prevents individuals from ingesting more food. Although we did not record from specific feeding motor neurons in *Melibe*, the *Melibe* feeding neural network likely undergoes a similar change, and our results from the anterior root recordings demonstrate that this reconfiguration leads to changes in swallowing, an outcome that was not measured in previous studies.

**Conclusions**

The *Melibe* posterior nerve communicates information about stomach distention to the buccal ganglia and CNS and this information appears to lead to cessation of feeding. The posterior nerve therefore appears to serve a role in *Melibe* analogous to that of the esophageal nerve in *Aplysia* (Kuslansky *et al.*, 1987) and the stomatogastric nerve in *Pleurobranchaea* (Croll *et al.*, 1987). In addition, stomach distention reduces signaling between the buccal ganglion and the brain, and terminates fictive swallowing rhythms in the anterior nerve of the buccal ganglion. This result demonstrates that stomach distention not only inhibits the activity of motor neurons (Elliot and Benjamin, 1989; London and Gillette, 1984), but also the rhythmic output of the swallowing network. Once individual feeding motor and interneurons in *Melibe* are
identified, we will be able to determine how stomach distention influences specific neurons to produce changes in feeding outputs.
APPENDICES
Appendix A: The role of SCP_B in feeding in *Melibe leonina*

Abstract

An important element in the study of satiation is the transmitters that influence the motivation to feed. The neurotransmitter serotonin has been demonstrated to exert a strong excitatory influence on feeding in invertebrates, and the molluscan peptide small cardioactive peptide B (SCP_B) also appears to influence feeding. SCP_B is of particular interest in feeding in *Melibe leonina*, as the *Melibe* buccal ganglion, which is comprised of only 30-40 neurons, and which is a target for stomach distention, contains an SCP_B staining cell. To determine the role of the peptide in feeding, we fed *Melibe* after injecting them with SCP_B, and also examined the influence of feeding on SCP_B content in the brain. Injections increased the duration of feeding, but did not enhance the initial responsiveness to food. Immunohistochemical processing did not reveal changes in SCP_B staining after feeding, but this qualitative technique may not be sufficient to detect changes in peptide concentration. These results suggest that SCP_B does not alter behavioral state, but rather enhances the response to food.

Introduction

In addition to post-ingestive cues from the stomach, the transmitters involved in feeding also regulate the motivation to feed. Ample evidence demonstrates that serotonin (5-HT) excites feeding in gastropods (Palovcik *et al.*, 1982; Lent, 1985; Rosen *et al.*, 1991), but the molluscan peptide small cardioactive peptide B (SCP_B) also appears to influence the decision to feed. SCP_B increases feeding responses in the snail *Limax maximus* (Prior and Watson, 1988), and alternatively inhibits them in *Lymnaea stagnalis* (Elliott *et al.*, 1991); although these are opposite results, they both demonstrate that SCP_B influences feeding. Additionally, SCP_B-staining cells are present in the buccal ganglia of many species (Watson and Willows, 1992; Murphy *et al.*, 1985), and these cells are active during fictive feeding rhythms (Watson and Willows, 1992), suggesting that SCP_B excites feeding in these species. The presence of SCP_B cells in the buccal ganglion also raises the possibility that stomach distention reduces the motivation to feed in part by inhibiting SCP_B transmission.

Consequently, an important element to our study of satiation in *Melibe* is an understanding of the impact of SCP_B on feeding. The experiments described in this appendix
work towards this end by addressing two questions. First, does SCP_B alter feeding in *Melibe*? If SCP_B excites feeding, then it should either increase the duration of feeding, increase the speed of feeding, or both. Second, can qualitative differences in SCP_B concentration be observed after a meal? If SCP_B is used in feeding circuits, and if stomach distention reduces the motivation to feed, then, as in the serotonergic metacerebral cells of gastropods (Hatcher et al., 2008) and retzius cells of leeches (Gaudry and Kristan, 2012), stomach distention should deplete SCP_B after a meal.

**Methods**

*The influence of SCP_B on feeding*

To assess the influence of SCP_B on feeding, two experiments were performed in which *Melibe* were fed after injections of SCP_B. Prior to each individual trial, subjects were placed in circular buckets (diameter of 30 cm) located within a larger tank of aerated seawater. Small mesh ‘windows’ in the buckets allowed water to flow through them, but prevented food from escaping. The tanks were kept in a 13 °C cold room that was on a 24 hour light/dark cycle, with 10 to 14 hours of light per day, depending on the season.

