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Behavioral and Molecular Analysis of Memory in the Dwarf Cuttlefish

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BEHAVIORAL AND MOLECULAR ANALYSIS OF MEMORY IN THE DWARF CUTTLEFISH

by

JESSICA BOWERS

(Under the Direction of Vinoth Sittaramane)

ABSTRACT

Complex memory has evolved because it benefits animals in all areas of life, such as remembering the location of food or conspecifics, and learning to avoid dangerous stimuli. Advances made by studying relatively simple nervous systems, such as those in gastropod mollusks, can now be used to study mechanisms of memory in more complex systems. Cephalopods offer a unique opportunity to study the mechanisms of memory in a complex invertebrates. The dwarf cuttlefish, *Sepia bandensis*, is a useful memory model because its fast development and small size allows it to be reared and tested in large numbers. However, primary literature regarding the behavior and neurobiology of this species is lacking. This research determined that juvenile *S. bandensis* exhibited short term memory (STM) and long term memory (LTM). To assess memory in dwarf cuttlefish, a memory test was conducted which utilized the predatory attack in cuttlefish. It was found that 4 week old dwarf cuttlefish retained memory of the experiment up to 4 days. Using an automated tracking software called DanioVision, this research found that cuttlefish selectively inhibit the tentacle striking phase of their predatory behavior, without inhibiting the attention and positioning phases. Determining the molecular mechanisms underlying memory is key to understanding how memory is manifested in the form of altered behavior. At the cellular level, memories are formed by altering the physical and chemical properties within specific neural circuits. The transcription factor, CREB, is responsible for transcribing genes required for initiating these long-term neuronal changes. Using immunohistochemistry, a molecular assay was developed to determine whether CREB is activated in cuttlefish arms during the memory experiment. Trained cuttlefish had a greater number of CREB positive cells in the epithelium of the arm than controls. Trained cuttlefish also had a greater average number of CREB-positive cells in positive suckers than untrained cuttlefish. These results suggest that CREB activation may result from behavioral training in cuttlefish. Lastly, it was found that

the distal tip of the arm contained more CREB-positive cells than the proximal part of the arm. Spatial activation of CREB may occur predominantly in the distal portion of the arm. By locating CREB for the first time in a cephalopod, this research presents dwarf cuttlefish as interesting models for studying the molecular mechanisms of memory formation.

INDEX WORDS: Cuttlefish, Cephalopod, Learning, Memory, CREB

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JESSICA BOWERS

B.S., Georgia Southern University, 2017

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CHAPTER 1

INTRODUCTION

Overview of Memory

Studying memory formation is fundamental to our understanding of how the brain stores and retrieves information. Charles Darwin (1859) suggested that the evolution of memory was related to fitness benefits gained from the ability to recollect salient events related to survival and reproduction. The molecular mechanisms underlying learning and memory remain a core focus of neurobiology, and while enormous progress has been made in the past century, much remains to be known about the processes governing memory formation and storage, particularly, the molecules responsible for facilitating these processes. Much of our knowledge about the human brain arises from memory studies using animals. Continued work with animal memory models could be the key to understanding the processes which ultimately lead to memory disorders such as Alzheimer's disease and dementia.

The terms learning and memory are often used interchangeably, because they are closely related and interdependent. Learning is defined as the acquisition of new information by using the senses, while memory is defined as the ability to store and retrieve the learned information (Cowan 2008). In an ethological sense, memory functions to alter future behavior based on what was learned. Learning and memory are interconnected, because learning cannot occur if information cannot be stored in the form of memory (Cowan 2008). Due to this complex relationship, learning and memory will be considered synonymous for this thesis.

Types of memory are categorized according to their duration. Short term memory (STM) lasts for minutes to hours, whereas long term memory (LTM) can last for days to a lifetime (Miyahsita et al 2007). The duration of memory types is related to changes at the neuronal level that occur when memories are formed (Miyashita et al 2007). The formation of STM and LTM occurs in separate, parallel processes, despite that they involve the same synapses and chemical messengers (Izquierdo et al 2002). STM begins

to form within minutes, while the formation of LTM requires a much slower “consolidation” process that can take hours to manifest as behavioral changes (Izquierdo et al 2002).

The prevailing hypothesis for the difference between STM and LTM is that LTM requires protein modifications and/or *de novo* protein synthesis, whereas STM does not require either process (Izquierdo 2002, Miyashita et al 2007). Protein synthesis inhibitors can block long term memories, but do not have an effect on short term memories (Lynch 2004). Izquierdo et al (2002) suggested that STM forms from transient, transcription-independent changes such as hyper-activation of AMPA receptors or exocytosis of glycoproteins that temporarily increase local cell adhesion. The events that result in a transient increase in communication strength are collectively known as short term facilitation. In contrast to LTP, wherein synaptic communication is strengthened for long periods, enhanced synaptic transmission from short term facilitation lasts less than a second (Jackman and Regehr 2017).

The mechanisms driving LTM are well-established in mammalian models, such as rodents (Baudry and Joel 1997). In vertebrates, memory formation and storage occurs in the hippocampus, a region near the center of the brain (Gärtner and Frantz 2010). While most regions of the brain cannot generate new neurons, neurogenesis occurs in the hippocampus throughout life, in a sub-region called the dentate gyrus (Bennarroch 2013; Gärtner and Frantz 2010). The ability of the hippocampus to generate new neurons is fundamental to the ability of organisms to create new memories throughout life. Memories are formed as a result of synaptic plasticity, or the ability of the connections between synapses to change in communication strength (Kaiser and Peters 2009). Synaptic changes can occur due to morphological shifts in neuron structure, such as axonal extension, or rapid growth of dendritic spines. Synaptic changes can also occur due to alterations of receptor densities on postsynaptic neurons, which modulates sensitivity to chemical messengers (Kasai et al 2010, Kaiser and Peters 2009). Strength between synapses can also degrade as a result of dendritic spine decay, which is thought to be related to the onset of memory disorders such as Alzheimer’s disease (Kasai et al 2009).

LTM results from changes in neuronal gene expression, which ultimately causes synthesis of new proteins and/or modifications to existing proteins (Lynch 2004). Changes in gene expression are mediated

by a process called long-term potentiation (Baudry and Joel 1997). Long-term potentiation (LTP) is a long-lasting increase in transmission strength between two neurons, and is initiated at the dendritic spines of post-synaptic neurons, or the neurons that receive chemical inputs from surrounding neurons (Kasai et al 2009). LTP occurs primarily in the hippocampus, implicating this brain region in memory formation (Gärtner and Frantz 2010). LTP is an activity-dependent process, meaning that information can be consolidated in specific, repeatedly activated neural circuits (Baudry and Joel 1997, Lynch 2004).

LTP was first described by Lomo and Bliss (1973), who subjected rabbit hippocampal neurons to repeated, high-frequency (tetanic) stimulation, and then observed sustained efficiency of transmission between the neurons. The two main receptors involved in vertebrate LTP are AMPA and NMDA, which are both activated by the neurotransmitter, glutamate (Baudry and Joel 1997, Lynch 2004). Hall (1992) describes the steps by which LTP occurs. Tetanic stimulation of hippocampal neurons causes glutamate release, which activates AMPA; AMPA, in turn, activates NMDA. This initiates a cascade of second messengers, namely the cyclic AMP (cAMP) pathway, which ultimately activate transcription factors in the post-synaptic nuclei. The upregulated gene expression may sustain the strength between the neurons by increasing the density of AMPA receptors on the post-synaptic neurons, thereby increasing the sensitivity towards neurotransmitter release.

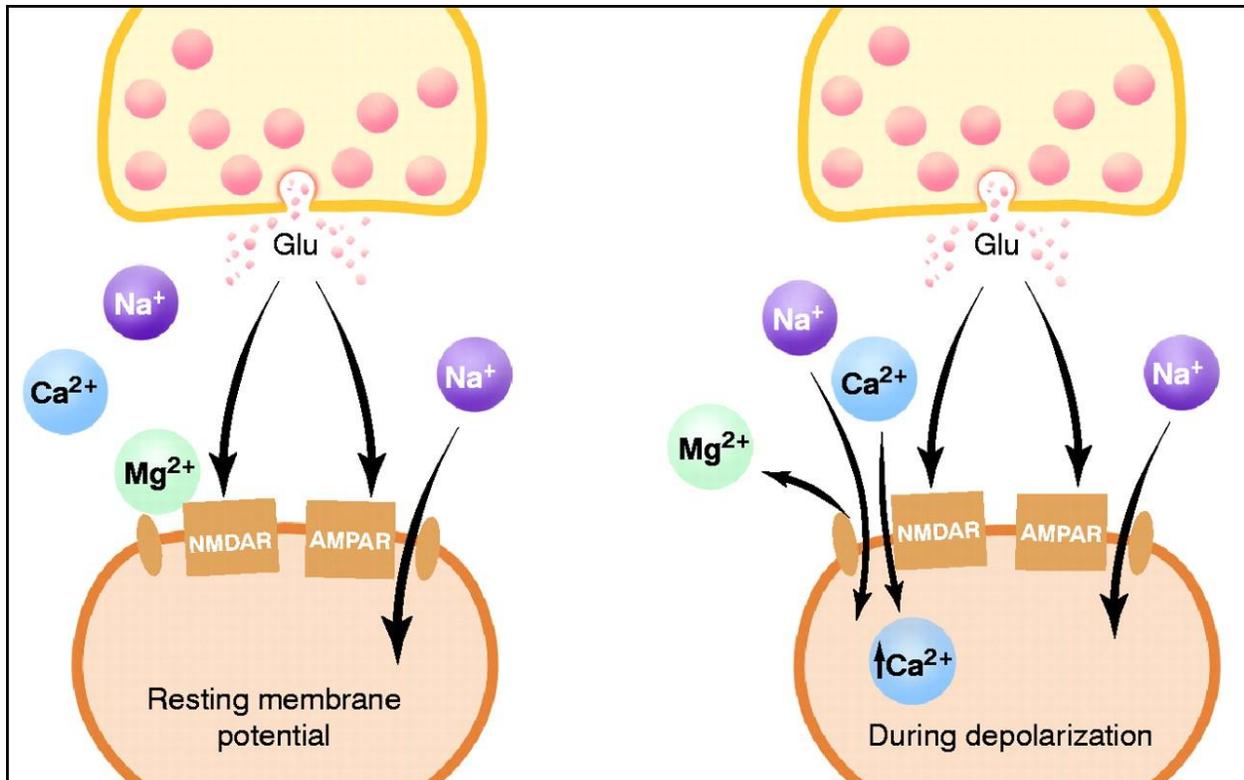


Figure 1. Synaptic events governing vertebrate LTP (Malenka and Nicoll 1999).

Figure 1 shows the steps by which LTP is activated in vertebrates. 1) Glutamate (Glu) is released by exocytosis in the presynaptic terminal and travels across the synaptic cleft. 2) Glutamate binds to AMPA receptors (AMPA) on the postsynaptic neuron, activating flow of extracellular Na^+ through the receptor channel. 3) Influx of Na^+ depolarizes the postsynaptic neuron, freeing Mg^{2+} from NMDAR. At resting potential, NMDAR activity is blocked by Mg^{2+} . 4) Extracellular Ca^{2+} flows through the NMDA receptor channel, increasing the concentration of intracellular Ca^{2+} . 5) Repeated stimulation of the postsynaptic neuron through these steps activates Ca^{2+} dependent signaling cascades that initiate LTP-related gene expression in the postsynaptic neuron, thereby increasing transmission strength at the synapse. While this shows the general receptors and neurotransmitters that govern LTP in vertebrates, similar calcium-dependent processes govern LTP in invertebrates, although the receptors and neurotransmitters may differ.

The transcription factor, CREB (cAMP response element binding protein), is a target of the cAMP pathway initiated by LTP (Albensi 2012), and is associated with the formation of LTM. CREB binds to CRE sequences on the DNA to induce transcription of LTM-related genes such as *c-fos* (Albensi 2012). The cAMP-CREB pathway is used in both vertebrates and invertebrates to activate LTM-related protein synthesis (Matsumoto et al 2006). CREB has been shown to participate in LTM formation of mice (Barco et al 2002; Lu et al 1999), rats (Rexach et al 2012; Zhang et al 2014), sea hares (Casadio et al 1999; Lee et al 2006; Lewin and Walters 1999; Rajasethupathy et al 2009), snails (Efimova et al 2007; Guo et al 2010; Ribeiro et al 2003; Sadamoto et al 2003), *Drosophila* (Yin et al 1995), honeybees (Eisenhardt et al 2003), wasps (van den Berg et al 2010), and crickets (Matsumoto et al 2006).

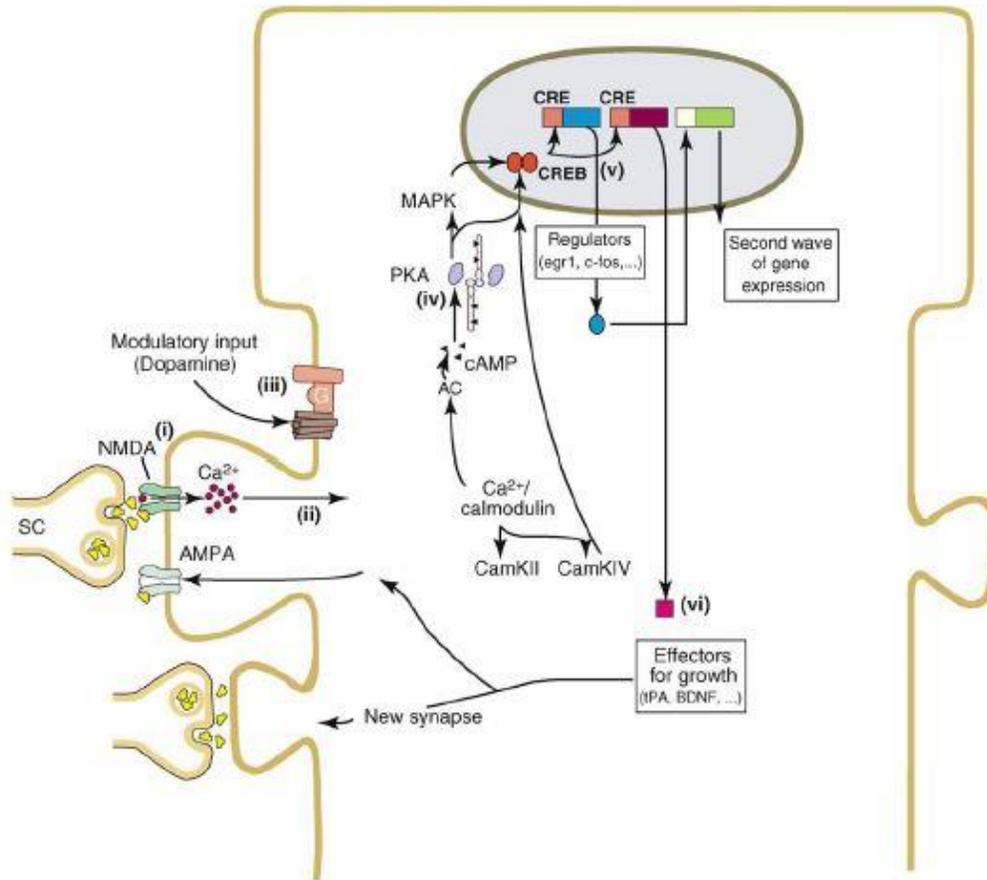


Figure 2. The CREB signaling pathway in neurons (Benito and Barco 2010).

Figure 2 describes the steps by which CREB is activated in neurons. When NMDAR channels open, an influx of calcium increases the intracellular calcium concentration of the postsynaptic neuron. Intracellular calcium then activates the Ca^{2+} sensor calmodulin, which activates Ca^{2+} /calmodulin dependent protein kinases, CaMKs. Adenylate cyclase, which catalyzes the conversion of ATP to cyclic AMP, is activated directly through the Ca^{2+} /calmodulin complex, or through CaMKs. cAMP goes on to activate various kinase pathways such as the mitogen activated kinase (MAPK) pathway, or the protein kinase A (PKA) pathway. These kinases activate CREB in the nucleus by phosphorylation. The inactive CREB protein contains a kinase-inducible domain (KID) with multiple phosphorylation sites; the best studied site is the serine-133 residue (Ribeiro et al 2003). Phosphorylated CREB binds to cAMP response elements (CREs) on the DNA, and enhance transcription of immediate early genes, ultimately resulting in protein synthesis which strengthens synaptic communication.

The CREB target *c-fos* and other genes such as *egr-1* and *arc* are termed immediate early genes (IEGs), because they are expressed rapidly following synaptic activation (Minatohara et al 2016). Upregulated expression of IEGs has been found in neurons populating regions where memories form (Gallo et al 2018), suggesting IEG activity can be used as a maker for LTM formation. Immediate early gene products (IEGPs) are proteins that in turn, activate transcription of “late genes” involved in the structural changes required for long term memory storage (Freeman and Rose 1999). IEG-related protein synthesis is fast, occurring within 1-3 hours of behavioral training in chicks (Freeman and Rose 1999).

The role of CREB in memory can be assayed by counting the number of CREB-positive neurons in a region or circuit after behavioral training. Kim et al (2013) used this assay to study fear conditioning in mice. By staining neurons with an anti-CREB antibody, they found an increase in the number of CREB-positive cells in a population of neurons called LA neurons within the amygdala, suggesting that LA neurons are important for establishing the memory trace during fear conditioning. Similarly, Brightwell et al (2007) found increased CREB staining in rat dorsal hippocampal neurons following a place learning task. These studies show that CREB immuno-labeling can be used to pinpoint parts of the nervous system undergoing memory-related synaptic plasticity.

CREB in Gastropod Mollusks

Gastropods, such as snails and the sea hare, *Aplysia*, have long acted as a simple molluscan systems for studying the properties of memory in invertebrates (Kemenes et al 2002, Welshhans and Rehder 2005, Susswein and Chiel 2012). Despite the availability of these well-understood molluscan models, much remains to be understood about the role of CREB in invertebrate memory. When treated with the convulsive agent PTZ, the snail, *Helix lucorum*, shows elevated immunoreactivity of CREB and Fos proteins in the pedal and cerebral ganglia, important regions for the modulation of feeding behaviors (Efimova et al 2007). Similarly, Ribeiro et al (2003) found elevated CREB immunoreactivity in the central nervous system of *Lymnaea stagnalis* after stimulation with forskolin, an activator of adenylate cyclase, the enzyme that catalyzes the cAMP-CREB cascade (Benito and Barco 2010).

While these studies provide evidence for CREB's involvement in the signaling pathway for memory, the synaptic proteins upregulated by CREB activity are less understood. Prior to operant conditioning in *Lymnaea stagnalis*, Guo et al (2010) used double stranded interfering RNA (dsRNAi) to knock down the CREB1 gene, and found reduced expression of syntaxin and dynamin mRNA and proteins. Syntaxin and dynamin are presynaptic proteins that facilitate transport and cycling of neurotransmitters through exocytosis and endocytosis, respectively (Guo et al 2010). Interestingly, this suggests that CREB1 may participate in presynaptic modifications, as well as the postsynaptic modifications traditionally associated with LTM. Similarly, Sadamoto et al (2003) injected dsCRE oligonucleotides into a CREB1 neuron of *L. stagnalis*, and found that cAMP dependent synaptic plasticity was inhibited.