Subjects adjusted to the buckets for 24 hours, and then were tested. Subjects were injected with SCP_B one hour before feeding, and were fed newly hatched *Artemia spp.* (brine shrimp). All trials began between 10 and 11 AM to ensure that the time of day did not affect the motivation to feed, and *Melibe* fed *ad libitum* for approximately 24 hours. A black and white camera suspended directly above the buckets captured feeding activity, and recorded from approximately one hour before *Artemia* addition to 24 hours post-addition. Camera outputs were digitized, time-stamped, and recorded on a computer using the video capture software Gawker,
which took one picture every second and streamed the images together at a rate of ten frames per second. In the first experiment Eight experimental and 15 control *Melibe* were tested, experimental individuals were injected with 1 mL of $10^{-3}$ SCP$_B$ and, and *Artemia* were added to yield a final concentration of 1000/L in the bucket. In the second experiment three experimental and ten control individuals were tested, 100 µL of $10^{-3}$ SCP$_B$ were injected, and *Artemia* yielded a final concentration of 3,000/L.

*The influence of feeding on SCP$_B$ concentration*

In order to determine if feeding depletes SCP$_B$, immunohistochemical staining was performed on brains of hungry and satiated *Melibe*. Hungry individuals (n = 1) those that had not fed for several days, whereas satiated individuals (n = 2) had finished feeding approximately 30 minutes before dissection. For the processing, brains were stained according to a protocol adapted from Watson and Willows (1992). Briefly, after feeding, brains were dissected out and fixed in 4% paraformaldehyde. Brains were then washed with phosphate buffered saline with triton, blocked with goat serum, and incubated for 48 hours in SCP$_B$ primary antibodies. After incubating in primary antibodies, brains were washed and blocked again, and then incubated in secondary antibodies. Lastly, brains were mounted on slides and viewed with a Zeiss fluorescent microscope.

**Results**

*The influence of SCP$_B$ on feeding*

To determine if SCP$_B$ influences the motivation to feed in *Melibe*, several experiments were run in which individuals were fed after injection with the peptide. In the first experiment, subjects were injected with 1 mL of $10^{-3}$ SCP$_B$, and *Artemia* were added to yield a final
concentration of 1,000/L (Fig. 1). Control *Melibe* (n = 15) began to feed immediately and reached their peak rate within three hours, whereas injected individuals (n = 8) responded slowly to food, gradually increasing their feeding rate for the first four hours after food addition. However, after this point they fed robustly, and continued to feed at this point for at least 12 hours. Additionally, their peak feeding rate was greater than in controls.

However, neither treatment group had satiated by the onset of darkness, and once the sun sets *Melibe* typically perform an activity bout that lasts for several hours (Newcomb *et al.*, 2014), a variable that could potentially confound these data. To remove this variable, we performed a second, modified experiment (Fig. 2), in which individuals were injected with only 100 µL of 10⁻³ SCP₉ and *Artemia* were added to yield a much greater concentration within the buckets (3,000/L); *Melibe* feeds much more quickly at a density of 3,000 *Artemia*/L (Watson and Trimarchi, 1992), so we hoped that with this greater concentration subjects would satiate before nightfall. The two groups fed similarly for the first four hours after food addition, but SCP₉ injected individuals (n = 3) fed at an elevated rate for longer than the controls (n = 10).
Figure 1: Feeding rate over time for trials in which *Melibe* were fed *Artemia* at a concentration of 1,000/L, and in which experimental individuals were injected with 1 mL $10^{-3}$ SCP$_B$. Black bar indicates darkness. Unlike controls, injected individuals responded slowly to food addition, but steadily increased their feeding rate (OHCs/min ± SEM) for several hours, and fed at an elevated rate for at least eights.
Figure 2: Feeding rate over time for trials in which *Melibe* were fed *Artemia* at a concentration of 3,000/L, and in which experimental individuals were injected with 100 µL $10^{-3}$ SCP$_B$. Black bar indicates darkness. Feeding rate (OHCs/min ± SEM) was similar between the two groups for the first four hours after *Artemia* addition, but after four hours injected individuals continued to feed at a somewhat elevated rate, whereas control *Melibe* satiated after this time.

**The influence of feeding on SCP$_B$ concentration**

In order to determine if feeding depletes SCP$_B$, the brains of hungry and satiated *Melibe* were stained for the presence of SCP$_B$. Immunohistochemical stains from a hungry individual (Fig. 3.A.), an individual that fed for approximately one hour (Fig. 3.B.), and an individual that had fed for approximately four hours (Fig. 3.C) revealed the same neuron in the buccal ganglion and the cerebral and pleural ganglia (not pictured), and, qualitatively, there was no difference in the degree of staining between the preparations.
Discussion

SCP_B appears to enhance feeding in *Melibe*. In the first experiment (Fig. 1), SCP_B injected individuals fed longer and at a faster rate than controls. Although the onset of darkness likely prolonged feeding in both groups, the stark contrast in feeding between them clearly demonstrates that SCP_B affected the motivation to feed. When we controlled for darkness by adding more *Artemia* and reducing the amount of SCP_B injected, the injected individuals did not feed at a faster rate, but still fed for a longer duration, supporting our hypothesis.

Unexpectedly, SCP_B did not increase the initial response or the general excitatory state of the subjects (the baseline OHC rate did not increase), and the large volume injections actually depressed the responsiveness to food (Fig. 2). This result suggests that SCP_B does not in fact alter behavioral state, but rather enhances an already-elicited behavior.

Figure 3: SCP_B staining in the buccal ganglion in A) an unfed subject, B) a subject that fed for 1 hour, and C) a subject that fed for 4 hours. The degree of staining did not differ between the individuals.
Immunohistochemical processing of the brain and buccal ganglion did not reveal differences in SCP<sub>B</sub> staining (Fig. 3). This result does not support our hypothesis, but does not necessarily reject it either. Immunohistochemistry is a qualitative method, and differences in staining will only emerge if feeding dramatically depletes SCP<sub>B</sub>; quantitative techniques, such as western blotting and qPCR, could more accurately determine if depletion occurs. In the gastropod <i>Pleurobranchaea californica</i>, feeding reduces the amount of 5-HT in the metacerebral cells fourfold, yet the cells still retain 5-HT (Hatcher <i>et al.</i>, 2008), and would likely still stain partially for the neurotransmitter. Based on our data feeding may not deplete SCP<sub>B</sub> to the same degree, but it might still deplete the peptide by a significant amount. In addition, if SCP<sub>B</sub> does not regulate behavioral state, then its abundance might not change in the same manner as 5-HT.

Although these data provoke interesting ideas, they ultimately need to be supported by further trials. Only three <i>Melibe</i> were tested with SCP<sub>B</sub> in the second trial, and none of these were tested without the peptide. Additionally, none of the control subjects were fed following a sham (water) injection, and thus we did not account for the possible effects from the procedure. Lastly, quantitative tests need to be performed to determine if feeding actually reduces the concentration of SCP<sub>B</sub>. With further trials, we will be able to more confidently provide our answers, and determine how SCP<sub>B</sub> influences the motivation to feed in <i>Melibe</i>, and if feeding and stomach distention influence SCP<sub>B</sub> transmission.
Appendix B: The influence of feeding on locomotion in *Melibe*

Abstract

Ample evidence demonstrates that light influences activity and circadian clocks, but research also suggests that feeding can also influence circadian patterns of activity. *Melibe leonina* is an organism that is ideally suited for research into circadian rhythms, so to help link our understanding of satiation in *Melibe* to circadian rhythms, we performed several experiments in which we fed individual *Melibe* and examined changes in locomotion. When food was offered at night individuals terminated their nightly crawling bout, but activity returned to normal the following night. Additionally, these bouts were not affected when *Melibe* was fed prior to nightfall. These results demonstrate that feeding interrupts locomotion, but also suggest that stomach distention, and thereby satiation, does not alter locomotion, a result that contrasts with those obtained in other species.

Introduction

Recently, our lab has begun to study circadian rhythms in *Melibe leonina*, with the specific goal of understanding how molecular clocks (i.e. proteins whose expression oscillates on a daily pattern) produces the changes in nervous system activity that ultimately underlie circadian activity patterns. *Melibe* is ideally suited to answer this question. The nervous system is amenable to neurophysiological analysis, and, uniquely, the eyes lie directly on the brain in *Melibe*, allowing one to relate changes in light to changes in fictive behaviors. Additionally, the swimming central pattern generator has been described in *Melibe* (Thompson and Watson, 2005), and several circadian clock proteins have been located within the brain (Unpublished data), providing an important background for our research.

Feeding influences activity in a number of species. The blowfly *Phormia regina* (Browne and Evans, 1960) and the gastropod *Aplysia californica* (Kupfermann, 1974) both move less after a meal, and the leech *Hirudo medicinalis* does not move at all after feeding (Gaudry and Kristan, 2012). Additionally, in *Hirudo* artificial distention inhibits swimming (Gaudry and Kristan, 2010), and as individuals gradually digest a meal they become increasingly more active (Gaudry and Kristan, 2012), demonstrating that it is a satiated state, and not merely the act of
feeding, that inhibits locomotion. Conversely, hunger enhances locomotion; in both rats (Strong, 1957) and Phormia (Green, 1964), locomotion increases as the time from the last meal increases. Additionally, in the pulmonate Lymnaea stagnalis locomotor neurons show increased excitability in hungry animals (Dyakonova et al., 2015).