The best studied molluscan model for CREB is the sea hare, *Aplysia*. The nervous system of *Aplysia* is small (<100,000 neurons) and many individual neurons responsible for eliciting specific behaviors are known (Hall 1992). Research on synaptic plasticity in *Aplysia californica* was pioneered by Eric Kandel, who studied habituation and sensitization of the gill withdrawal reflex. During this behavior, a light touch to the siphon causes the gills to retract (Purves et al 2001). Repeated touching of the siphon

reduces the intensity of the withdrawal response, known as habituation. Pairing of the touch with a noxious stimulus (i.e. a tail shock) reinstates the original gill withdrawal response, which is called sensitization. Intense gill withdrawals resulting from repeated siphon-tail shock pairing last several weeks, due to synaptic modifications at the synapse between the siphon sensory neuron and gill motor neuron. These synaptic changes are mediated by facilitatory interneurons stimulated by tail shocking (Figure 3). The neural properties of the gill withdrawal reflex has propelled many CREB-related studies in *Aplysia*. Since it is the best known invertebrate memory model, the functions of CREB in *Aplysia* are important to consider for comparative analyses with other invertebrates. While CREB's role in memory has been studied extensively in *Aplysia*, new insights could still be gained by studying CREB in more complex relatives of *Aplysia*, such as cephalopods.

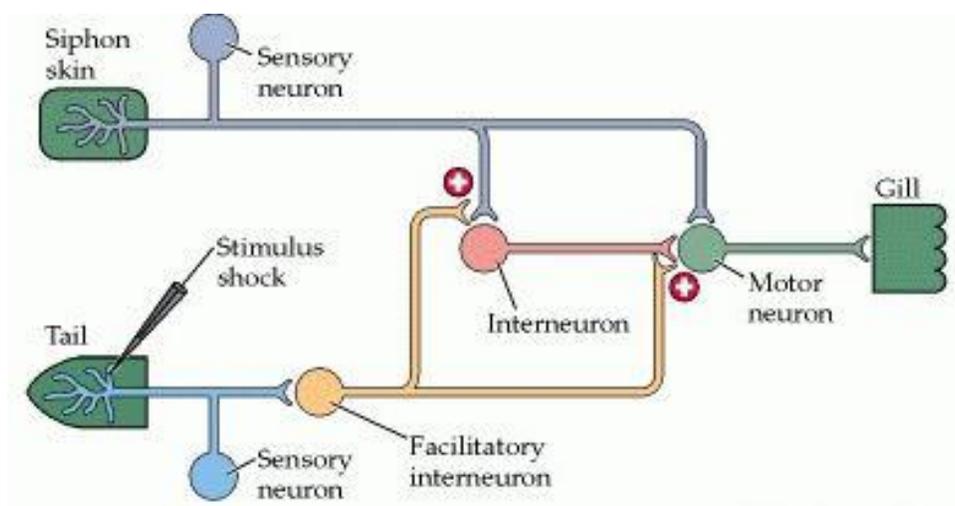


Figure 3. Schematic of the gill withdrawal reflex circuit in *Aplysia* (Purves et al 2001).

The gill withdrawal circuit in *Aplysia* is shown in Figure 3. A touch to the siphon activates the siphon sensory neuron, which synapses onto the gill motor neuron, causing gill withdrawal. After habituation by repeated siphon touches, a shock to the tail reinstates the original gill withdrawal response. Tail shocking activates facilitatory interneurons, which synapse onto siphon sensory neurons. The interneurons release serotonin, which binds to G-coupled receptors on the siphon sensory neurons. G-

coupled receptors activate the cAMP pathway; during short term sensitization, cAMP causes an increase in neurotransmitter release to the gill motor neuron from the siphon sensory neuron, increasing excitability. Repeated siphon-tail shock pairings result in activation of the cAMP-CREB signaling pathway in the siphon sensory neuron, leading to long term synaptic changes, and sustained sensitization of the gill withdrawal reflex.

Several studies have shown that cAMP-CREB activation is serotonin dependent in *Aplysia* (Casadio et al 2009, Lewin and Walters 1999, Rajasethupathy et al 2009). Serotonin may be a key player in memory formation of various invertebrates, but additional animal models are needed. This differs from conventional vertebrate LTP, which primarily uses glutamate to activate the cAMP-CREB pathway (Baudry and Joel 1997, Lynch 2004). Work with *Aplysia* has also attempted to identify potential regulators of CREB activity. The transcription factor ApAF (*Aplysia* activating factor) induces long term facilitation by interacting with CREB-dependent IEGs, as well as alleviating repression mediated by CREB2 (Lee et al 2006). While CREB1 has been shown to promote long term facilitation in gastropods (Guo et al 2010; Sadamoto et al 2003), other CREB family proteins such as CREB2 are repressive (Bartsch et al 2000), preventing long term facilitation. Relief of CREB2 repression by downstream effectors such as ApAF is needed to convert short term facilitation into long lasting synaptic structural changes, or long term facilitation (Lee et al 2006). Micro RNAs (miRNAs) have also emerged as negative regulators of CREB activity in *Aplysia*. Using cDNA libraries obtained from the central nervous system, Rajasethupathy et al (2009) determined the miRNA, mi-R124, was specific to the sensory neurons where long term facilitation is known to occur in *Aplysia*. Western blotting showed that inhibition of mi-R124 led to an increase of CREB dependent IEGPs. Lastly, mi-R124 levels were reduced when the neurons were repeatedly pulsed with serotonin. This provides further evidence that serotonin is needed to initiate long term facilitation in mollusks.

Serotonin may play a dynamic role in the memory of invertebrates, activating long term facilitation through several avenues, such as (1) binding to G-coupled receptors, inducing the cAMP-CREB pathway (Purves et al 2001), and (2) downregulation of synaptic plasticity repressors such as

miRNAs (Rajasethupathy et al 2009). The neurotransmitters used by other invertebrates to facilitate CREB signaling are not well known, and new animal models are needed to understand these mechanisms. Ultimately, work with *Aplysia* has shown that the mechanisms governing memory are complex and multifaceted, even in relatively simple nervous systems. Gastropod studies have laid the groundwork for beginning to understand memory mechanisms in more complex invertebrates, namely the cephalopods.

The cephalopods, including octopus, squid, and cuttlefish, offer a special opportunity to study mechanisms of memory in a complex molluscan nervous system. There is much to be learned from the use of cuttlefish as behavioral and neurobiological models, as they share many analogies with vertebrates, including a centralized nervous system, gravity-sensing statocysts, and other highly developed sensory systems, most notably the visual system (Mather and Kuba 2006, Shigeno et al 2018).

Cuttlefish as Invertebrate Memory Models

Cuttlefish possess a highly organized brain and peripheral nervous system (Boycott 1961). They demonstrate advanced, LTM-related processes such as spatial learning (Alves et al 2007; Grasso and Basil 2009; Purdy et al 1999), contour completion (Zylinski et al 2012), and visual equivalence (Lin and Chiao 2017). The foundational evidence for memory in cuttlefish comes from the “prawn in a tube” experiment, developed by Sanders and Young (1940). In this procedure, cuttlefish are presented with a prey item enclosed in a clear glass or plastic tube. The predatory attack in cuttlefish is visually prompted, and results in the rapid ejection of two specialized feeding tentacles (Messenger 1968). Cuttlefish strike the tube with feeding tentacles, and, unable to obtain the prey, learn to inhibit their predatory behavior (Messenger 1971). The number of strikes decrease within trials (acquisition), and also across consecutive days of presentation (retention), showing that cuttlefish store information in both STM and LTM (Agin et al 1998; Dickel et al 2001; Messenger 1971; Messenger 1973a; Purdy et al 2006).

LTM of the task occurs in adult European cuttlefish, *Sepia officinalis* (Agin et al 1998; Cartron et al 2013; Dickel et al 1998; Dickel et al 2001), the pharaoh cuttlefish, *Sepia pharaonis* (Purdy et al 2006),

and the bobtail squid, *Euprymna scolopes*, which still retained the task 10 days after the initial training session (Zepeda et al 2017). Retention in cuttlefish improves with increased vertical lobe development, the center for memory processing in cephalopods (Dickel et al 2001; Mather and Kuba 2006).

Research Questions

Short and Long Term Memory in Dwarf Cuttlefish

Using the “prawn in a tube” procedure, this research first aimed to determine whether short and long term memory functioned in 4-week old dwarf cuttlefish, *Sepia bandensis*. The dwarf cuttlefish is a tropical species native to the Indo-Pacific (Jereb and Roper 2006), and its behavior and learning abilities are poorly documented in the primary literature. The first goal of this research was to determine if juvenile dwarf cuttlefish were suitable memory models, by subjecting them to a learning experiment based on the “prawn in a tube” procedure.

The learning procedure consisted of a training and retention phase. It was hypothesized that 4-week old cuttlefish would exhibit STM of the procedure by reducing tentacle strikes within trials. Short term memory was evaluated from the training phase, which consisted of 5 consecutive, 10 minute learning sessions with 20 minute resting periods between sessions. Each trial was divided into 2 continuous, 5 minute intervals to assess STM acquired within the trial. Strikes were counted for each interval. It was predicted that there would be less strikes in the second interval when compared to the first interval. It was also predicted that the number of strikes would decrease across trials. The average total number of tentacle strikes was used to measure learning across trials.

It was hypothesized that 4-week old cuttlefish would also exhibit LTM of the “prawn in a tube” procedure. To assess LTM, trained cuttlefish were subjected to a single, 10 minute retention test, 4 days after the training phase. The same test was performed on naive cuttlefish that had not undergone the training phase. It was predicted that the trained cuttlefish would retain the memory acquired during the training phase, and that the strike response rate would resemble the response rate from the last trial of

training (Trial 5). It was also predicted that the response rate of naive cuttlefish would be similar to the response rate of trained cuttlefish during the first trial of training. The average total number of strikes from each group/trial was used to make statistical comparisons.

The Effect of Learning on the 3 Phases of the Predatory Response

Although learning via strike reduction has been well documented, it is unknown whether the “prawn in a tube” procedure inhibits other phases of the predatory sequence. The three phases of cuttlefish predatory behavior are attention, positioning, and finally the strike (Shinzato et al 2018; Wells 1958). Messenger (1971) posited that learning occurred via strike contingent pain from tentacles hitting the tube. This implies that only the striking phase becomes inhibited, as pain is only experienced when the strike occurs, and not while the cuttlefish faces or moves towards the prey. However, the cues that cuttlefish use to learn the procedure remain debated, and may also include visual cues such as seeing the tube (Cartron et al 2013).

The next goal of this research was to determine whether learning the “prawn in a tube procedure” selectively inhibited the striking phase. To do this, cuttlefish activity was recorded with DanioVision, a high-throughput organismal tracking system. It was hypothesized that the procedure would also inhibit prey interest, i.e. the amount of positioning near prey, and the duration of time spent facing prey. Positioning was measured as the total distance moved by each cuttlefish. It was predicted that a reduction in total movement across sessions would occur as a result of learning. To measure the amount of time spent near prey, experimental arenas were divided into equal two zones, one of which contained the inaccessible prey. Time spent facing prey was measured as the time the eyes of the cuttlefish were oriented towards the zone containing the prey tube. It was predicted that cuttlefish would reduce the amount of time spent facing the prey zone across trials, as a result of learning.

Activation of CREB in Cuttlefish Arms During Learning

Messenger (1971) proposed that cuttlefish stop striking during the “prawn in a tube procedure” due to pain experienced when tentacles hit the tube. During the learning experiments in this research, cuttlefish would repeatedly propel themselves forward when striking, bringing all of the arms into contact with the tube. They would then sit still for extended periods, attached to the tube with their suckers. From this observation, it was asked whether tactile cues obtained by the 8 arms also contributed to memory of the procedure. The suckers lining the arms contain mechanoreceptors and chemoreceptors, and play a large role in manipulating prey towards the mouth after capture (Halm et al 2003, Mather and Dickel 2017). Although strikes are made with the feeding tentacles, it is possible that sensory neurons within suckers of the arms also obtain tactile cues from the tube, which may allow cuttlefish to learn the tube is an obstacle. With this in mind, the last goal of this research was to determine whether the memory-related transcription factor, CREB, was present in cuttlefish arms, and whether CREB became activated in the nervous system of the arms during the learning procedure. Before molecular mechanisms can be considered, including potential locations for CREB activation, the major anatomical aspects of the cuttlefish nervous system must be understood.

Neuroanatomy of the Cuttlefish Brain

While the typical molluscan nervous system is described as a simple “rope-ladder” of concentrated cell bodies (ganglia) linked by large axonal tracts (commissures), the cephalopod mollusks possess the most complex nervous system of all invertebrates (Mather and Kuba 2006). The cephalopod brain is a centralized mass of approximately 30 fused lobes, found between the two eyes (Shigeno et al 2018). The brain is encased in cranial cartilage, and contains two major divisions, based on their position relative to the esophagus. The esophagus lies at the center of the two masses; the supraesophageal mass lies dorsal to the esophagus, and the subesophageal mass is ventral (Boycott 1961). The supraesophageal

mass controls “higher functions” such as learning, and movement of the head and eyes. The subesophageal mass is largely composed of lower motor centers associated with the arms and tentacles, as well as regions controlling visceral functions and inking (Boycott 1961; Mather and Kuba 2006). The vertical lobe is the foremost dorsal structure and contains over 25 million neurons, making it the largest learning and memory center of all invertebrates. The vertical lobe alone comprises more than half of the neurons in the supraesophageal mass (Shigeno et al 2018).

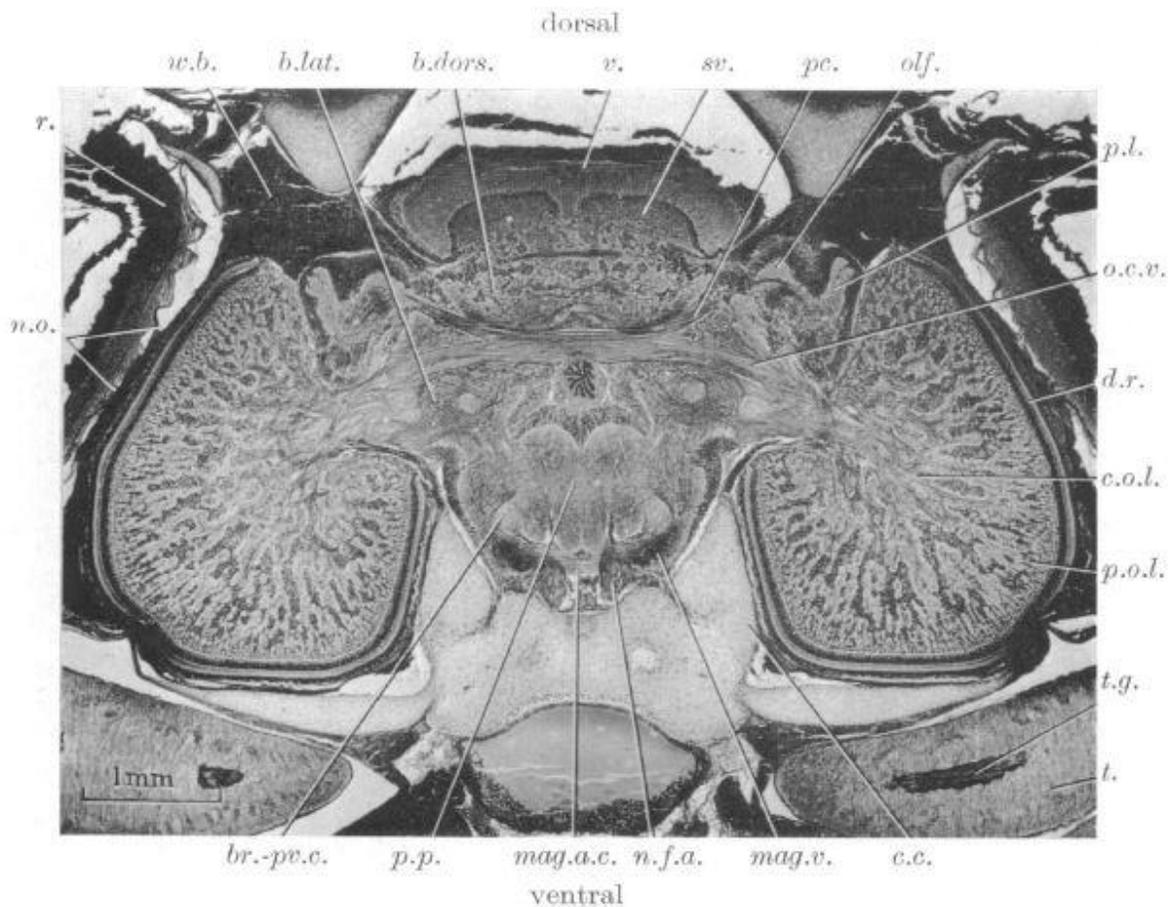


Figure 4. Transverse section of the brain in *S. officinalis* (Boycott 1961).

In Figure 4, the major anatomical features of the cuttlefish brain are shown. The esophagus lies near the center of the brain, beneath the optic commissure (o.c.v). The subesophageal mass is found ventral to the esophagus, and contains lower motor centers for the arms and tentacles, as well as regions

controlling visceral functions such as inking behavior. The two round optic lobes comprise a large portion of brain mass and are connected to the retinas (r.). Dorsal to the esophagus lies the supraesophageal mass, which controls higher motor functions associated with head and eye movement. The most dorsal region of the supraesophageal mass is the M-shaped vertical lobe (v.), which is the learning center in cephalopods. The cranial cartilage (c.c.) encases the brain.

Neuroanatomy of the Arms and Tentacles

Decapodian cephalopods (squid and cuttlefish), have 8 muscular arms and 2 retractable feeding tentacles, while the octopods have 8 arms, and lack feeding tentacles (Mather 2006). The arms form a crown around the mouth and aid in prey capture and manipulation, as well as locomotion (Halm et al 2003). The arms are largest in diameter at their base, and taper towards the tips. Rows of grasping suckers line the oral side of the arms and relay both chemosensory (Wells 1963) and tactile (Wells and Wells 1956) information to the arms and the brain. In cuttlefish and squid, the two feeding tentacles terminate into enlarged clubs used to seize prey when ejected (Wells 1958). The suckers on the feeding tentacles are confined to the clubs (Rorbach and Schmidtberg 2006).

Despite having a large brain mass, the majority of cephalopod neurons lie in the peripheral nervous system, within the arms (Mather and Kuba 2006). While the brain contains 170 million neurons, the 8 arms contain approximately 350 million neurons combined (Young 1961). Arm innervation from the brain originates in subesophageal mass, in the anterior brachial lobe, which contains the brachial ganglia (Boycott 1961). Some of the nerves in the brachial ganglia arise from the pedal lobe (subesophageal mass), which controls the tentacles, and also gives inputs to the arms (Boycott 1961). Efferent fibers from the brachial ganglia are organized into 8 brachial nerves, one for each arm. At the base of the arm crown, the interbrachial commissure forms a connecting ring around the brachial nerves, allowing the arms to transmit information to each other, and coordinate movement (Sakaue et al 2014, Shigeno et al 2018). Past the interbrachial commissure, each brachial nerve cord becomes an axial nerve

cord, which runs through the center of each arm, innervating muscle and transmitting sensory information obtained from suckers (Shigeno et al 2018). The axial nerve cord acts as the integrative center for each arm, projecting large fibers into the muscle layers as well as innervating individual suckers (Bellier et al 2017).