Thus, an important element to our research on circadian rhythms in Melibe is an understanding of the relationship between feeding and locomotion. Melibe exhibits strong nocturnal behavioral patterns. Individuals move infrequently during the day, but after the sun sets crawl robustly for several hours (Newcomb et al., 2014). Interestingly, individuals simultaneously perform oral hood casting motions for most of their crawling episode, even in the absence of prey, suggesting that they move at night to search for food. The goal of the experiment in this appendix was to determine the impact of feeding on nocturnal locomotion in Melibe. If feeding inhibits nocturnal locomotion in Melibe, then the addition of food to individuals at various points in the day should reduce crawling. Answering this question will reveal if feeding reduces not only short bursts of activity, but also circadian patterns of locomotion. Additionally, this experiment will provide important data for our understanding of the neural mechanisms of circadian rhythms in Melibe.

Methods

To assess the influence of feeding on locomotion, we performed two experiments in which we recorded the activity of individual Melibe continuously for 5-6 days, and fed them on the penultimate day. At the beginning of each trial, subjects were placed in individual buckets within a larger tank of aerated seawater. The buckets had holes to allow water to flow through
them, and the tanks were kept in a 13 °C cold room that was on a 24 hour light/dark cycle (lights on from 7 AM to 7 PM). A black and white camera suspended directly above the buckets captured activity for the duration of the trial. Camera outputs were digitized, time-stamped, and recorded on a computer using the video capture software Gawker, which took one picture every second and streamed the images together at a rate of 15 frames per second. Afterward, activity was analyzed with the motion tracking software ethovision. Lastly, the resulting data were used to generate actograms, which were created in ImageJ with the ActogramJ plugin.

In the first experiment, seven *Melibe* were fed the brine shrimp *Artemia* at 9:30 PM, with *Artemia* added to yield a final concentration in the tank of 3,000 individuals/L. At this time of night *Melibe* were in the middle of their night time bout of locomotion, which allowed us to determine if feeding interrupts locomotion. In the second experiment, four *Melibe* were fed at approximately 12:45, which allowed us to determine if a recent meal influences nightly bouts of activity. To quantifiably determine how feeding influenced locomotion, the nightly activity of each *Melibe* was averaged for feeding nights and non-feeding nights, and the averages were compared using a paired t-test.

**Results**

To determine if feeding can interrupt locomotion, seven *Melibe* were fed 2.5 hours after nightfall, when they were approximately halfway through their nightly bout of activity. Feeding caused subjects to essentially stop moving (Fig. 1.A), and they moved significantly less for the rest of the night than they did in the absence of food (Fig. 2; P = 0.004). The following day, subjects showed a brief bout of activity at 9 AM (approximately 12 hours after food addition),
but otherwise did not behave differently, and performed a regular crawling bout the following night (Fig. 1.B.).
Figure 1: *Melibe* locomotion over time. A) Percent of the time spent moving for 4-5 days without feeding (gray trace) and 1 with a nighttime feeding (black trace). Data are plotted as percent of time spent active per 30 minutes ± SEM. Black bar indicates period of darkness; star indicates the time when food was added. B) Representative actogram showing daily activity over the course of a trial. Both figures demonstrate that *Melibe* begins moving rapidly at the onset of darkness, and remains generally active throughout the night. When fed, however, locomotion ceases.
Figure 2: Average percentage of the night (± SEM) spent moving after 9:30 PM, the time at which food was added on the feeding night. Subjects spent significantly more time moving on non-feeding nights than on feeding ones.

In order to determine if a recent meal influences the nightly bout of locomotion, four individuals were fed at 12:45 PM. The results from the first chapter of this thesis reveal that *Melibe* typically satiates after five hours of feeding, so individuals should have satiated less than two hours prior to the onset of darkness. There was no significant difference between locomotion on feeding and non feeding days (Fig. 3; P = 0.26), and individuals actually moved slightly more in the night after feeding than on the non feeding days.
Fig. 3: Average locomotion over the course of a day for several *Melibe* on days with and without feeding. Black bars indicate night, star indicates food addition. Average activity (percent of time active per 30 minutes ± SEM) did not differ between feeding and non-feeding days.