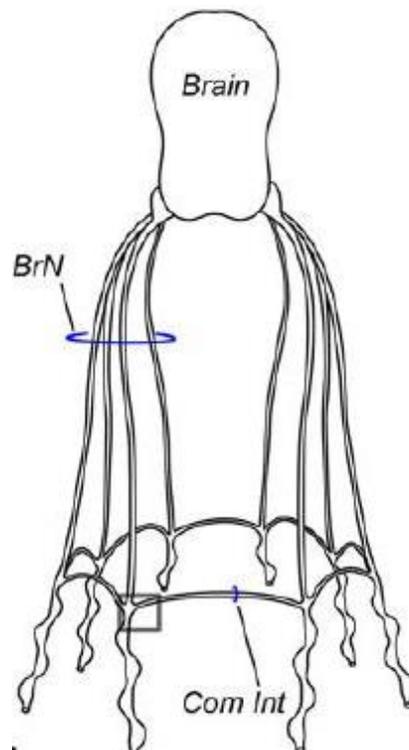


Figure 5. Schematic of the cephalopod peripheral nervous system organization (Sakaue et al 2014).

Figure 5 displays the organization of the peripheral nervous system of cephalopods. Brachial nerves (BrN) extend from brachial ganglia attached to the anterior brachial lobe of the brain. The interbrachial commissure (Com Int) connects the brachial nerves at the base of each arm. Beyond the interbrachial commissure, each brachial nerve becomes an axial nerve cord, which runs through the center of each arm.

The feeding tentacles also contain axial nerve cords (Boycott 1961). The tentacles are innervated by the pedal lobe, and do not respond when electricity is applied to the brachial lobe, suggesting that separate circuits may control the arms and tentacles (Boycott 1961). The tentacles expand and eject very quickly due to the presence of specialized cross-striated and helical muscles wrapped around the axial nerve cord (Grimaldi et al 2004).

The musculature, neural organization, and receptors of the arms are all summarized in a body of work by P. Graziadei (1965, 1976). The musculature of the arms is divided into 3 main types: the intrinsic muscle of the arm, the intrinsic muscle of the suckers, and the acetabulo-brachial muscles, which unite the muscles of the arm and the muscles of the sucker (Graziadei 1965). The intrinsic muscle of the arm contains 3 layers, which are innervated by 4 intramuscular nerve cords. The intramuscular nerve cords are integrated by large lateral fibers emanating from the central axial nerve cord (Graziadei 1965).

The suckers contain two muscular components: the infundibulum and the acetabulum. The infundibulum is the external, disc-like portion of the sucker, while the acetabulum is the internal muscular cavity which mediates the pressure differentials needed for grasping and adhesion (Bellier et al 2017). Each sucker is innervated by a sucker ganglion, dorsal to the acetabular roof (Bellier et al 2017; Graziadei 1965). Two lateral fibers project from the sucker ganglion; the ventral ganglion fiber innervates the sucker itself, while the dorsal fiber meets the ventral roots emanating from the axial nerve cord. The ventral roots from the axial nerve cord also directly innervate each sucker (Graziadei 1965).

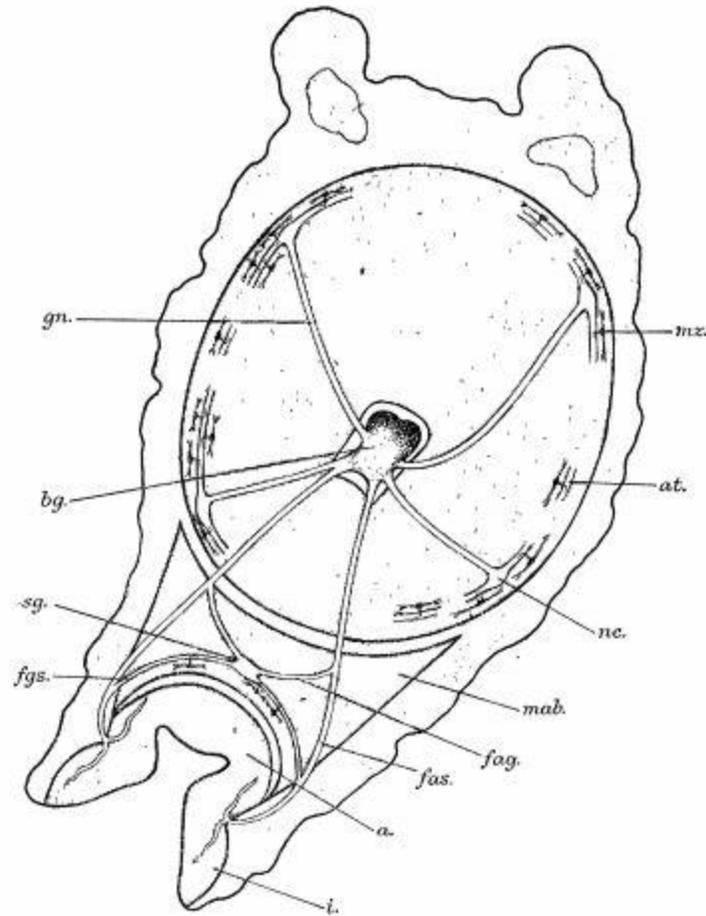


Figure 6. Cross-sectional view of innervation within a cephalopod arm (Graziadei 1965).

Shown in Figure 6, four intramuscular nerve cords (nc.) innervate the muscle layers of the arm. The brachial ganglia (bg.), or axial nerve cord, lies at the center of the arm and innervates each intramuscular nerve cord with large lateral fibers (ng.). Ventral roots of the axial nerve cord innervate the muscles of the acetabulum (a.) and infundibulum (i.) in each sucker. Dorsal to the acetabular roof lies the sucker ganglion (sg.). The dorsal sucker ganglion fiber joins with the axial nerve cord ventral roots (fag.), while the ventral ganglion fiber innervates the sucker itself (fgs.).

The two main types of neurons in cephalopod arms are motor neurons, which control the muscles of the arms and suckers, and sensory neurons, which obtain tactile and chemical information from the environment. The surface of cephalopod skin contains many sensory neurons. These neurons are most

densely concentrated on sucker epithelia, making each individual sucker a sensitive organ (Graziadei and Gagne 1976). Four morphological types of primary sensory cells have been described in the sucker epithelium, most of which are ciliated. Graziadei and Gagne (1976) posited that these ciliated cells serve chemoreceptive and mechanosensitive functions. Sensory information obtained by suckers is transmitted to the brain learning centers (i.e. the vertical lobe) by the axial nerve cord (Graziadei and Gagne 1976). Therefore, suckers likely play a large role in the neuronal processes underlying learning of chemical and tactile cues in cephalopods.

Despite that the anatomy of arm innervation has been well documented, few studies have attempted to uncover the molecular function of neurons within the arms. The axial nerve cords in arms and tentacles, as well as suckers, contain cholinergic nerves, which utilize the neurotransmitter acetylcholine (Sakaue et al 2014). Serotonin also participates in sensory transmission in the octopus arms, as 5HT immunoreactivity is detected in the axial nerve cord, and 5HT-immunoresponsive cells project fibers into the suckers (Bellier et al 2017).

While sensory information from the suckers reaches the brain, it is possible that the suckers also modulate activity in the arms independent of brain activity, due to the high level of integration among the arm muscles, axial nerve cord, and each individual sucker. Many cephalopod researchers have contemplated the level of autonomy that exists in cephalopod arms, owed to their coordinative ability. Graziadei (1965) observed that the 8 arms of octopuses are still able to make coordinated movements when separated from the brain, and that isolated arms likewise share this ability. Given this complexity, repeated activation of sensory neurons within the suckers may permit synaptic plasticity signals to occur in the arm itself. This is not unlike the process of long term sensitization in *Aplysia*, wherein repeated activation of sensory neurons in the siphon changes the signaling properties of the gill withdrawal circuit. Synaptic changes in the arms may serve to modulate transmission of information to the vertical lobe during learning, while also modulating local activity within the arm.

The neurons in cephalopod arms share several similarities with plastic neurons in gastropods. These similarities suggest that cephalopod arms are capable of synaptic plasticity, and may use CREB

signaling. The neurons in cephalopod suckers contain fibers that use serotonin, the same neurotransmitter known to activate and regulate cAMP-CREB signaling in *Aplysia* sensory neurons (Purves et al 2001; Rajasethupathy et al 2009). Moreover, CREB immunoreactivity occurs in the pedal ganglia of the snail, *Helix lucorum* (Efimova et al 2007). In gastropods, the pedal ganglia control the muscular foot and tail (Bargmann 1930, Chase 2002). Embryological studies have determined that cephalopod arms evolved from the muscular foot of ancestral gastropod mollusks (Shigeno et al 2008). Likewise, the pedal lobe in the cephalopod brain, along with the brachial lobe, innervates the arms (Boycott 1961). Given the physiological and evolutionary connections between cephalopod and gastropod nervous systems, it is possible CREB signaling is utilized to mediate synaptic plasticity in cephalopod arms, similar to CREB signaling in gastropod pedal ganglia and gill withdrawal reflexes.

During the learning experiments described in 1.5, it was observed that cuttlefish would propel themselves forward during tentacle strikes, bringing their arms into contact with the prey tube, where they would often adhere themselves with their suckers. From this observation, it was asked whether tactile cues obtained from the arms activated CREB signaling during the learning experiment. It was hypothesized that CREB signaling could be activated in cuttlefish suckers due to sensory neurons obtaining tactile cues from contacting the prey tube. To test this hypothesis, tissue sections of trained cuttlefish arms were stained with an anti-pCREB antibody. The pCREB antibody specifically stains against the phosphorylated CREB, which is the active version of the protein used to transcribe immediate early genes (Benito and Barco 2010). The number of CREB-positive cells were counted in trained cuttlefish were compared to untrained controls. Previous studies using rodents have assayed the role of CREB in learning by counting the number of CREB-positive cells in the nervous system after a learning experiment (Brightwell et al 2007, Kim et al 2013). In mice, CREB immunoreactivity increases in LA neurons populating the amygdala (Kim et al 2013). In rats, CREB immunoreactivity increases in the dorsal hippocampus following a place learning task (Brightwell et al 2007). It was predicted that cuttlefish trained with the learning procedure would have more pCREB immuno-positive neurons within the suckers of the arms than untrained controls.

CHAPTER 2

MATERIALS AND METHODS

Cuttlefish Husbandry

Sepia bandensis eggs were reared in two 20 gallon tanks in the animal care facility located within the Statesboro campus of Georgia Southern University. Published information on the optimal rearing conditions of *S. bandensis* is limited, but helpful insights for *S. banensis* care were personally offered by Brett Grasse (Manager of Cephalopod Operations, Marine Biological Laboratory, Woods Hole, MA), and Theresa Gunn (graduate student, Georgia Southern University). Tanks were filled with artificial seawater made with Instant Ocean sea salt mix (Spectrum Brands, Inc.). Salinity was maintained at 30-35ppt, temperature at 25 ± 2 °C and pH at 8.0-8.2. A refractometer was used to monitor salinity, and an API master saltwater test kit was used to monitor ammonia, nitrite, and pH. An overhead light was operated on a 12 hour light:dark cycle for both tanks. To maintain salinity, tank water was frequently topped off with freshwater dechlorinated with water conditioner (Natural Rapport). Tanks were equipped with Tetra Whisper EX Power filters (20-30 gal), and carbon filter pads were changed monthly. Tanks were heated with Tetra HT30 100W submersible heaters, and were constantly aerated with air stones, supplied by gas lines within the animal care facility. Changes of ~30% tank volume were carried out bi-weekly to control for pH and buildup of ammonia/nitrite.

Sepia bandensis eggs were obtained from Blue Zoo Aquatics (Hawthorne, CA), or SeaDwelling Creatures (Los Angeles, CA). During acclimation, eggs masses and water were removed from the plastic shipping bags and placed in buckets (one bucket per tank). Eggs were drip acclimated with tank water for at least 2 hours using small airline tubing. Drip rate from tanks was maintained at ~1 drop per second by tying several loose knots in the tubing. After drip acclimation, egg masses were placed inside a floating mesh basket within each tank. A second air stone was placed in each tank to aerate the egg masses, supplied by a Tetra 10-100 gallon air pump. The air stones were placed underneath the baskets containing the egg masses to aerate the eggs and prevent biofouling of egg capsules. The eggs were checked daily for

hatchlings. Each day, new hatchlings were removed from the egg basket and placed in a new basket within the tank. Cuttlefish were transported between baskets using a kitchen ladle, to ensure they remained submerged in water during handling. Cuttlefish were separated by baskets according to age. Cuttlefish were considered 0 days old on the day of hatching. The age of each basket was updated daily. Cuttlefish were not used in experiments until they were 21 days old.

Cuttlefish were fed live *Mysis* shrimp daily. *Mysis* were obtained from Sachs Systems Aquaculture Inc. (St. Augustine, FL) and were maintained in a separate, 20 gallon tank. Salinity was maintained at 25-20ppt, and the tank was constantly aerated with an air stone. *Mysis* were fed a mixture of live brine shrimp hatchlings and ground goldfish flakes (Tetra). Water changes were carried out bi-weekly for the *Mysis* tank.

DanioVision Tracking System

Behavioral experiments were conducted using a DanioVision Observation Chamber with accompanying EthoVision XT computer tracking software (Noldus Information Technology). The DanioVision Observation Chamber was designed for controlled, high-throughput behavioral tracking of zebrafish larvae, but has been adapted for use on other small organisms such as anemones (Oren et al 2015), tadpoles (Stanley et al 2015), and *Drosophila* larvae (Graham et al 2016). The chamber is a closed system equipped with an infrared sensitive camera for recording subjects. Hardware settings within the chamber such as white lighting, temperature, and humidity can be modified using EthoVision XT to create different experimental stimuli. EthoVision XT is also used to create detection settings for identifying and tracking subjects, designing trials, and to collect subject data during experiments. Data collected by the software includes components of subject activity such as distance moved, velocity, and duration of movement.



Figure 7. The DanioVision Observation Chamber.

A photograph of the DanioVision Observation Chamber is shown in Figure 7. The chamber is equipped with an infrared camera for recording subjects (top). Subject arenas are placed on a platform on the floor of the chamber (arrow). The arena platform can be illuminated using the built in white light.

Experimental Arenas

Six experimental arenas to be fitted in the DanioVision Observation Chamber were designed for use in all experiments. The arenas were created by modifying a six well culture plate (Corning), so that each well represented an individual cuttlefish arena (Figure 7). Each arena measured 3.5 cm in diameter. A clear plastic tube measuring 2 cm in height was glued into the floor of each arena using J-B Weld clear epoxy (W.W. Grainger, Inc.). After setting overnight, water was pipetted into each tube to ensure the seal was waterproof. This was done to eliminate the chance that chemical cues from the prey would influence cuttlefish behavior. The outside walls of each well were painted opaque with gray acrylic paint to provide isolation for each cuttlefish. The floor remained transparent to allow the arenas to be illuminated by the built in white light inside the chamber.

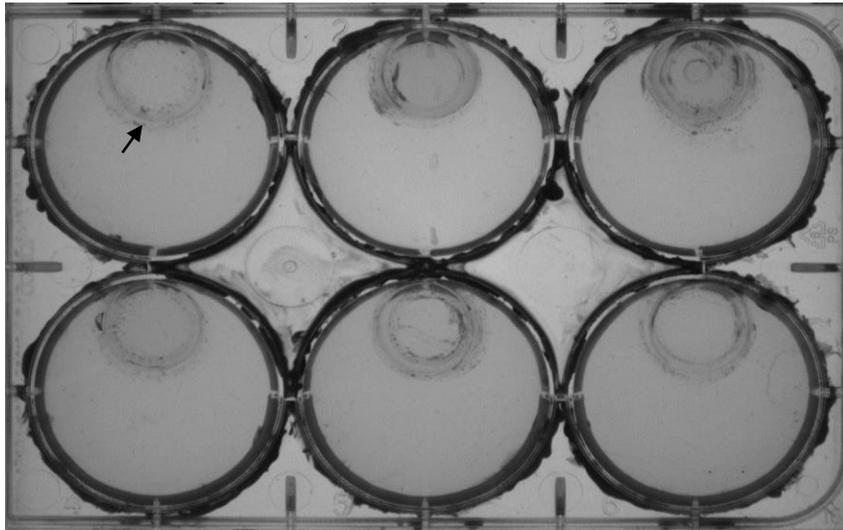


Figure 8. The experimental arenas.

Figure 8 shows a photograph of the experimental arenas taken from the infrared camera inside the DanioVision Observation Chamber. Tubes for holding *Mysis* (arrow) were fixed into the top center portion of each arena. The built-in white light sat directly beneath the plate.

Experiment 1: Assessment of Short and Long Term Memory in Dwarf Cuttlefish

In the first chapter, it was asked whether juvenile dwarf cuttlefish are suitable models for studying memory. This experiment was designed to determine whether juvenile dwarf cuttlefish exhibit behavioral evidence for short and long term memory. It was hypothesized that 4 week old cuttlefish would exhibit behavioral evidence for both short and long term memory. Past studies have shown that *S. officinalis* exhibit short term memory as young as 8 days (Agin et al 1998; Dickel et al 1998), and exhibit long term memory around 30 days (Agin et al 1998; Dickel et al 2001).

When experiments began, cuttlefish were not fed until trials were completed each day. To assess short and long term memory in *S. bandensis*, a learning paradigm was designed based on the “prawn in a tube” procedure. The paradigm consisted of a training and retention phase. During the training phase, 4 week old cuttlefish underwent 5 consecutive, 10 minute trials, with a 20 minute resting period between each trial. Cuttlefish were removed from the housing tanks and placed in a large Tupperware container filled with tank water. Each arena of the experimental plate was filled with 15 mL of tank water using a 50 mL syringe. Cuttlefish were placed back into the container during resting intervals. The container was aerated with a portable air pump during resting intervals. Water was replaced in the container and the arenas for each trial.

To begin each trial, cuttlefish were placed individually in each arena, and acclimated inside the DanioVision Observation Chamber for 5 minutes. Each of the shrimp tubes were filled with water from the *Mysis* tank, and a single mysid shrimp was added to each tube using six plastic transfer pipettes. After adding the shrimp, the chamber was closed and the trial was recorded using Ethovision XT. The built-in white light operated at 15% intensity during trials to ensure prey visibility.

To assess short term memory of the task, each trial was divided into two continuous, 5 minute intervals, and the strikes carried out by each cuttlefish were tallied during each interval. Strikes were not tallied until the experiment was recorded in its entirety. During the experiments, cuttlefish often extended

their feeding tentacles to touch the tube without completing the ejection; these were not counted as strikes. Only instances when the tentacles visibly completed a full strike against the tube were counted. The strikes made by each cuttlefish during the first and last interval were averaged, and the averages for each interval were compared within each trial. It was predicted that cuttlefish would learn within each trial, and that the average number of strikes would decrease between the first interval and last interval of each trial. I also predicted that cuttlefish would learn between trials, and the average total number of strikes (combined intervals) would decrease from Trial 1 to Trial 5.

To determine whether dwarf cuttlefish exhibited long term memory, a retention test was performed on the trained cuttlefish. Four days after the training day, the trained cuttlefish underwent a single, 10 minute retention test, with procedures identical to the training sessions. For control, the same test was performed on naive cuttlefish on the retention day. The naive cuttlefish were the same age as the trained cuttlefish, but had never been exposed to the learning experiment. It was predicted that trained cuttlefish would retain memory acquired during training, and would produce a total average strike count similar to Trial 5. It was also predicted untrained cuttlefish would produce a total average strike count similar to Trial 1.

Experiment 2: Assessment of Predatory Response Phases Using DanioVision

Prior to designing our learning paradigm, preliminary trials were conducted with the experimental arenas to assess differences in cuttlefish activity resulting from the presence or absence of prey. These trials occurred with different cuttlefish than those used in the learning paradigm. Cuttlefish in preliminary trials were 2 weeks old when trials began. Each trial consisted of 3 cuttlefish presented with a shrimp in the prey tube, and 3 control cuttlefish with tubes filled with water only. Trials lasted 20 minutes. Cuttlefish were tested once a day for 5 days, for a total of 20 trials. Cuttlefish were not fed until trials were completed each day. There was not a significant learning effect via strike reduction across these trials. This was attributed to the age of the cuttlefish tested, as cuttlefish do not show significant long term

retention until they are approximately 4 weeks old (Dickel et al 2001). However, it was observed that cuttlefish presented with inaccessible shrimp moved a greater total distance than control cuttlefish with no shrimp present. This was attributed to the cuttlefish's repeated attempts to position themselves near the prey.

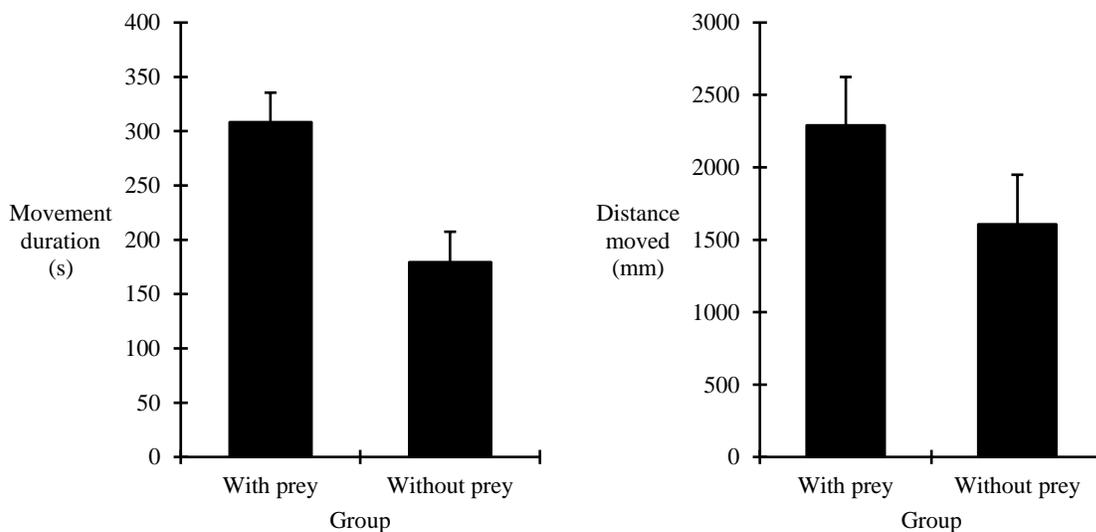


Figure 9. Preliminary cuttlefish tracking data.

Figure 9. Preliminary cuttlefish tracking data collected from a total of 20 trials lasting 20 minutes each. The data in figure 9 shows tracking collected from a total of 20 trials lasting 20 minutes each. Data for both figures presented at mean + SE. Cuttlefish presented with prey ($n = 12$) spent more time moving than control cuttlefish ($n = 12$) without prey (Wilcoxon test; $X^2 = 16.7914$; $p < 0.0001$). Cuttlefish presented with prey also moved a greater total distance than control cuttlefish (Wilcoxon test; $X^2 = 9.2936$; $p = 0.0023$).

From this observation, it was asked whether the learning experiment would affect prey interest in the 4 week old cuttlefish tested in 2.4. While the learning experiment assays learning via the reduction in tentacle strikes, it does not take into account the entire predatory response of the cuttlefish. The predatory response of cuttlefish is cued by visual detection of prey, and occurs in three distinguishable phases

(Shinzato et al 2018; Wells 1958). The three phases of cuttlefish predatory behavior are attention, positioning, and finally the strike (Shinzato et al 2018; Wells 1958). The attention phase begins when the cuttlefish orients its eyes towards the prey (Shinzato et al 2018; Wells 1958). During positioning, the cuttlefish moves closer to the prey, while the tentacles begin emerging to prepare for the strike (Wells 1958). While the predatory response is easily observed, it is not well known how the feedback elicited by failed prey capture affects predatory behavior as a whole. Repeated failure to capture prey may also temporarily disrupt the cuttlefish's visual interest and motor response to the prey, however, these components of the predatory response are difficult to accurately quantify by manual observation. By coupling the "prawn in a tube" procedure with DanioVision high-throughput tracking, this experiment aimed to determine whether the learning process selectively inhibited the striking phase. It was hypothesized that learning would also inhibit positioning near the prey, and reduce the amount of time eyes were oriented towards the prey. Positioning and orientation of the eyes were measured using DanioVision subject tracking.

DanioVision Subject Tracking

EthoVision XT software accompanies the DanioVision Observation Chamber, and is used to obtain automated tracking data from animal subjects. Subject tracking occurred from saved video files after the experiment was recorded in its entirety. EthoVision XT uses center point tracking to record subject movement within the arena. (Figure 10a). The movement path of the cuttlefish is measured from the center point as a continuous line (Figure 10b). In addition to center point tracking, another subject tracking method was used called "head to tail" tracking. Head to tail tracking places a tracking point on the head and tail of the animal, such that orientation and movement of the whole body within the arena can be analyzed. Separate detection settings were used to track the movement of each mysid shrimp (Figure 10c). Size calibration of the experimental plate was set at 11.5 cm (length of the plate). Subject tracking occurred at a rate of 30 frames/sec. Subject tracking occurred via the static subtraction method,

in which changes in each frame are compared to a static background reference image, in this case, the image of the empty experimental arenas (Figure 8).

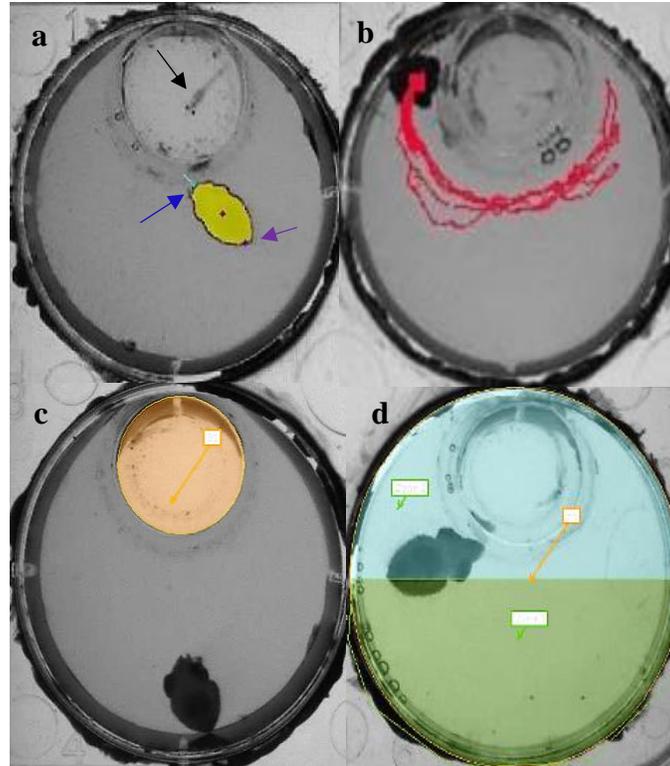


Figure 10. Experimental setup designed with EthoVisionXT.

Figure 10 shows the arena settings generated with EthoVision XT software. a) Detection of cuttlefish inside the experimental arenas. The red center point marker appeared on the dorsoanterior portion of the mantle. The head point appeared on the tip of the arm crown (blue arrow), while the tail point appeared on the posterior tip of the mantle (purple arrow). Movement of the center point is used to generate subject data such as distance moved, velocity, frequency of rotations, and movement paths. Mysid shrimp were present simultaneously within the arena (black arrow) but did not interfere with

cuttlefish tracking. (b) Movement path of cuttlefish acquired by center point tracking. (c) Arena settings for detecting mysid shrimp. The orange shading indicates that arena boundaries were confined to the inside of each tube. Separate detection settings (not shown) were used to measure activity of mysids. (d) Arena settings for cuttlefish. Each arena was divided into two zones. The top zone (blue) contained the shrimp tube; the bottom zone (green) was empty.

Prey interest was assayed by the amount of time the cuttlefish spent facing the prey. To assess the time the cuttlefish spent facing the prey, EthoVision XT was used to divide each cuttlefish arena into two zones. The arenas were divided in half, with the top zone containing the shrimp tube (Figure 10d). The time the eyes of the cuttlefish were oriented towards each zone was measured by using the head tracking point, which appeared between the eyes, on the tip of the arm crown (Figure 10a). The duration the head tracking point spent facing each zone was measured for each cuttlefish during training, and again during the retention test. Total distance moved by each cuttlefish was also recorded to measure the amount of positioning near prey. Distance moved was measured by center point tracking, which generates a complete movement path per trial for each subject (Figure 10b). It was predicted that duration spent facing the prey, and total distance moved by the cuttlefish would both decrease across consecutive trials, as a result of learning the prey was inaccessible.

Experiment 3: Assessment of CREB Activation in Cuttlefish Arms

During the learning experiments, it was observed that, when striking, cuttlefish would often propel themselves forward, wrap their arms around the tube, and sometimes remain adhered for long periods with their suckers. Examples of this behavior are shown in Figure 10.

Past studies involving similar learning experiments have discussed the potential cues used by cuttlefish used to memorize the procedure. Messenger (1971) posited that strike reduction was caused by pain experienced with the feeding tentacles hit the tube, but no available research has confirmed nociception in cuttlefish tentacles. Other cues independent of the tentacles may also be used to learn the procedure.

Cartron et al (2013) argued that, due to their ability to detect transparent objects with polarized vision, cuttlefish used visual cues from the tube itself to learn the tube was a physical barrier. Supporting this, they found a decrease in learning ability when the transparent tube was covered by a depolarizing filter.

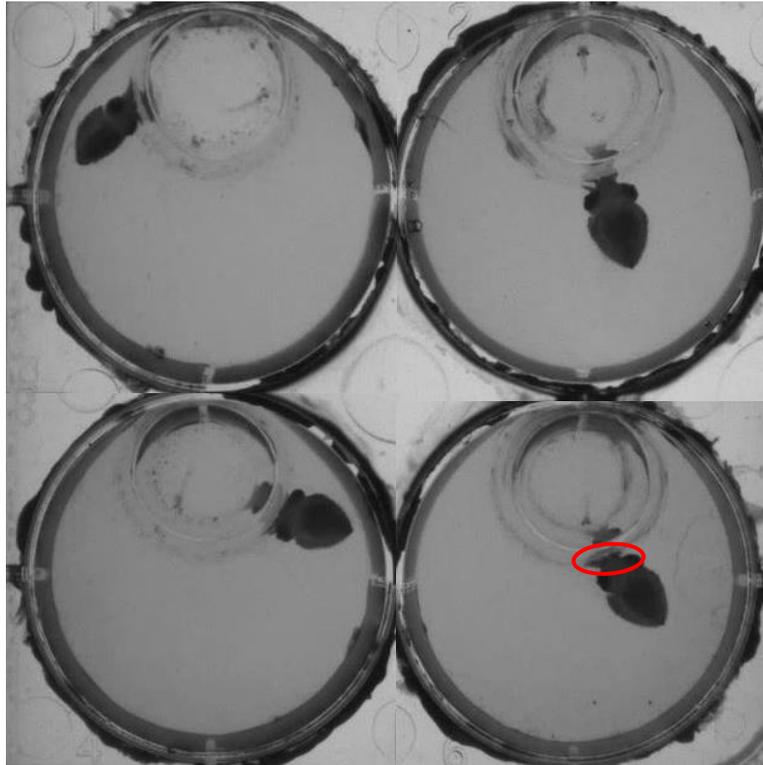


Figure 11. Examples of arm contact with the prey tube during learning experiments.

In Figure 11, the arms of the cuttlefish can be seen spreading across the tube (red oval). Each of the 4 images was taken of a different individual during Trial 1 of training. Despite that several studies have discussed possible cues and sensory systems used by cuttlefish to learn the procedure, no studies have considered the role of the arms, which contain many sensitive suckers. Additionally, no studies have determined whether synaptic plasticity occurs within cuttlefish sensory systems during “prawn in a tube” training. Chapter 1 discussed the relevance of the transcription factor, CREB, as a marker for synaptic plasticity and learning in broad animal groups, including the gastropod mollusks, close relatives of cuttlefish (Casadio et al 1999; Efimova et al 2007; Guo et al 2010; Lee et al 2006; Lewin and Walters

1999; Rajasethupathy et al 2009; Ribeiro et al 2003; Sadamoto et al 2003; Shigeno et al 2008). It is already known that cephalopods use suckers for tactile discrimination learning (Wells and Wells 1956) and chemoreception (Wells 1963). Graziadei and Gagne (1976) described 4 types of ciliated sensory neurons on the sucker epithelia, providing morphological evidence for sensory transmission by suckers. Given that cuttlefish frequently touched the prey tubes with their arms, it was asked whether tactile cues obtained from the arms activated CREB signaling during training. Although CREB is activated in the sensory neurons of gastropod relatives (Purves et al 2001), CREB has not yet been identified in the nervous system of any cephalopod. It was hypothesized that CREB could be activated in cuttlefish arms due to sucker sensory neurons obtaining tactile cues from contacting the prey tube. CREB activation was assayed using a phosphorylated CREB (pCREB) antibody on arms of trained and naive cuttlefish.

Cuttlefish were trained using the same procedure described in 2.4.1, except that cuttlefish were euthanized for dissection after training, and no retention test was performed. Strikes made by cuttlefish during training were counted and analyzed as described in 2.4.1, and results were included in Chapter 3. After training, cuttlefish were returned to their housing tanks for a period of one hour before dissections occurred. A waiting period of one hour was chosen because CREB-dependent immediate early gene expression occurs within a time sensitive window, i.e. 1-3 hours after behavioral training in chicks (Freeman and Rose 1999).

After one hour, cuttlefish were euthanized for dissection. Control cuttlefish (untrained) were euthanized at the same time. Euthanasia was conducted in an effort to minimize stress. Cuttlefish were submerged in regular tank water within individual petri dishes. Euthanasia was carried out using a seawater solution containing 7.5% MgCl₂ and 5% ethanol, which are often used in combination for cephalopod surgical anesthesia and humane killing (Gleadall 2013). The solution was added to each petri dish in 10-drop increments until the cuttlefish paled and no longer responded to a gentle tactile stimulus with a Pasteur pipette. Euthanasia was confirmed by placing each cuttlefish under a dissecting microscope and observing no respiratory contractions of the mantle, no body movement, and no chromatophore

activity. The cuttlefish were then fixed overnight in 4% paraformaldehyde (PFA) dissolved in 1X phosphate buffered saline (PBS).

After fixing overnight, the arms were dissected from each cuttlefish. A dissecting tray was created by lining the inside of a large petri dish with hardened 4% agarose. 1X PBS was used as a dissection buffer. The mantle of the cuttlefish was pinned to the dissecting tray, and the arms were removed at their base with micro surgical scissors. The arms were separated according to pairs into microcentrifuge tubes filled with 4% PFA. Cuttlefish have 4 pairs of arms that differ in size and dorso-ventral arrangement, with arm pair 1 being the most dorsal pair, and arm pair 4 being the most ventral. The head and tentacles of each cuttlefish were also preserved in 4% PFA. The arms were stored in 4% PFA at RT until tissue sectioning occurred.

Arms were prepared for sectioning by suspending them in square molds of 6% low melting agarose, then rapidly freezing the mold on an ice pack. Each block contained only one suspended arm, which was oriented so that arms were cut dorsoventrally, with the dorsal epidermis being cut first by the vibratome blade, and the ventral suckers cut last for each section. Once frozen, the agarose blocks were cut out of the mold and trimmed with a razor. Arms were stored by pairs at RT in 1X PBS until sectioning. Tissue sections were obtained using a Leica VT1000S vibratome. Agarose blocks were super glued (Gorilla Glue) onto metal chucks, and submerged within a PBS bath to prevent desiccation. Longitudinal tissue sections were cut to a thickness of 150 μM , and were collected from the PBS bath using a small paintbrush. Tissue sections were cut out of agarose slices using a dissecting pin, and were stored in 8-chambered cell culture slides containing 4% PFA. Each chamber contained sections from only one arm, so that one whole slide contained sections from all 8 arms of each cuttlefish. The tissue sections were refrigerated in 4% PFA until antibody staining began.

In addition to pCREB, tubulin was also stained. Tubulin is a cytoskeletal protein found in neurons, and tubulin staining can be used to view axons and overall neural organization (Miller and Joshi 1996). Tubulin and pCREB staining occurred simultaneously, using a procedure modified from Efimova

et al (2007), who used pCREB antibodies in the nervous system of *Helix* snails, a mollusk relative.

Cuttlefish were stained using the solutions and antibodies listed in Table 1.

Table 1. Solutions needed for cuttlefish pCREB and tubulin immunohistochemistry.

Solutions	Components
PBS (1X)	10X PBS stock DH ₂ O
PFA (4%)	1X PBS PFA (w/v)
IB	1X PBS 2.5% horse serum (v/v) 2.5% bovine serum albumin (w/v) 0.5% Triton X-100 (v/v)
MeOH (20%, 50%, 70%, 80%)	100% methanol stock DH ₂ O
pCREB primary antibody (1:500)	IB Rabbit polyclonal anti-Phospho CREB IgG (Ser 133)
pCREB secondary antibody (1:500)	IB Alexa Fluor 488 chicken-anti-rabbit IgG
Tubulin primary antibody (1:500)	IB Mouse monoclonal anti-acetyl alpha tubulin IgG (Lys 40)
Tubulin secondary antibody (1:500)	IB Alexa Fluor 555 goat-anti-mouse IgG
Glycerol (20%, 50%, 70%)	100% glycerol stock DH ₂ O

Cuttlefish arms were stained within the chambered slides over a period of 3 days. The arms were stored in 4% PFA for at least one night prior to staining. A glass Pasteur pipette was used to transfer solutions into the chambers. On the first day, the 4% PFA was removed, and the chambers were gradually incubated with increasing methanol concentrations of 20%, 50%, 70%, and 80% MeOH, respectively. Tissues were incubated at 20%, 50%, and 70% concentrations for 10 min, and stored overnight in 80% MeOH. This was done to increase the access of the pCREB antibody across nuclear membranes. The second day, the chambers were washed 3 times for 5 minutes each in 1X PBS. The chambers were then washed for 30 minutes in incubation buffer (IB). IB contained a detergent (Triton X-100) to permeate

tissues for easier antibody access, and also contained protein sera (horse and bovine albumin) to prevent non-specific antibody binding. After 30 minutes, chambers were refrigerated overnight with 500 μ L of 1:500 primary antibodies in IB. 4 mL of 1:500 antibody solution was made for each slide by pipetting 8 μ L of primary antibody into a glass vial containing 4 mL IB, and vortexing the vial. 500 μ L of the 1:500 stock was pipetted into each chamber. 1:500 secondary antibody solutions were made the same way. The CREB antibody was rabbit polyclonal anti-Phospho CREB IgG (Ser 133; Invitrogen). The tubulin antibody was mouse monoclonal anti-acetyl alpha tubulin IgG (Lys 40, Sigma Aldrich).

The second day, chambers were washed once in IB, then refrigerated overnight in 500 μ L of 1:500 secondary antibodies. The pCREB secondary antibody was Alexa Fluor 488 chicken-anti-rabbit IgG (Invitrogen). The tubulin secondary antibody was Alexa Fluor 555 goat-anti-mouse IgG (Invitrogen). The secondary antibodies were conjugated with fluorescent dyes that emitted either green light (488) or red light (555) when viewed under a fluorescent microscope.

The third day, chambers were washed 3 times for 5 minutes each in 1X PBS, then fixed in 4% PFA for a minimum of 4 hours. The chambers were then incubated with increasing glycerol concentrations of 20%, 50%, and 70% glycerol, respectively. Glycerol concentration was increased when the tissue samples equilibrated, and sank to the bottom of the chamber. The preserved tissues were refrigerated and kept away from light until imaging.

Images were obtained with a confocal microscope (ZEISS LSM-710). Fluorescent images were obtained at 100X and 200X magnification. The Z-stacking method was used to obtain a 3D image from multiple stacked images of the tissue, i.e. one image per every 3 μ M of 150 μ M total tissue thickness; ~50 images per tissue section. After imaging, raw images were processed to reduce background noise, and the number of pCREB fluorescent cells were manually counted for each tissue section. It was predicted that pCREB would be found in the suckers, and that the number of pCREB-positive cells would be greater for trained cuttlefish than naive controls.

Data Analysis

JMP Pro 13.1.0 (SAS, Inc.) was used to analyze data from all three experiments. Data generated by DanioVision automated tracking were exported from EthoVision XT software as Microsoft Excel files for analysis. Nonparametric tests were used when data did not meet the assumptions for parametric tests. Significance was tested at $\alpha = 0.05$ for all analyses.

CHAPTER 3

BEHAVIORAL ANALYSIS

While cephalopods have been used for behavioral experiments, research documents only a handful of species. The learning abilities of the dwarf cuttlefish, *Sepia bandensis*, have not been well studied. There were two goals for the behavioral analysis. The first goal was to determine whether short and long term memory functioned in 4-week old dwarf cuttlefish. It was hypothesized that cuttlefish would exhibit behavioral evidence of both short and long term memory, as has been shown for other species. To test this hypothesis, cuttlefish underwent a memory experiment, in which they were presented with an inaccessible prey item enclosed in a plastic tube. It was predicted that cuttlefish would show short term memory by reducing tentacle strikes within trials. To assess short term memory, training sessions were divided into two continuous, 5 minute intervals and the number of strikes per interval were counted. To assess long term memory, cuttlefish underwent a single retention test 4 days after the initial training sessions. It was predicted that cuttlefish would maintain a reduction in tentacle strikes during the retention test, and would strike less compared to untrained controls.

The second goal of the behavioral analysis was to determine whether the memory experiment affected other parts of cuttlefish predatory behavior, beyond tentacle striking. Cuttlefish predatory behavior is categorized into 3 observable phases: Attention towards the prey, positioning near the prey, and finally the tentacle strike (Shinzato et al 2018). While learning has been assayed by the reduction in strikes over time, it is not well known how the memory experiment affects the other two phases of the predatory response. This is because attention and positioning are difficult to quantify by manual observation. It was hypothesized that attention and positioning towards prey would also be inhibited by the memory procedure. This hypothesis was tested by using the DanioVision automated tracking system to measure the amount of attention and positioning by each cuttlefish. Using EthoVision XT software, each arena was divided into two equal zones, one zone containing the prey tube, the other zone empty. Positioning was measured by a tracking point placed on the center of each cuttlefish. Attention was

measured by placing an additional tracking point on the front of each cuttlefish, to track the orientation of the eyes relative to the zone containing the prey tube. It was predicted that total positioning would be reduced across trials, and attention towards the zone containing the prey tube would also be reduced across trials.

Nonparametric analyses were used on the following data, which failed to meet the assumptions for parametric tests (normally distributed data and equal variances). The Kruskal-Wallis test is the nonparametric equivalent of an ANOVA for two or more groups (test statistic = χ^2). The sign test is the nonparametric equivalent of a paired t-test (test statistic = M).

Cuttlefish Show Behavioral Evidence for Short and Long Term Memory

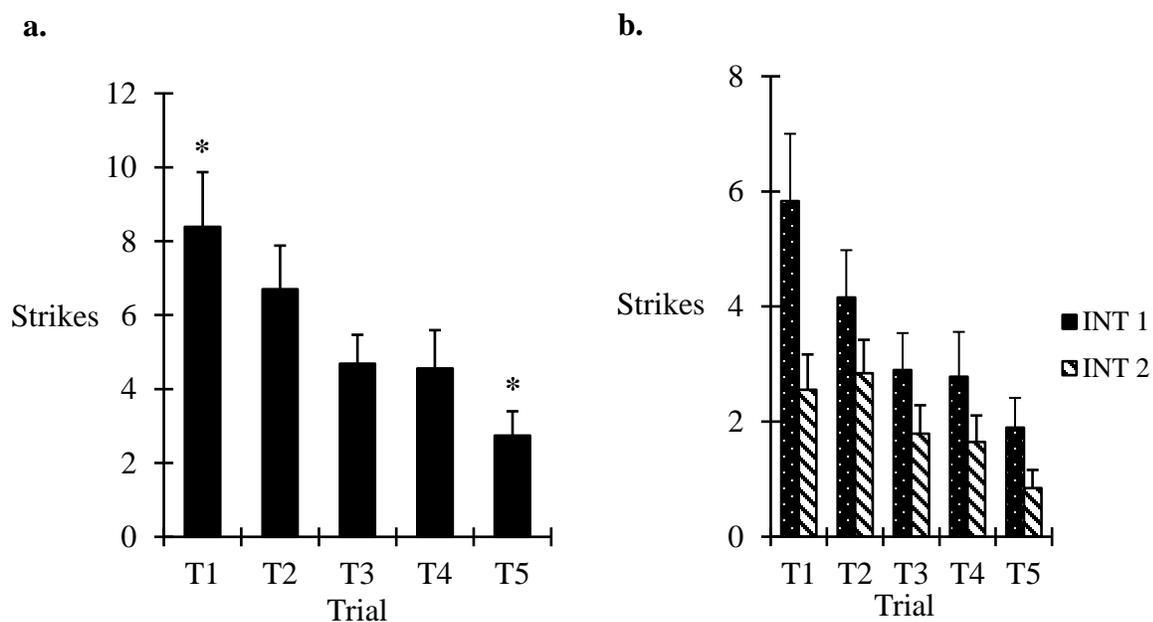


Figure 12. Comparison of strikes made for each training session. (a) Total strikes made during each training session. (b) Strikes made during Interval 1 and Interval 2 for each training session. (INT 1 = Interval 1; INT 2 = Interval 2). Data presented as mean + SEM (n = 19).

More strikes occurred during Interval 1 than Interval 2 (Figure 12b, Sign test; $M = -12.5$, $p = 0.0020$). Total strikes made during training (INT 1 and INT 2 combined) also differed across sessions (Figure 12a, Kruskal-Wallis test; $\chi^2 = 11.7$, $p = 0.0194$). Total strikes for T5 decreased when compared to T1 (Figure 12a, Steel-Dwass multiple comparisons; $Z = 2.7$, $p = 0.0282$).

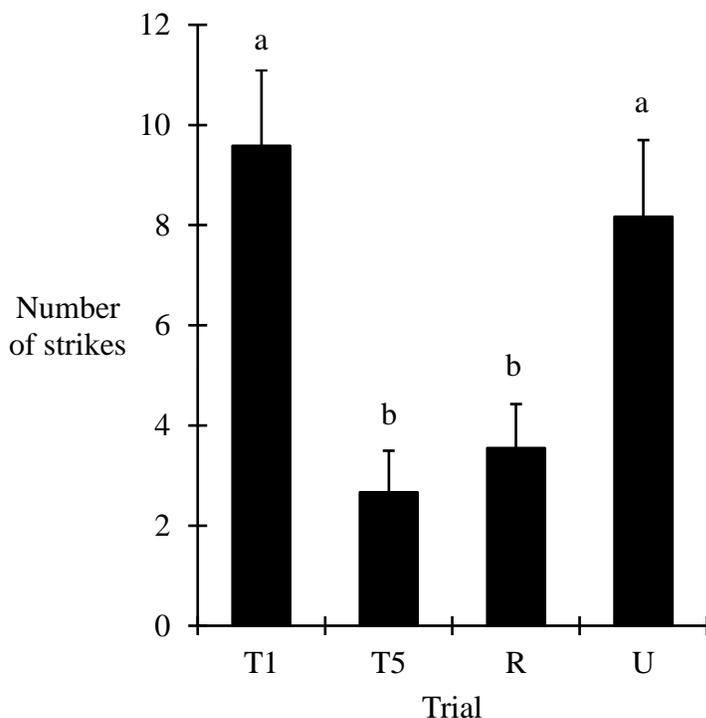


Figure 13. Comparison of total strikes for T1, T5, retention (R; $n = 12$) and untrained cuttlefish (U; $n = 12$). Letters represent significant differences between trials. Data presented as mean + SEM.

Four days after training, a single retention test was used to evaluate long term memory. Untrained controls also underwent the retention test. Total strikes differed among sessions (Figure 13, Kruskal-Wallis test; $\chi^2 = 12.982$, $p = 0.0020$). Retention differed from Trial 1 (Figure 13, Steel-Dwass multiple comparisons; $Z = 2.8$, $p = 0.0233$), but not from Trial 5 ($Z = -0.75$, $p = 0.8774$). Total strikes for untrained cuttlefish differed from Trial 5 ($Z = 2.348$, $p = 0.0398$), but not from Trial 1 ($Z = -0.492$, $p = 0.9608$).

Training Inhibits Striking, but not Positioning or Prey Interest

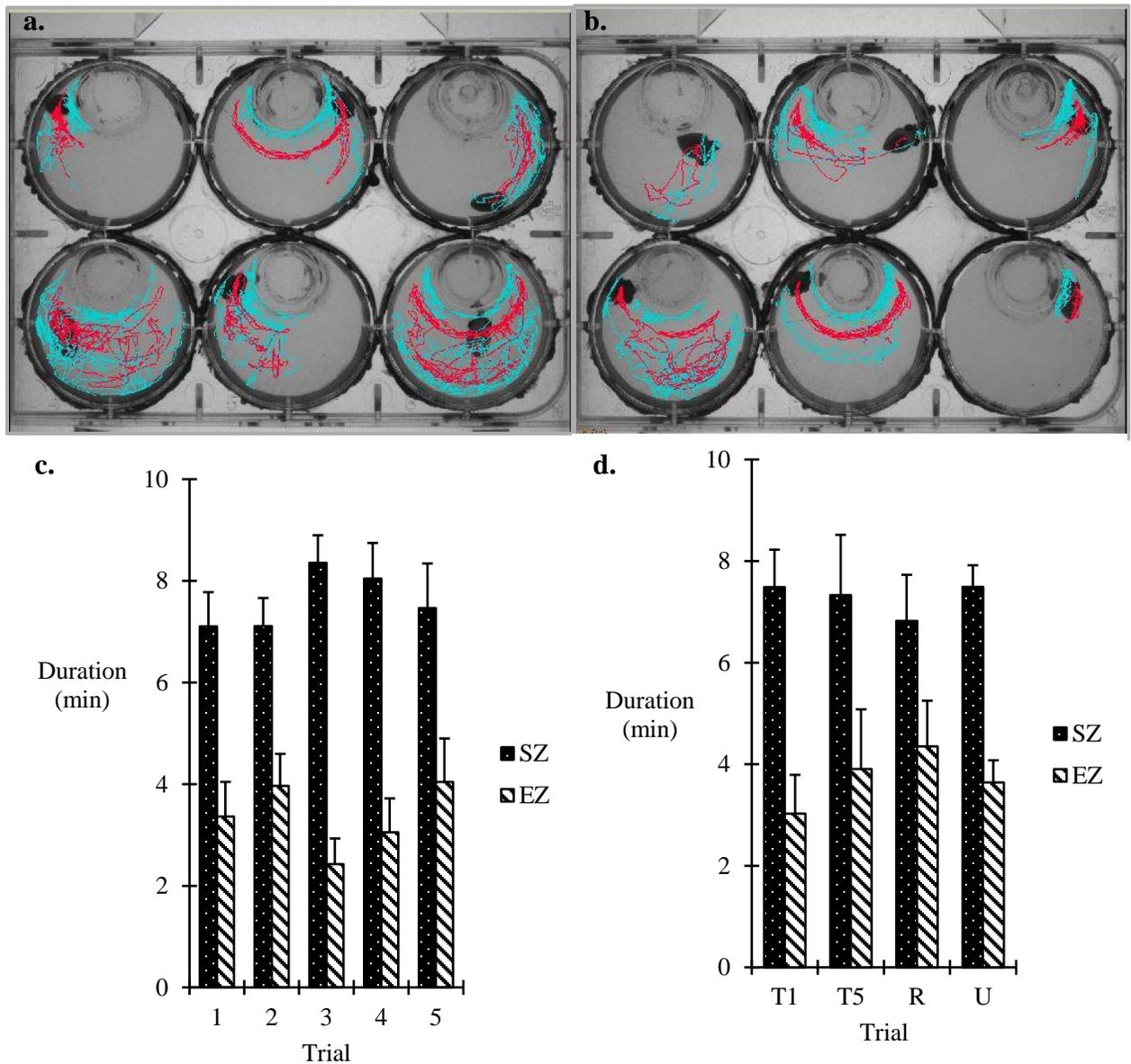


Figure 14. Orientation of the eyes during training and retention.

Figure 14 shows orientation data generated from EthoVision XT tracking. (a) Cuttlefish tracking for Trial 1 of training. (b) Tracking for the same individuals during Trial 5. The lines represent movement paths and anterior-posterior orientation of each cuttlefish during training. The blue line was generated

from the head tracking point placed anteriorly on the arm crown, and was used to measure the orientation of the eyes relative to the shrimp tube. (c) Total time cuttlefish spent facing the shrimp zone and the empty zone during training (SZ = shrimp zone; EZ = empty zone). Data presented as mean duration + SEM (n= 19). (d) Total time cuttlefish spent facing the shrimp zone and empty zone during the retention test (R; n = 12). T1 and T5 responses for the same cuttlefish are also shown, as well as untrained controls (U; n = 12). Data presented as mean duration + SEM.

There was not a strong visual difference in the attention towards prey (blue line) during the beginning of training (Figure 14a) versus the end of training (Figure 14b). Cuttlefish spent more time facing the shrimp zone than the empty zone during training (Figure 14c, Sign test; $M = -24.5$, $p < 0.0001$). Duration facing the shrimp zone did not differ across training trials (Figure 14c, Kruskal-Wallis test; $\chi^2 = 6.434$, $p = 0.1691$), nor did duration facing the empty zone (Kruskal-Wallis test; $\chi^2 = 3.224$, $p = 0.5212$). Duration facing the shrimp zone did not differ across training, retention, or untrained controls (Figure 14d, Kruskal Wallis test; $\chi^2 = 2.7279$, $p = 0.4355$), nor did duration facing the empty zone (Figure 14d, Kruskal Wallis test; $\chi^2 = 1.6534$, $p = 0.6474$).

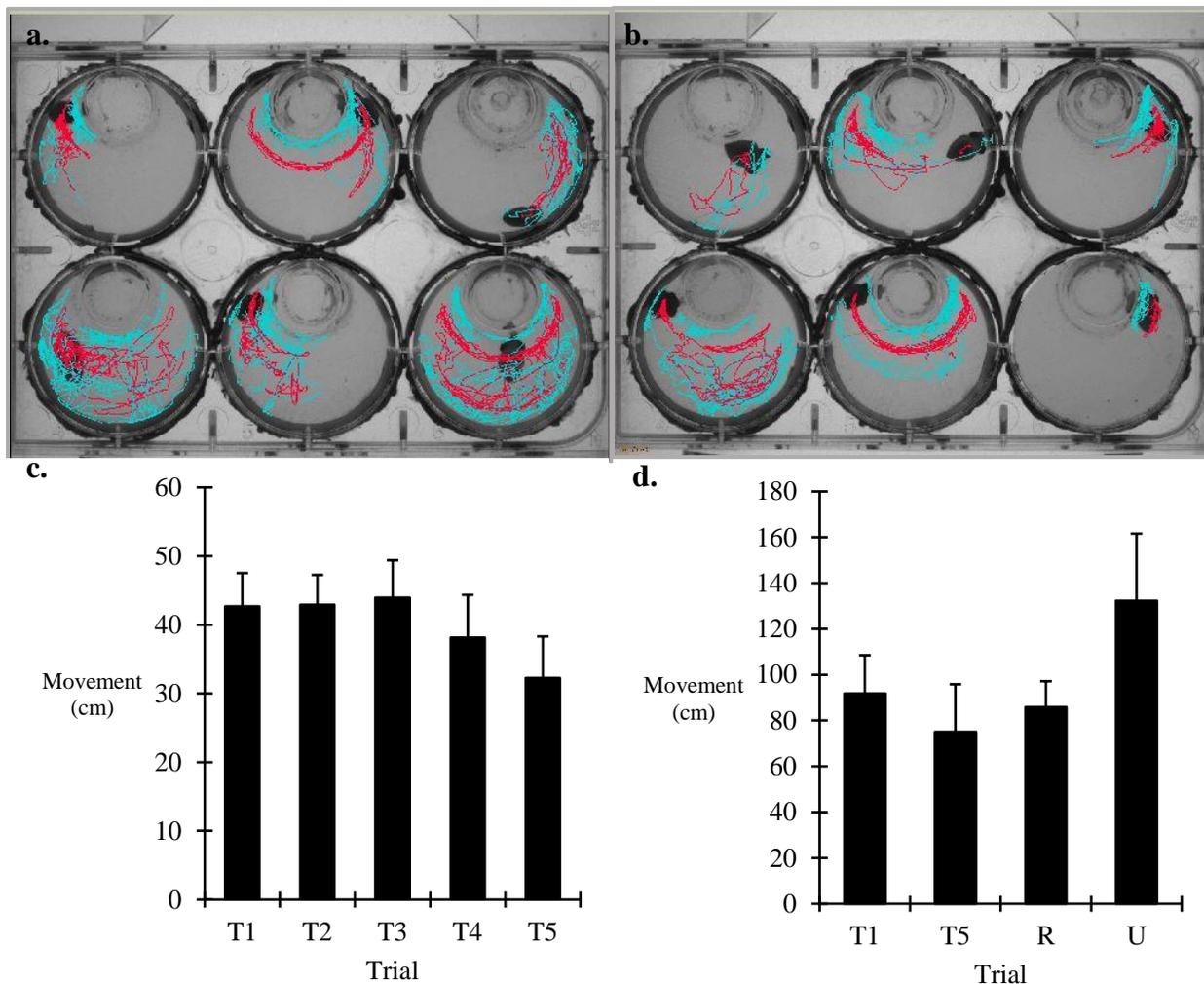


Figure 15. Positioning of the cuttlefish during training and retention.

Figure 15 shows positioning data of the cuttlefish generated by EthoVision XT subject tracking. (a) Cuttlefish tracking for Trial 1 of training. (b) Tracking for the same individuals during Trial 5. The lines represent movement paths and anterior-posterior orientation of each cuttlefish during training. The red line was generated from the tracking point placed on the center of each cuttlefish, and was used to measure total movement. (c) Total positioning by cuttlefish during training. Data presented as mean positioning + SEM ($n = 19$). (d) Total positioning by cuttlefish that underwent the retention test ($n = 12$). T1 and T5 responses for the same cuttlefish are also shown, as well as untrained controls (U; $n = 12$). Data presented as mean movement + SEM.

There was not a strong visual difference in the positioning towards prey (red line) during the beginning of training (Figure 15a) versus the end of training (Figure 15b). Total positioning did not change across training sessions (Figure 15c, Kruskal-Wallis test, $\chi^2 = 7.2$, $p = 0.1256$). Positioning also did not differ for the retention test, nor for untrained controls (Figure 15d, Kruskal-Wallis test; $\chi^2 = 7.67$, $p = 0.0534$). The p-value of 0.0534 could be an issue of sample size; at $n = 12$, power was 0.2. Power below 0.5 is weak for determining the level of effect with a given sample size (Whitlock and Schluter 2015). A larger sample size could be required to determine the differences between trained and untrained cuttlefish.

Discussion

The first goal of this research was to determine whether dwarf cuttlefish, *Sepia bandensis*, were suitable models for studying memory. It was asked whether juvenile dwarf cuttlefish exhibited behavioral evidence for short and long term memory. Cuttlefish were given a memory test which assayed learning as the reduction of tentacle strikes made against an inaccessible prey item enclosed in a plastic tube. It was found that STM functioned in 4 week old *S. bandensis*, as the number of strikes was significantly greater for Interval 1 than Interval 2 (Figure 12b, $p = 0.0020$). This is consistent with findings using *S. officinalis*, for which short term memory operates as early as 8 days old (Agin et al 1998; Dickel et al 1998). A significant reduction in strikes occurred between Trial 1 and Trial 5, which suggests that learning also occurred across trials (Figure 12a, $p = 0.0282$). Messenger (1971) showed that establishment of memory in cuttlefish is biphasic, with STM decaying after 20 minutes, and LTM appearing after 1 hour. The biphasic memory curve has also been described for the chambered nautilus, *Nautilus pompilius*, a shelled cephalopod (Crook and Basil 2008). The reduction in strikes between T1 and T5 of training may represent the activation of LTM stores in *S. bandensis*, as approximately 80 minutes elapsed between T1

and T5. The 20 minute resting interval may have been too long for STM to be maintained between each trial, which could explain why a significant reduction in strikes did not occur until T5.

S. officinalis does not exhibit 24 hour retention until about 30 days of age (Dickel et al 2001). Similarly, this experiment found that 4-week old *S. bandensis* retain memory of the “prawn in a tube” procedure for up to 4 days (Figure 13). In cuttlefish, improvement in retention is correlated with increased vertical lobe maturation, the center for memory in cephalopods (Dickel et al 2001). The performance of trained cuttlefish during the retention test differed from T1 of training ($p = 0.0233$), but was similar to T5 ($p = 0.8774$). This suggests that the cuttlefish did not revert to their original response rate, despite that 4 days elapsed between training and the retention test. Concomitant with this, the response of untrained cuttlefish was similar to T1 ($p = 0.9608$) but differed from T5 ($p = 0.0398$). The retention performance also suggests that the reduction in strikes during training is due to learning, and not fatigue acquired across multiple trials. This experiment established that juvenile *S. bandensis* are suitable behavioral models for studying memory.

Little is known about the behavioral feedback generated by the success or failure of prey capture attempts. Although the reduction in strikes can be used to assay learning, it does not take into account the other predatory behaviors of the cuttlefish. Next, this research asked how learning affected the other phases of the predatory response in dwarf cuttlefish. Cuttlefish predatory behavior is categorized into 3 phases: attention, positioning, and the strike (Wells 1958, Shinzato et al 2018). While all phases can be easily observed during the experiment, the amount of attention and positioning by the cuttlefish are both difficult to quantify manually. For this reason, it is not well known how learning the “prawn in a tube” task may affect predatory behaviors besides striking. Automated tracking systems such as DanioVision can be used to alleviate the difficulty of measuring the multiple variables that make up complex behaviors. Cuttlefish are an interesting candidate for automated tracking due to their learning abilities and dynamic behaviors. Despite this, little to no literature is available regarding attempts to quantify cuttlefish behavior with automated tracking methods. Since juvenile cuttlefish display many adult behaviors, it is possible that results from DanioVision can also be used to characterize adult behaviors. In this

experiment, DanioVision automated tracking was used to analyze how the attention and positioning phases changed across training.

The analysis of predatory behavior with DanioVision showed that neither positioning nor the time spent watching prey changed as a result of learning. This suggests that the “prawn in a tube” procedure selectively inhibits the striking phase, and not attention and positioning phases. Trained cuttlefish maintained similar total positioning across trials, despite that they struck less with tentacles (Figure 15c, $p = 0.1256$). Positioning also did not differ across trials for untrained controls (Figure 15d, $p = 0.0534$). During training, cuttlefish also spent the same amount of time facing prey across sessions, suggesting they did not lose interest in the prey (Figure 14c, $p = 0.1691$). The duration spent facing prey during training also did not differ when compared to the retention test and untrained controls (Figure 14d, $p = 0.4355$). Cuttlefish were fed immediately after training and retention, and they readily captured and ate freely swimming mysids. Similar observations were made in Dickel et al (1998, 2001). This is consistent with the finding that interest in prey is not inhibited by the procedure. However, it is not known how cuttlefish respond to freely swimming prey when cues are associated with attention and positioning, rather than striking. Inhibition of predatory response phases (attention, positioning, striking) may only occur when a sensory cue is associated with the specific response phase.

Once an assay for learning is established, the cues governing learning can be considered. Several authors have questioned why cuttlefish stop striking, and have offered potential cues that may be responsible for reinforcement. Messenger (1971) posited that learning occurred via strike contingent pain from tentacles hitting the tube. This could explain why only the striking phase becomes inhibited, as pain is only experienced when the strike occurs, and not while the cuttlefish faces or moves towards the prey. While cues may be obtained by the tentacles, it is unknown whether the tentacles are nociceptive. Furthermore, it has been reported that cuttlefish can still learn the task when their tentacles are cut, even though it takes longer (Cartron et al 2013). Given this, cues obtained independent of the tentacles could also contribute to learning. Cartron et al (2013) suggested that cuttlefish also use visual cues obtained from the prey tube to learn the prey is inaccessible. They hypothesized that the tube is not invisible to

cuttlefish due to their ability to see polarized light. Cuttlefish use polarized vision to increase contrast between object borders and the background, aiding target detection (Pignatelli et al 2011; Temple et al 2012; Shashar et al 2000). Moreover, polarized vision aids the detection of transparent prey, which has been shown in squid (Shashar et al 2000) and has been suggested for pelagic fish (Johnsen et al 2011). Carton et al (2013) found that, by covering the transparent tube with a depolarizing filter, cuttlefish took longer to learn the task. Cues obtained from the prey tube could be important factors in the rate of learning.

While Messenger considered the cues obtained by the tentacles, no studies have considered the role of the arms, which play a large role in tactile learning (Wells and Wells 1956). During the behavioral experiments, it was observed that cuttlefish often contacted the prey tube with their arms, either after striking or while positioning themselves near the tube. Although behavioral evidence for learning has been shown, few studies have investigated the molecular mechanisms underlying the learned behavior. The next chapter discusses whether CREB, a memory-related transcription factor, is activated in the arms during training. The behavioral assay discussed in this chapter was used to investigate the role of arms in the learning process. It was hypothesized that tactile cues obtained from the tube during training could activate CREB signaling in the arm sensory neurons.

CHAPTER 4

MOLECULAR ANALYSIS

The goal of this experiment was to determine whether the memory-related transcription factor, CREB, was activated in the nervous system as a result of behavioral training in dwarf cuttlefish. Specifically, this experiment examined whether CREB was activated in the arms of cuttlefish. While cuttlefish strike the tube with their feeding tentacles, it is possible that the arms also obtain important sensory cues from the tube, which may contribute to inhibition of predatory attacks. During behavioral experiments, cuttlefish would often contact the prey tube for long periods with their suckers, which contain tactile receptors (Graziadei and Gagne 1976). It was hypothesized that tactile cues from the tube could activate CREB in the sensory neurons of cuttlefish suckers. To test this hypothesis, cuttlefish underwent 5 training sessions using the memory experiment described in Chapter 3, and arm tissues were stained using pCREB and tubulin antibodies. One hour after training, the cuttlefish were euthanized and tissues were fixed in paraformaldehyde. Each arm was cut dorsoventrally using a vibratome blade to obtain sections for tissue staining. Each longitudinal section contained the dorsal epidermis on the aboral side of the arm, the intrinsic muscles of the arm, and ventral suckers on the oral side of the arm. Sections were then stained with antibodies and imaged using confocal microscopy. It was predicted that CREB would be found in the suckers of cuttlefish, and that trained cuttlefish would have a greater CREB-positive cell count than untrained controls. Lastly, this experiment characterized the spatial activation of CREB in the arms, by assessing the difference in the number of CREB-positive cells between the distal tip and proximal base of the arm.

Nonparametric analyses were used on the following data, which failed to meet the assumptions for parametric tests (normally distributed data and equal variances). The Kruskal-Wallis test is the nonparametric equivalent of an ANOVA for two or more groups (test statistic = χ^2). The sign test is the nonparametric equivalent of a paired t-test (test statistic = M).

CREB is Activated in the Suckers and Ventral Epithelium of Cuttlefish Arms

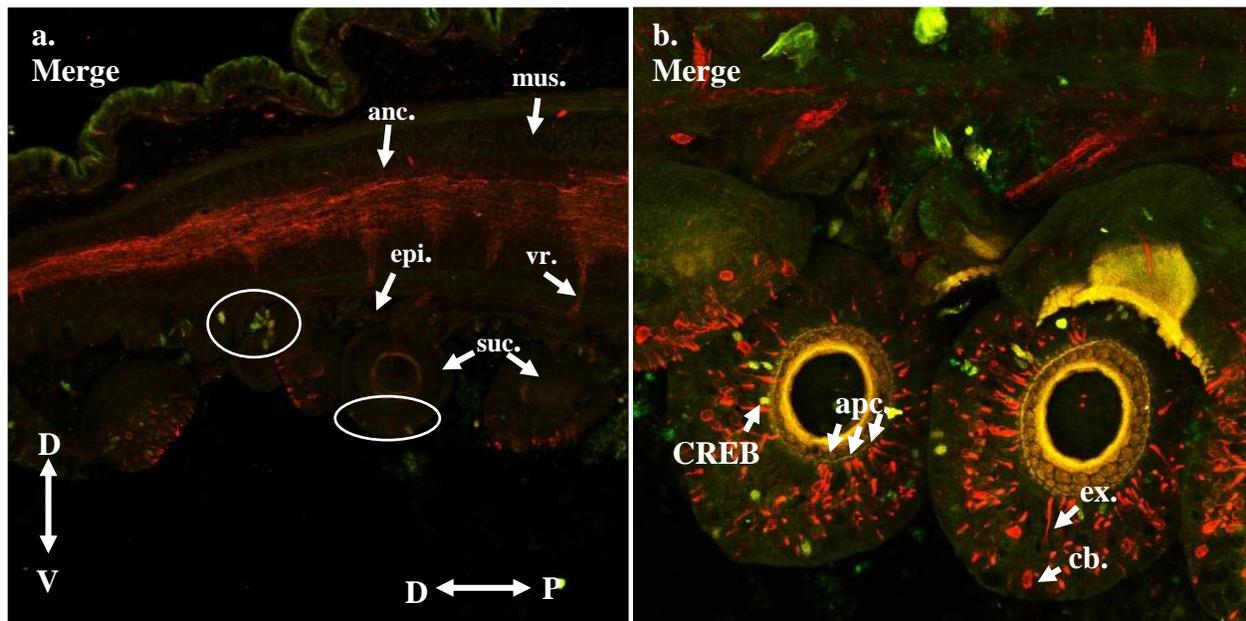


Figure 16. Tubulin and CREB staining in a section of a cuttlefish arm.

Figure 16 shows tubulin (red) and CREB (green) staining in a cuttlefish arm. (a) 100X image of a 150 μ m longitudinal section of a cuttlefish arm stained with tubulin and CREB fluorescent antibodies. The axial cord (anc.) is shown running through the center of the arm, between muscle layers (mus.) Ventral roots (vr.) projecting from the axial nerve cord are also shown innervating the suckers (suc.) White circles indicate CREB-positive cells in suckers, and in ventral epithelium (epi) of the arms, which forms folds between suckers. Double arrows indicate dorsal-ventral and distal-proximal orientation. (b) 200X magnification of CREB and tubulin staining in cuttlefish suckers. Cell bodies (cb.) and extensions (ex) of neurons were stained in the suckers. Neuronal extensions were seen projecting towards the sucker epithelium. This is similar to descriptions of epithelial sensory neurons in octopus suckers given by Graziadei and Gagne (1976). Populations of these neurons resemble apical clusters (apc.) of Type 2 receptors, which contain spherical cell bodies and send bundles of ciliated projections to the surface of the epithelium (Graziadei and Gagne 1976). Cells stained with anti-pCREB are also visible in the suckers.

Tubulin antibodies were used to visualize the neuroanatomy of cuttlefish arms. A schematic of the arm neuroanatomy is given in Figure 16. Features of the arm described by Graziadei (1965, 1976) are included in the schematic. The axial nerve cord (anc.) is the central nerve in each arm, derived from brachial nerves originating in the brain (Boycott 1961). The axial nerve cord integrates the arm, innervating the intrinsic muscle layers (mus.) with intramuscular nerve cords. Ventral roots (vr.) project from the axial nerve cord to innervate each individual sucker (suc.). The suckers contain a dense array of sensory receptors near the sucker epithelium. These sensory receptors are neurons that send ciliated projections directly to the surface of the epithelium. The sensory cells occur in 4 morphological types, and obtain chemical and tactile cues from the environment. The sensory cells stained in Figure 16b generally had spherical cell bodies (cb.), and sent extensions (ex.) towards the surface of the sucker epithelium. These extensions may represent primary cilia, the part of the of sensory neuron which receives and processes sensory signals at the surface of the epithelium (Graziadei and Gagne 1976). The morphology and arrangement of the cells resembles Type 2 receptors described by Graziadei and Gagne (1976). Type 2 receptors are closely arranged into structures called apical clusters (apc.), which are clusters of cell bodies that send bundles of ciliated projections to the epithelial surface. Cells stained with CREB were visible amongst sensory cells in the suckers (Figure 16b). The rows of suckers are surrounded by an epithelium (epi) lining the ventral boundary of the arm muscle (Figure 16a). The properties of this epithelium have not been well described. The epithelium lines the dorsal edge of the suckers, and forms folds irregular folds between suckers (Figure 16a). Clusters of CREB-positive cells were found in the epithelium (Figure 16a). Tubulin staining revealed neurons in the epithelium, which morphologically resembled neurons found in the suckers (Figure 16b). The neurons had spherical cell bodies and sent projections to the outer edge of the epithelium, suggesting that the epithelium may have sensory properties similar to suckers.

Training Activates CREB in Cuttlefish Arms

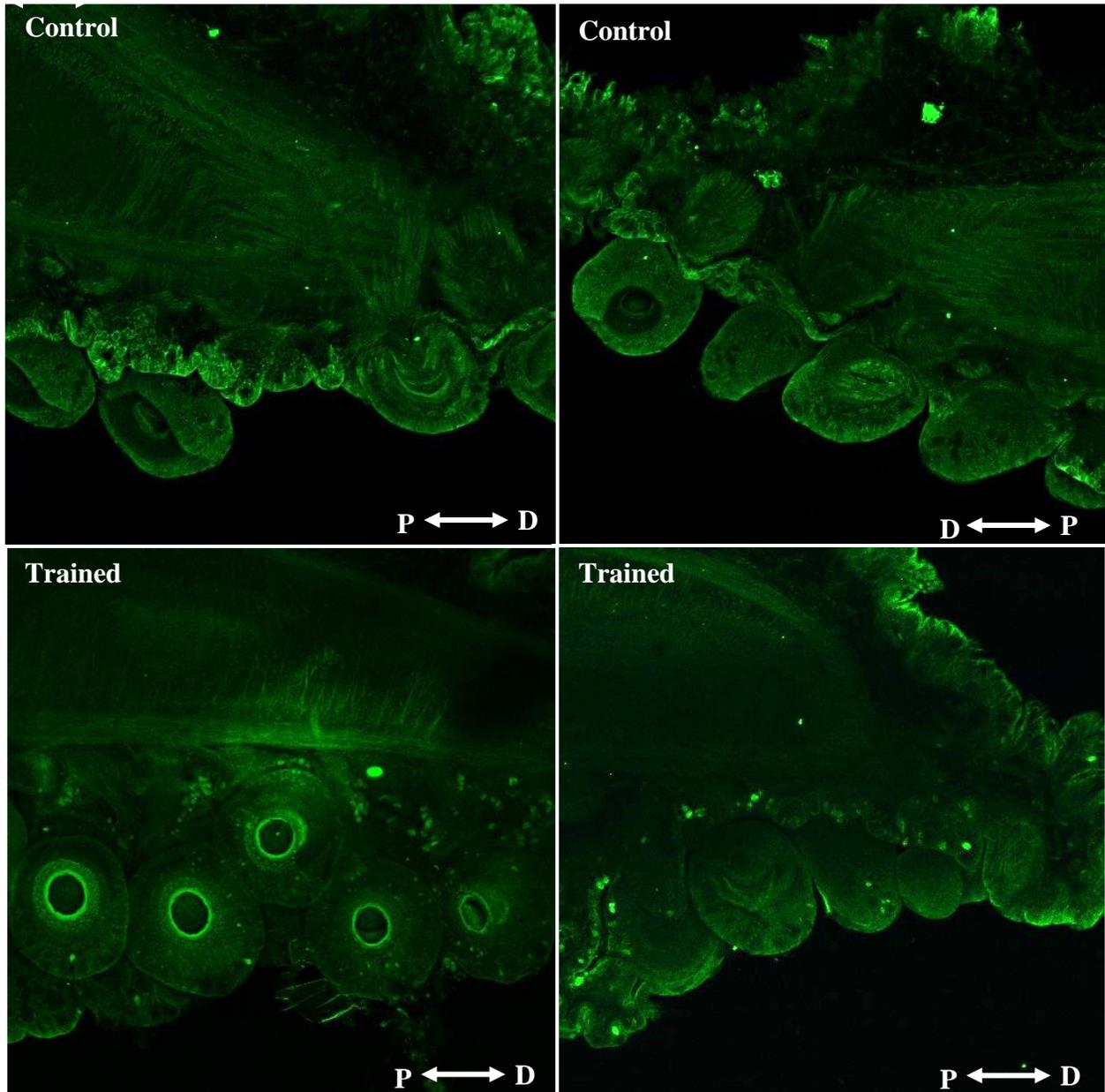


Figure 17. Comparison of CREB positivity for trained and control arms. Trained arms had an abundance of CREB-positive cells in the suckers and epithelia. A reduction of CREB-positive cells was visible in controls. Images taken at 100X magnification.

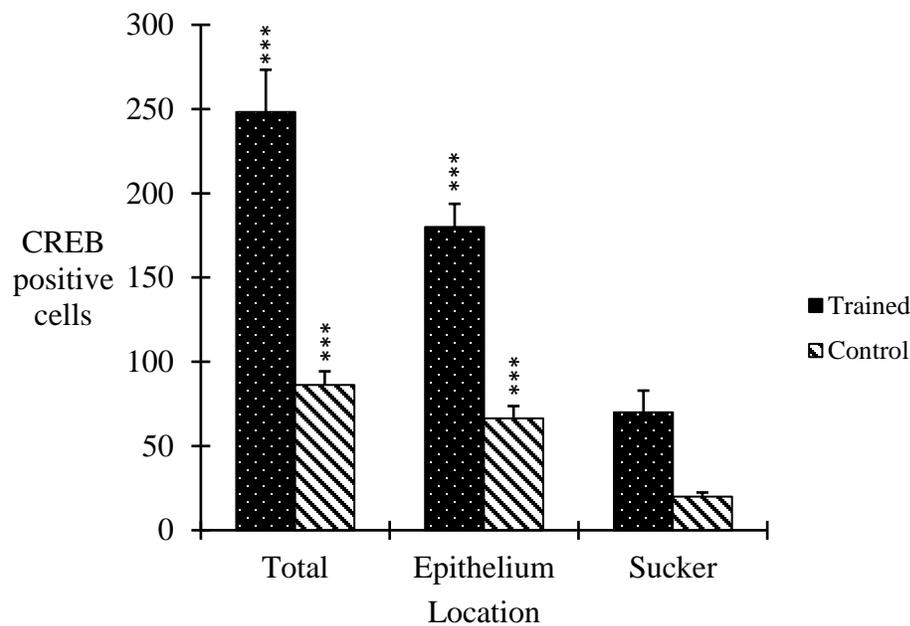


Figure 18. CREB-positive cell counts in trained and control cuttlefish arms. Asterisks represent significant differences between trained and control counts. Data presented as mean counts per individual + SEM (n = 8).

CREB-positive cells were counted for the first arm pair of each cuttlefish. Trained cuttlefish had a greater total CREB-positive cell count than controls (Figure 18, Kruskal-Wallis test; $\chi^2 = 11.18$, $p = 0.0008$). Trained cuttlefish also had a greater CREB-positive cell count in the epithelium (Figure 18, Kruskal-Wallis test; $\chi^2 = 13.19$, $p = 0.0003$). CREB-positive cell counts in the suckers did not differ between groups (Figure 18, Kruskal-Wallis test; $\chi^2 = 0.821$, $p = 0.3686$).

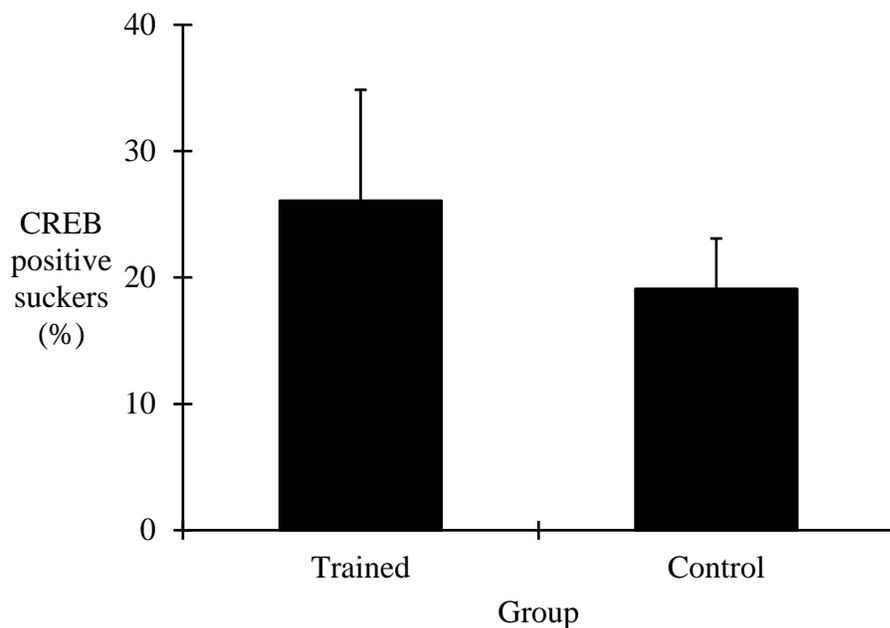


Figure 19. Proportion of CREB-positive suckers in trained and control cuttlefish arms. Data presented as mean proportion of positive suckers per individual + SEM (n = 8).

A total of 195 suckers were examined for trained cuttlefish, and 284 suckers were examined for controls. There was an average of 49 suckers present per trained individual (n = 4), and 71 total suckers present per control individual (n = 4). An average of 26% of suckers examined were CREB-positive per trained cuttlefish, and an average of 12% of suckers were positive per control cuttlefish. The proportion of CREB-positive suckers did not differ between trained and controls (Figure 19, Kruskal-Wallis test; $\chi^2 = 2.134$, $p = 0.6440$).

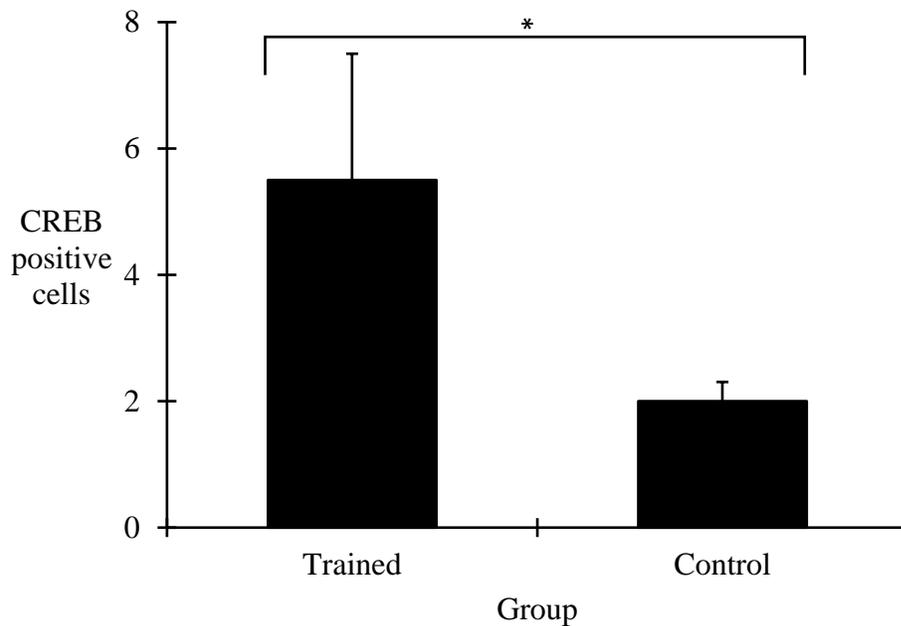


Figure 20. Number of CREB-positive cells found in positive suckers for trained and control cuttlefish. Asterisks represent significant differences between trained and control. Data presented as mean positive cells per sucker + SEM (n = 8).

Trained cuttlefish had a total of 51 CREB-positive suckers, with an average of 5.5 cells per positive sucker. Control cuttlefish has a total of 37 CREB-positive suckers, with an average of 2 cells per positive sucker. Trained cuttlefish had a greater average cell count in positive suckers than control cuttlefish (Figure 20, Kruskal-Wallis test; $\chi^2 = 5.4$, $p = 0.0202$).

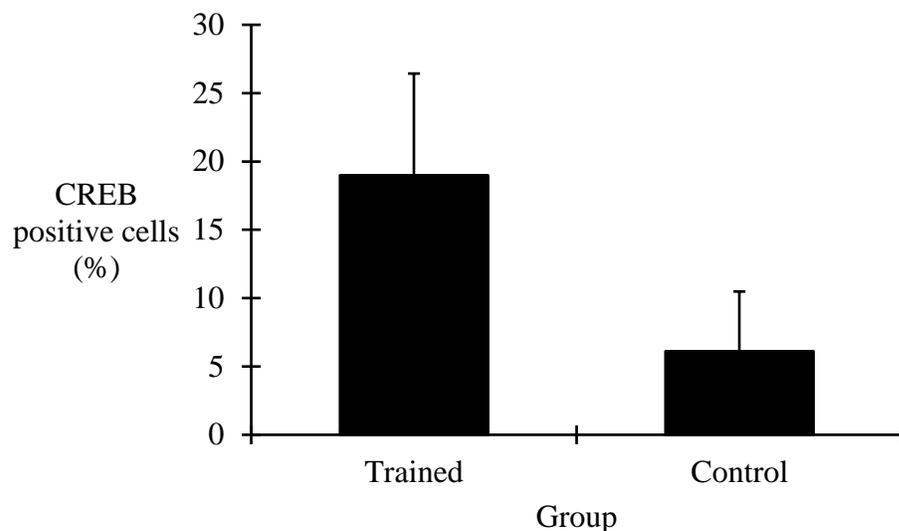


Figure 21. Percentage of grand total of CREB positive cells found in trained and control cuttlefish. Data is shown as mean percentage contributed to the grand total for each control and trained cuttlefish + SEM (n = 8).

In total, 1433 CREB-positive cells were found. 1088 of 1433 CREB-positive cells were found in trained cuttlefish, comprising 76% of positive cells overall, while control cuttlefish contributed 24% of positive cells (345 of 1433). Trained cuttlefish contributed an average of 19% each to the grand total, while control cuttlefish contributed an average of 6% each. (Figure 21). The average percentage of CREB-positive cells increased about 3-fold as a result of training.

Differential Activation of CREB Along the Longitudinal Axis of the Arm

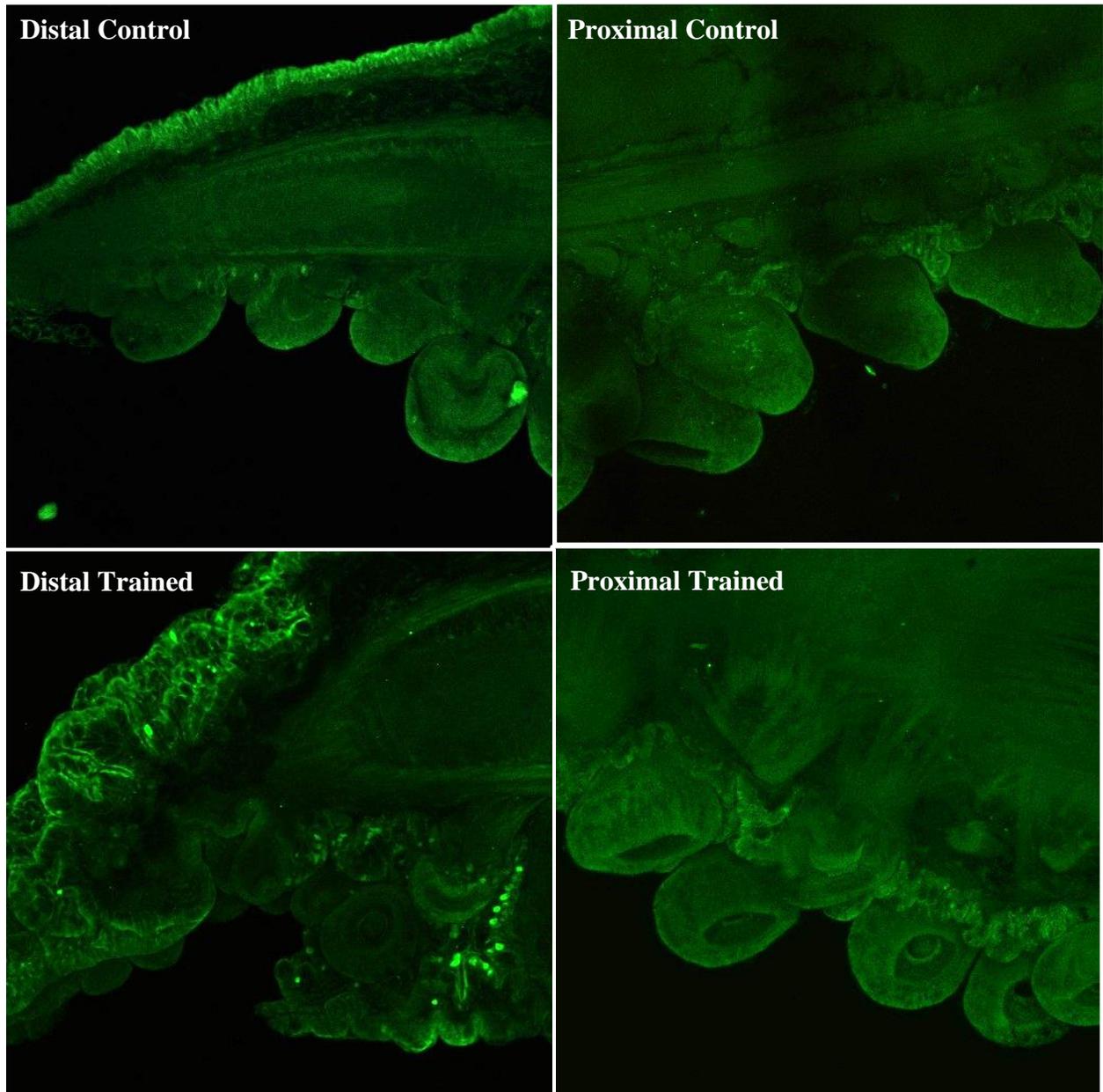


Figure 22. Comparison of CREB-positive cells in the distal and proximal portions of the first arm pair. Overall, CREB was found in greater abundance towards the tip of the arm (distal), and positivity was reduced towards the base of the arm (proximal).

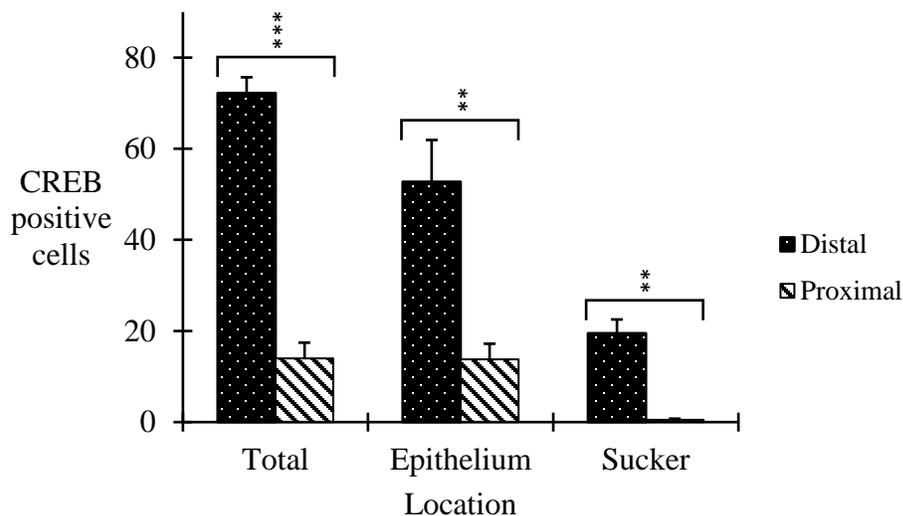


Figure 23. CREB-positive cell counts in the distal and proximal portions of the first arm pair for control cuttlefish. Asterisks represent differences between distal and proximal portions. Data presented as mean counts per individual + SEM (n = 4).

To assess differences in CREB positivity between distal and proximal portions of the arm, each tissue section was divided in half along its longitudinal axis and the number of CREB-positive cells in each half were counted. The distal portion of the arm was the half the furthest from the head of the cuttlefish and included the tip of the arm. The proximal portion of the arm was the half closest to the head of the cuttlefish. For control cuttlefish, the distal portion contained an average of 72 total positive cells per individual, while the proximal portion contained only an average of 14 positive cells (Figure 23). The distal portion of the arm contained more total CREB-positive cells than the proximal portion (Figure 23, Sign test; $M = -8.0$, $p < 0.0001$). The distal portion of the arm also contained more positive cells in the epithelium (Figure 23, Sign test; $M = -6.5$, $p = 0.0010$), and in the suckers (Figure 23, Sign test; $M = -5.5$, $p = 0.0010$).

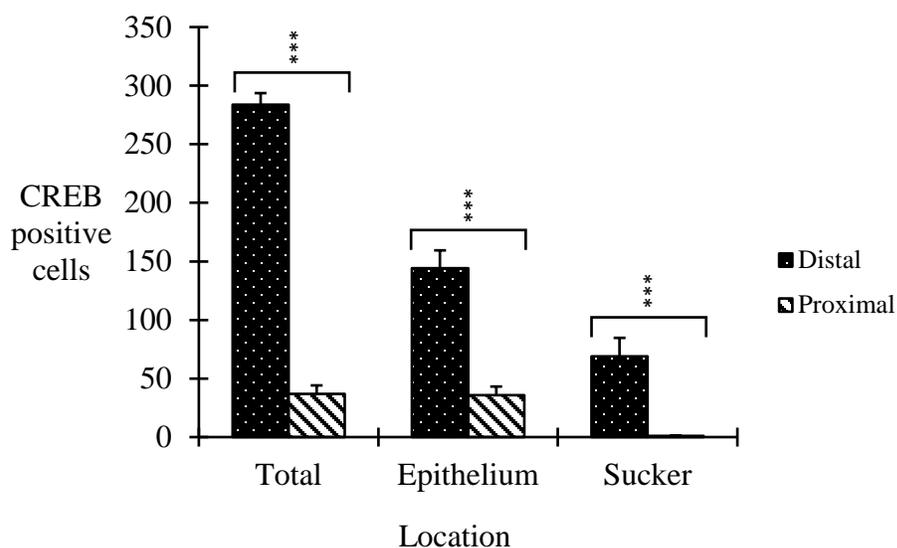


Figure 24. CREB-positive cell counts in the distal and proximal portions of the first arm pair for trained cuttlefish. Asterisks represent significant differences between distal and proximal portions. Data presented as mean counts per individual + SEM (n = 4).

For trained cuttlefish, the distal portion contained an average of 284 total positive cells per individual, while the proximal portion contained only an average of 37 positive cells (Figure 24). The distal portion of the arm contained more total CREB-positive cells than the proximal portion (Figure 24, Sign test; $M = -11.0$, $p < 0.0001$). The distal portion of the arm also contained more positive cells in the epithelium (Figure 24, Sign test; $M = -10.0$, $p < 0.0001$), and in the suckers (Figure 24, Sign test; $M = 7.5$, $p < 0.0001$).

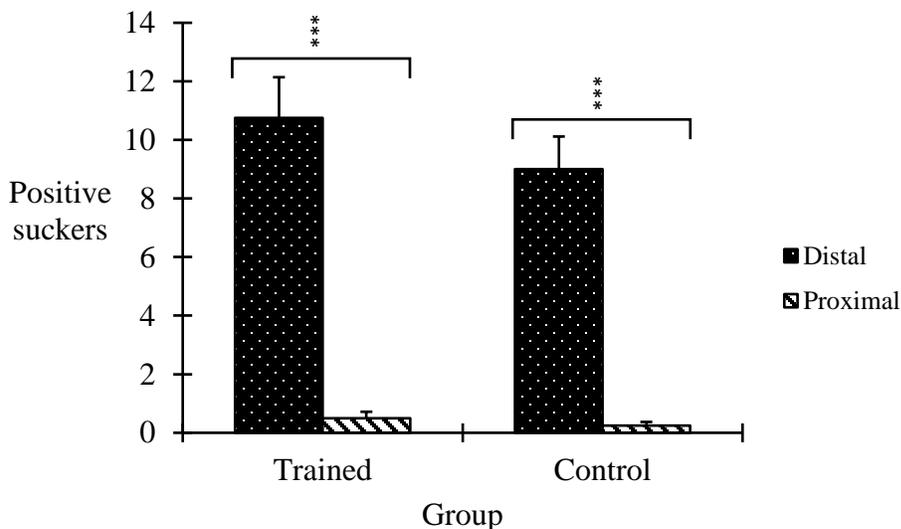


Figure 25. Number of CREB-positive suckers in the distal and proximal portions of the first arm pair for trained and control cuttlefish. Data shown as mean positive suckers per cuttlefish + SEM (n = 8).

The distal portion of the arm contained 96% of positive suckers for trained cuttlefish, and 97% of positive suckers for controls. The distal portion had an average of about 11 positive suckers per trained individual, and 9 positive suckers per control individual (Figure 25). The proximal portion of the arm contained about 4% of positive suckers for trained cuttlefish, and 3% for controls. The distal portion of the arm contained significantly more CREB-positive suckers than the proximal portion for both trained cuttlefish (Figure 25, Sign test; $M = 6.5$, $p = 0.0002$) and control cuttlefish (Figure 25, Sign test; $M = 6.0$, $p = 0.0005$).

Discussion

The second goal of this research was to develop a molecular assay to identify cellular changes related to learning and memory in dwarf cuttlefish. Specifically, it was asked whether CREB was activated in the arms of the cuttlefish during training. In the first chapter, the relevance of CREB as a molecular marker for memory was discussed for both vertebrate and invertebrate models. Prior to the

work in this thesis, CREB had never been identified in any cephalopod. The key findings of this experiment were that (1) CREB is present in the suckers and epithelia of cuttlefish arms (Figure 16), (2) Training increased the number of CREB-positive cells in the arms ($p = 0.0008$, Figure 18), and (3) the distal portion of the arm contained more CREB-positive cells than the proximal portion, regardless of training ($p < 0.0001$, Figure 23, Figure 24).

At the molecular level, memory is defined as an increase in communication strength within specific neural circuits (Kaiser and Peters 2009). Changes in communication strength between neurons is known as synaptic plasticity. Synaptic plasticity is induced in neural circuits that are repeatedly stimulated, such as when an animal repeatedly performs the same task during a learning experiment. Repeated stimulation activates transcription factors in the neural circuit, resulting in gene expression. The transcription factor, CREB, initiates memory-related gene expression and protein synthesis in neurons (Albensi 2012). The proteins lead to long-lasting structural changes in the neurons, increasing the communication strength within the circuit. CREB has been used as a marker for memory in both vertebrates and invertebrates. The best known invertebrate models for CREB are gastropod mollusks, such as *Aplysia*. Although these models are available, much remains to be known about the signaling mechanisms, downstream targets, and regulators of CREB. While cephalopod mollusks show behavioral evidence for memory, no studies have identified CREB in the cephalopod nervous system, or its potential role in memory. By showing evidence for CREB activation in cuttlefish, this research offers cephalopods as potential new models for studying the role of CREB in memory formation.

The behavioral experiments in this research established that dwarf cuttlefish exhibit both short and long term memory. When presented with inaccessible prey enclosed in a plastic tube, cuttlefish reduced their tentacle strikes against the tube within trials (short term), and maintained the reduction in strikes 4 days after training (long term). It was then asked whether CREB was activated in the nervous system of cuttlefish during behavioral training. Approximately 2/3 of total neurons in cuttlefish are found in the arms (Mather and Kuba 2006), so it is possible that the arms play a large role in various types of learning. Although strikes are made with the feeding tentacles, it is possible that sensory neurons within

the arms also obtain tactile cues from the tube, which may allow cuttlefish to learn the tube is an obstacle. Cuttlefish repeatedly contacted the prey tube with their arms, either after striking or while positioning themselves near the prey tube. Cues obtained by the arms could play a role in memory of the “prawn in a tube” procedure.

Figure 16b showed the neuroanatomy of the suckers. The suckers lining cephalopod arms contain many sensory neurons (Graziadei and Gagne 1976), which are used for tactile learning (Wells and Wells 1956) and taste discrimination learning (Wells 1963). Activation of CREB in sensory neurons has been shown in tactile experiments with *Aplysia*, a gastropod relative (Purves et al 2001). With this in mind, it was hypothesized that tactile cues obtained from the prey tube could activate CREB in the sensory neurons of cuttlefish suckers. To test this hypothesis, cuttlefish arms were stained with pCREB antibodies 1 hour after behavioral training. pCREB (phospho-CREB) is the activated version of CREB which binds to DNA in the nucleus of neurons. Tubulin antibodies were also used to co-locate CREB within the neural organization of the arms. It was predicted that CREB would be found in sensory neurons of the suckers, and that trained cuttlefish would have a greater number of CREB-positive cells than untrained controls. The first arm pair was selected for analysis because it is the most dorsal, and would likely have the greatest chance of contacting the prey tube as the cuttlefish faces the prey.

Neurons stained with anti-tubulin were found in the suckers (Figure 16b), and epithelial tissue of the arm. Neurons found in the suckers resemble Type 2 sensory receptors described by Graziadei and Gagne (1976), which contain spherical cell bodies, and project extensions directly to the surface of the sucker epithelium. The bundled arrangement of these sucker neurons could represent apical clusters, which may transmit chemical or mechanical information to the arms (Graziadei and Gagne 1976). Neurons were also found in the epithelium surrounding the suckers. These neurons also had spherical cell bodies, and projected extensions towards the edge of the epithelium. The neurons in the epithelium may also serve a sensory function, similar to the neurons of the suckers. CREB-positive cells were found in the suckers of cuttlefish arms, among the sensory neurons (Figure 16b). Unexpectedly, large clusters of

CREB-positive cells were also found in the epithelial folds between suckers, and the epithelium lining the dorsal edge of suckers (Figure 16a).

CREB is found in the pedal ganglia of gastropods, which controls the muscular foot (Efimova et al 2007). Cephalopod arms are thought to be derived from the molluscan foot (Shigeno et al 2008), so the presence of CREB in the arms may represent a shared trait with ancestral mollusks. This research was unable to identify the type of cell stained with CREB. While CREB-positive cells were found amongst sensory neurons in the suckers and epithelium, it was unclear whether any of the extensions or cell bodies were co-labeled with CREB. Although these CREB-positive cells may still serve some sensory function, other molecular markers may be required to identify the specific cell type.

Nonetheless, training had a significant effect on the number of CREB-positive cells found in the arms. Figure 17 showed a visible difference in CREB staining in trained vs. control cuttlefish. Trained cuttlefish had more total CREB-positive cells than controls (Figure 18, $p = 0.0008$). While more CREB was found in the suckers for trained cuttlefish, the number of CREB-positive cells in the suckers did not differ between trained and control cuttlefish (Figure 18, $p = 0.6440$), nor did the number of positive suckers (Figure 19, $p = 0.3686$). However, the average number of CREB-positive cells found in positive suckers was significantly greater for trained cuttlefish (Figure 20, $p = 0.0202$). 76% of total CREB positive cells were found in trained cuttlefish, and 24% in controls (Figure 21). Each trained cuttlefish contributed an average of 19% of cells to the total CREB count, while controls contributed an average of 6% each (Figure 21). Training resulted in about a 3-fold increase in the percent of CREB-positive cells (Figure 21).

This research only took the first arm pair into account. The first arm pair was chosen for analysis because it is the most dorsal pair, and could have the greatest chance to contact the tube when the cuttlefish is facing the prey. Some cuttlefish may have contacted the prey tube more often with other arms besides the first pair. Given this, it is possible that differences in the suckers could be uncovered if all of the arms are taken into account, rather than just the first arm pair.

Surprisingly, trained cuttlefish had significantly more CREB-positive cells in the epithelium of the arm than controls (Figure 18, $p = 0.0003$). This suggests that the epithelium could play a role in detecting the cues used to learn the “prawn in a tube” procedure. Although the characteristics of the suckers have been described in detail, there is little information regarding the properties of the epithelium surrounding the suckers, or its role in sensory transmission. This research found neurons in epithelium, which morphologically resembled sensory neurons in the suckers (Figure 16b). The neurons in the epithelium may play a sensory role, similar to that of the suckers. Other markers, such as antibodies for primary cilia, could be used in future experiments to distinguish the role of neurons in the epithelium of the arms. Ciliated projections are a key characteristic of epithelial sensory neurons, and cilia are found in the sensory neurons of the suckers (Graziadei and Gagne 1976).

Very few studies have investigated the cellular mechanisms driving memory in cuttlefish. After training cuttlefish with the “prawn in a tube” procedure, Agin et al (2003) injected cuttlefish with cycloheximide (CXM), a protein synthesis inhibitor. They found that injections given 1 to 4 hours after training inhibited 24 hour retention of the procedure, suggesting that *de novo* protein synthesis is required for LTM in cuttlefish. Given that CREB is a transcription factor responsible for protein-dependent LTM formation, the differences in CREB positivity between trained and control cuttlefish could still represent functional significance for CREB in memory. For this research, cuttlefish were fixed in paraformaldehyde 1 hour after training, which falls within the time-sensitive window of 1 to 4 hours for protein synthesis given by Agin et al (2003). A similar timeframe of 1 to 3 hours is given for memory-related CREB detection in chicks (Freeman and Rose 1999). Future studies that manipulate the time before tissue fixing could further delineate the timeframe for post-training CREB activation in cuttlefish.

In addition to identifying differences in CREB activation between trained and untrained cuttlefish, this research also attempted to characterize spatial activation of CREB in the arms. During cell counts, it was visibly apparent that more CREB-positive cells occurred towards the distal tip of the cuttlefish arm, and less in the proximal base, closest to the head of the cuttlefish (Figure 22). For control cuttlefish, the distal portion of the arm contained more CREB-positive cells in the epithelium (Figure 23 p

= 0.0010) and in the suckers (Figure 23, $p = 0.0010$). For trained cuttlefish, the distal portion of the arm also contained more CREB-positive cells in the epithelium (Figure 24, $p < 0.0001$), and in the suckers (Figure 23, $p < 0.0001$). Furthermore, nearly 100% of positive suckers were found distally (96% for trained and 97% for control, Figure 25). The distal portion of the arm contained significantly more CREB-positive suckers than the proximal portion for trained cuttlefish (Figure 25, $p = 0.0002$), and control cuttlefish (Figure 25, $p = 0.0005$). CREB signaling in cephalopod arms may occur predominately in the tip of the arm. An examination of other arm pairs would be needed to confirm whether distal activation of CREB is consistent for all the arms. If CREB is activated via sensory transmission in the arms, the distal half of the arm may be more salient in cue detection than the proximal half.

The remaining question is how CREB activated in the arms contributes to memory. It is possible that CREB activation in the arms could result in changes in behavior unrelated to the arms, such as tentacle strikes. This is owed to the large degree of integration between the peripheral nervous system of the arms, and the brain. Signals are transmitted between the arms and brain via large nerve tracts called the brachial nerves (Boycott 1961, Sakaue et al 2014). In the brain, the brachial nerves originate as brachial ganglia from the brachial lobe. Each of the 8 brachial ganglia become an axial nerve cord, which runs through the center of each arm (Figure 16). The axial nerve cord acts as the integrating center for each arm, innervating the muscle layers as sending nerve roots to each individual sucker (Figure 16a). Signals obtained from the environment by the suckers are transmitted back to the axial nerve cord, to the brachial nerves, and ultimately the brain (Boycott 1961). CREB-dependent protein synthesis in the arms may alter the signaling properties of neurons within the arms, modulating the transmission of signals back to the brain. The brachial lobe of the brain also controls activity of the tentacles (Boycott 1961), so it is possible that signals from the arms play a role in modulating activity of the tentacles and other behaviors.

CHAPTER 5

CONCLUSION

Cephalopods are intriguing animal models, because they exhibit dynamic behaviors and have the most complex nervous system of all invertebrates. Although cephalopods have been used for behavioral studies, research currently documents only a handful of species, and knowledge of cellular memory mechanisms is lacking. The establishment of new animal models to study memory is important, because much of what is understood about memory in humans is derived from animal studies. This research developed novel behavioral and molecular assays for memory in *Sepia bandensis*, a poorly studied species of cuttlefish. The dwarf cuttlefish was chosen as a memory model because it is a small cephalopod, making it easy to rear and test in large numbers. Dwarf cuttlefish also exhibit many adult behaviors at juvenile stages, so useful insights can be gained throughout the lifespan. Molecular assays for studying memory in cuttlefish could lead to new understanding of memory formation in broad animal groups.

This research found that juvenile dwarf cuttlefish exhibit behavioral evidence for memory both in the short term and the long term. Cuttlefish underwent a memory experiment called the “prawn in a tube” procedure, which assays memory as the reduction in tentacle strikes against inaccessible prey over time (Messenger 1971). It was hypothesized that dwarf cuttlefish would exhibit short and long term memory of the procedure, as has been shown by other cuttlefish species. Dwarf cuttlefish showed short term memory by reducing tentacle strikes within each trial. Dwarf cuttlefish also showed long term memory by maintaining the reduction in strikes for up to 4 days after the initial training sessions. These results indicated that dwarf cuttlefish were suitable models for experiments aimed at studying potential cellular changes related to memory.

Behavioral memory experiments were conducted in a DanioVision Observation Chamber, which is an automated animal tracking system. Attempts to use automated tracking for studying cephalopod behavior are lacking. Dwarf cuttlefish are great candidates for the DanioVision system, owed to their small size and learning abilities. While memory has been assayed via strike reduction, little is known

about how learning the “prawn in a tube” procedure affects the predatory response as a whole. The predatory response of cuttlefish consists of 3 phases: attention, positioning, and the strike (Shinzato et al 2018). Attention and positioning phases are difficult to quantify manually, stressing the need for systems optimized to track these behaviors in cuttlefish. It was hypothesized that cuttlefish would also lose visual interest in the prey, and inhibit positioning towards prey. Using DanioVision automated tracking, this research found that cuttlefish inhibit tentacle strikes without inhibiting attention or positioning towards the prey. Future work with DanioVision tracking could reveal more information about the behavioral changes displayed by cuttlefish when tested under different experimental conditions or when exposed to novel cues.

After establishing dwarf cuttlefish as behavioral memory models, the next goal of this research was to develop a molecular assay for memory. The molecular experiments targeted CREB, a transcription factor related to memory-dependent protein synthesis in neurons (Albensi 2012). CREB has been shown extensively in the nervous system of gastropods, close relatives to cephalopods (Casadio et al 1999, Efimova et al 2007, Ribeiro et al 2003, Sadamoto et al 2003). However, CREB has never been identified in the nervous system of any cephalopod. The molecular experiments aimed to determine whether CREB was activated in the cuttlefish nervous system during behavioral training, specifically, the nervous system of the arms. It was hypothesized that tactile cues obtained by the suckers in the arms could activate CREB in the sucker sensory neurons. Using immunohistochemical techniques, an anti-pCREB antibody was used to locate CREB in the first arm pair of trained cuttlefish. This experiment found that trained cuttlefish had a greater number of CREB-positive cells than untrained cuttlefish. Trained cuttlefish had a greater number of CREB-positive cells in the epithelium of the arms, but not as expected in the suckers. A difference in CREB activation may be seen if all of the arms are examined, and not just the first pair. Future work examining all 8 arms could further illuminate the effect of training on CREB activation in the suckers. The epithelium surrounding the suckers may also play a role in obtaining cues from training, but little information is available regarding the sensory characteristics of this tissue. Lastly, this research found that the distal portion of the arm contained the majority of CREB positive cells, while the proximal

portion contained very few positive cells. This suggests that CREB signaling in cuttlefish arms predominately occurs near the tip of the arm. Future work examining other arm pairs is needed to characterize the spatial activation of CREB in each arm.

This research demonstrated CREB activation for the first time in any cephalopod. However, it was unable to identify the type of cells containing phosphorylated CREB. While the molecular experiments showed a difference in CREB activation between trained and untrained cuttlefish, the CREB positive cells were not confirmed to be sensory cells. Molecular markers specifically targeting sensory epithelial cells could help determine if CREB activation depends on sensory transmission within the suckers and epithelium of the arms. Beyond this, much remains to be known about the activation of CREB in cephalopods, and its signaling mechanisms. CREB activation in gastropod mollusks is serotonin-dependent (Casadio et al 1999). Serotonin is found in the arms and suckers of cephalopods (Bellier et al 2017) making it a potential neurotransmitter required for CREB activation.

All trained cuttlefish were fixed 1 hour after training. Future experiments that manipulate the time of tissue fixing may elucidate the time window during which CREB is activated after behavioral training. The results of this research open up many potential avenues for further experimentation using the dwarf cuttlefish as a model for memory.

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