**Discussion**

The addition of prey to tanks interrupted nighttime crawling in subjects. Thus, in *Melibe*, as in *Aplysia* (Kupfermann, 1974), *Phormia* (Browne and Evans, 1960), and *Hirudo* (Gaudry and Kristan, 2012), feeding terminates locomotion. *Melibe* likely crawls at night to search for prey, and once it finds food it ceases searching and begins to feed. Crawling ceased almost as soon as food was added, suggesting that external sensory cues, rather than post ingestive information from the stomach, inhibit locomotion. The daytime feeding experiment supports this inference, as individuals that had recently satiated did not show differences in crawling at night. Furthermore, in both experiments activity returned to normal the day after feeding, even though
the *Melibe* stomach is still partially distended 24 hours after a meal (personal observation). Lastly, in preliminary neurophysiological experiments, stomach distention did not the rhythmic output from the pedal ganglion. We did not directly test this idea, but our results also suggest that neither stomach distention nor prey stimuli influence clock neurons, because regular activity resumed the day after feeding. Instead, sensory receptors may connect to locomotor interneurons and outweigh the input from clock neurons.

Although we expected prey addition to interrupt feeding, we also expected stomach distention to inhibit crawling. Stomach distention reduces locomotion in *Hirudo* (Gaudry and Kristan, 2010), and it would make sense for it to do so in all animals given the risk associated with foraging. Movement to a new location increases the risk of predation, and if an animal does not need to eat, it should have no reason to assume this danger. However, there are several possible reasons for this logic to not apply to *Melibe*. First, *Melibe* like many nudibranchs, produces a noxious chemical that prevents attack (Barsby, 2002), and thus does not have to worry about predation. Second, whereas *Hirudo* feeds approximately once a year (Gaudry and Kristan, 2012), and thus does not need to worry about finding a meal for a long time, *Melibe* digests its food quickly, and thus needs to be ready to find a new meal before long.

Additionally, several factors could have confounded our data. First, although the daytime feeding subjects appeared healthy and fed robustly when given *Artemia*, they moved little during trials, and in particular did display the characteristic nighttime activity bout. These individuals were tested in July, the end of the life cycle for most *Melibe*, and they may not have been motivated to search out food. Second, the procedure in the preliminary neurophysiological tests likely influenced the rhythms obtained from the pedal nerves. Without inhibitory input from the foot, the isolated *Melibe* brain produces almost non-stop fictive swimming, and in this artificially
excited state may not respond to stomach distention the way it should. Both of these experiments bear repeating, and with stronger data we will be able to conclusively determine if stomach distention inhibits locomotion.

In light of the observations made in this study, we propose a simple circuit to explain the regulation of nighttime behavior in *Melibe* (Fig. 4). Shortly before sunset clock neurons begin to depolarize crawling pattern generators and oral hood pattern generators, but inhibitory signals from light prevent these behaviors from occurring (See Newcomb *et al.*, 2014 for a description of this phenomenon). Once darkness falls inhibition is removed, and the *Melibe* begins to crawl and perform oral hood movements. If food is encountered then prey stimuli inhibit crawling while exciting the oral hood pattern generator, likely doing so via the metacerebral cells. However, if the individual has recently consumed a satiating meal, then stomach distention inhibits the metacerebral cells, preventing the inhibition of crawling.
Figure 4: Proposed circuit of nighttime behavioral regulation in *Melibe*. As evening approaches clock neurons begin to excite oral hood pattern generators and crawling pattern generators, but ambient light induces weak inhibitory signals that prevent activity. However once the sun sets inhibition is removed, and individuals begin to crawl and cast about with the oral hood. If prey is encountered then the food arousal system, likely regulated by the metacerebral cells, inhibits crawling while exciting the oral hood pattern generator, causing individuals to stop moving and feed robustly. However, if the stomach is distended from a prior meal, then gastric stretch receptors prevent food arousal, removing the inhibition of crawling.

There are several new experiments we can perform to more thoroughly tease out the relationship between stomach distention and locomotion. The most logical next step is to artificially distend the stomach with a non-nutritive bulk, and see how locomotion is affected. This experiment will remove the added variable of prey stimuli, and allow us to determine how
stomach distention itself influences circadian rhythms. Once we have conclusively identified clock neurons in *Melibe*, it will also be important to explicitly determine if feeding stimuli influence these neurons, as well as the already identified swimming interneurons. The answers to these questions will help reveal how the *Melibe* central nervous system integrates feeding and locomotion to produce circadian rhythms and changes in behavioral state.
References:


