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SIMULTANEOUS DISCRIMINATION LEARNING AND ITS NEURAL CORRELATES IN THE CUTTLEFISH *SEPIA OFFICINALIS* (CEPHALOPODA:MOLLUSCA)

By

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ABSTRACT

SIMULTANEOUS DISCRIMINATION LEARNING AND ITS NEURAL CORRELATES IN THE CUTTLEFISH *SEPIA OFFICINALIS* (CEPHALOPODA:MOLLUSCA)

By

Miranda Alderson Karson

Highly controlled behavioral experiments, immunohistochemistry, and western blot analysis were used to examine visual and spatial discrimination learning in the common cuttlefish (*Sepia officinalis).* A simultaneous discrimination maze was designed and cuttlefish were trained against preference to exit a two-choice maze using either visual or spatial cues. Maze performance was then compared to that of a motor control group, which received visual and spatial cues, but were not reinforced for choosing between the cues. Cuttlefish in both the visual and spatial groups yielded improved exit performance relative to control subjects after repeated training in the maze, indicating that cuttlefish can learn to make simultaneous discriminations based on either type of cue. Both visual- and spatial-cue trained cuttlefish also yielded significantly improved performance compared to cuttlefish previously trained in a similar experiment, where both visual and spatial cues were relevant to selecting the appropriate exit. After maze training, cuttlefish were sacrificed and brains prepared for either immunohistochemistry or western blotting to examine calexcitin expression. Calexcitin is a protein that yields increased expression during associative learning in molluscs as well as some vertebrates. Cuttlefish central nervous systems immunolabelled for calexcitin yielded calexcitin distribution within

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learning related-brain areas including the optic lobes, vertical lobe, subvertical lobe, inferior frontal lobe, and superior frontal lobe as well as motor regions including the median and dorsal basal lobes. Cell counts, comparing intensely calexcitin stained cells in cuttlefish from the visual, spatial and control groups indicated greater levels of intracellular calexcitin within the optic lobes of visual-cue-trained cuttlefish than did spatial-cue trained or control cuttlefish. Control cuttlefish yielded greater levels of intracellular calexcitin in the subvertical lobe than in the visual and spatial cue trained cuttlefish. Western blotting was used to further quantify calexcitin expression in the supraoesophageal and optic lobes of all cuttlefish using homogenate, cytosolic and microsomal fractions. Similar to the immunohistochemical results, greater quantities of calexcitin were found in the optic lobes of visual trained cuttlefish than in spatial cue trained or control cuttlefish. Experiments also indicated differences in expression between the right and left optic lobes. However, these differences did not appear to relate to the initial preference of the cuttlefish nor to differences in size between the optic lobes. This may suggest lateralization of function between the optic lobes. Overall, it appears that calexcitin expression is involved in visual discrimination learning in cuttlefish. The experiments here indicate that cuttlefish show both spatial and visual discrimination learning ability, and calexcitin expression may relate directly to associations of visual stimuli.

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ACKNOWLEDGEMENTS

This project would not have been possible without the help of a large number of people. Many thanks to my advisor, James Atkinson as well as the rest of my graduate committee, Heather Eisthen, Thomas Getty, Roger Hanlon, and Robert Root-Bernstein for their helpful comments and support. Additional thanks to Roger Hanlon and the Marine Resources Center (Marine Biological Laboratory, Woods Hole, MA) for use of laboratory space and technical support during experimentation. Particularly, I am thankful to Janice Hanley, Bill Mebane, Mollie Tubbs, and Nicole Gilles and for their daily assistance in animal care and tank maintenance. I am very appreciative of Dan Alkon, Deanna Buck and Tom Nelson at the Blanchette Rockefeller Neurosciences Institute, Gaithersberg Maryland, for their incredible generosity and assistance in training me in various laboratory techniques. I am grateful to Jean Boal for her continued guidance, much of my knowledge and interest in cephalopods is a direct result of working with her. I am also appreciative of recent correspondence with John Messenger related to interpretation of the results of these experiments. Finally, I would like to thank my family and friends for their support and encouragement over the years. Financial support for this project was provided by Sigma Xi, the Marine Biological Laboratory and the Blanchette Rockefeller Neurosciences Institute.

To all of the above, my heartfelt appreciation.

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Chapter One

Introduction to the Cephalopods

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1.1 Classification

Cephalopods are members of the phylum Mollusca, an ancient group characterized by an evolutionarily plastic body plan that allows for a tremendous diversity of form. The cephalopods are interesting molluscs in that several groups resemble modern teleost fish in terms of their morphology, physiology, ecology and behaviors (Hanlon and Messenger, 1996). Thus, the cephalopods are considered "advanced" invertebrates.

The cephalopods appeared in the late Cambrian, several million years before the first primitive fish (Teichert, 1988). There are two extant divisions of cephalopods, the ancient Nautiloidea (the nautiluses) and the more modern Coleoidea. The coleoids diverged from the nautoloids in the Ordovician and include three major extant orders, the octopods (octopuses), teuthoids (squid), and the sepioids (cuttlefishes) (Teichert, 1988). The evolution of the cephalopods has been largely linked to adaptations allowing for low-pressure gas spaces used to maintain neutral buoyancy (Packard, 1972). Such adaptations include the formation of chambered shells (Nautilodea) and those observed in the coleoids including drastic reductions in the external shell, dynamic lift, chemical lift, swim bladders, and various behavioral modifications (Packard, 1972).

Neutral buoyancy has yielded a number of interesting behavioral consequences. First, reductions in the shell have allowed for more rapid locomotion as well changes in defensive strategies (Packard, 1972). Coleoid cephalopo

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cephalopods rely heavily on camouflage, speed, or shelter use to evade predators, compared to the nautiluses, which like many other shelled molluscs pull their entire body into a shell when threatened (Wells et al., 1992). Directly related to reductions in shell size are changes in predatory behaviors. Coleoid cephalopods are fast-swimming, active predators, whereas slow-moving nautiluses are primarily scavengers (reviewed in Hanlon and Messenger, 1996). Thus, there are a number of obvious differences in foraging habits between these two orders and there are a number of related differences in the brain and sensory structures that are evident (Budelmann, 1994). Specifically, nautiloids have very large olfactory lobes, and lens-less "pinhole camera" eyes interpreted as adaptations associated with scavenging (Young, 1965 and 1988). In contrast, coleoids have large optic lobes, complex camera eyes, increased eye musculature, and greatly reduced olfactory lobes (Budelmann, 1994). In this way, coleoid cephalopods are well suited for a highly visual, predatory lifestyle. However, many octopuses have well-developed chemoreceptors on their suckers and complex chemotactile memory systems (for review see Young, 1988; Budelmann, 1994).

The complexity of the coleoid sensory and processing systems relative to that observed in the nautiluses correlates with the coleoids' increased reliance on complex external stimuli (visual, chemosensory, tactile, etc.) used to locate food (Sanders, 1975; Young, 1988). For instance, the coleoid eye shows a remarkable convergence with the vertebrate eye and is able to discern much more detail (of objects at a variety of distances) than the eyes of other invertebrat

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invertebrates (Young, 1963). Additionally, a mechano-sensory lateral line system that is sensitive to local water movement in particular directions has developed in the coleoids, but is not present in the nautiluses (Budelmann et al., 1991). The lateral line system allows for prey detection (or predator avoidance) at night, in deep water, or in turbid water when normal visual processing is difficult (Budelmann et al, 1991; Budelmann, 1994). Thus, it is clear that the coleoid cephalopods receive a much broader range of sensory input than nautiluses. Details of the cuttlefish brain and mechanisms for processing of sensory input are described in Chapter 3.

1.2 Evolution of the Coleoid Cephalopods

Many hypotheses address the potential influences driving the advent of a shell-less, active, carnivorous life-style in the coleoid cephalopods. In the early Paleozoic, the buoyant, chambered-shell forms (somewhat similar to modern *Nautilus*) were likely the most mobile and successful cephalopods (Teichert, 1988). There has been a great deal of speculation that intense competition existed between cephalopods and vertebrates. Specifically, it has been suggested that ancient fish exerted strong predatory pressure on cephalopods during the Devonian period, forcing them to occupy niches in deeper water (Packard, 1972). This may have favored morphological changes allowing significant reductions in shell size. Shell-less cephalopods may have later returned to shallower water and competed directly with the teleost fishes during the Triassic (Packard, 1972). Support for this comes from Aronson (1991) who hypothesized that predatory teleost fishes directly affected the distribution of

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octopuses. In addition, it is likely that predation pressure from the teleost fish further favored adaptations promoting greater speed and maneuverability in the cephalopods (Packard, 1972).

Teleost fish and cephalopods probably shared a number of marine niches because they share similar size ranges, predatory styles and other behaviors (Packard, 1972). However, the extent to which fish and cephalopods were in direct competition with one another has been argued extensively (O'Dor and Webber, 1986). Regardless, there are certain cephalopod adaptations that are almost certainly directly related to the presence of fish. For instance, skin camouflage on color-blind cephalopod bodies is probably "aimed at fooling vertebrate eyes" (Packard, 1988).

As mentioned before, the ability to avoid teleost fish (or to eat them) may have required increased speed and maneuverability in the cephalopod body plan, which in turn required a decrease in the shell and increased coordination between various sensory and motor structures and muscles (Budelmann, 1994). This coordination was achieved in large part by the development of a sophisticated centralized processing unit (a complex brain). As cephalopod brain size and complexity increased, behavioral repertoires also increased (Young, 1988; Budelmann, 1994). The ability to locate and relocate shelters or foraging patches, acquire new hunting skills, and generally gain knowledge about the physical world are all significantly aided by diverse forms of complex learning (e.g. associative learning, spatial learning, discrimination learning, etc.).

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The cuttlefish (order Sepiodea) and squids (Order Teuhthoidea) each have eight arms and two tentacles are often referred to as the "decapods". There are several other notable morphological and anatomical differences between the octopuses and decapods including body shape, fins, and sensory structures. Thus, anatomical and behavioral comparisons between octopuses and decapods may shed further light on the adaptive radiation of the coleoid line.

1.3 Natural History of *Sepia officinalis*

The common cuttlefish*, Sepia officinalis* Linnaeus, 1758, is distributed throughout the North Sea, English channel, in the Mediterranean Sea and the Atlantic Ocean along the west coast of Africa extending to the southern-most point of Africa (Boletsky, 1983*)*. Adult *Sepia* are primarily benthic organisms, living in coastal waters and on the continental shelf in relatively shallow waters (no deeper than 150 meters). During the summer, temperature increases induce many populations of *S. officinalis* to migrate to shallower waters of about 30-40 meter depth where they aggregate with conspecifics for spawning (Boletsky, 1983; Boucaud-Camou and Boismery, 1991). The orientation mechanism used during these migrations has not yet been described.

After a female cuttlefish has mated, she lays her eggs, putting each egg into a separate case. Egg cases are then attached in clusters to objects near the substrate (Boletsky, 1983). Eggs take about two months to develop depending on the water temperature: development is more rapid in warmer water. Female cuttlefish can lay eggs several times toward the end of their life, but most begin to senesce shortly after spawning. Male and female cuttlefish have a short

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lifespan of about one to two years. Hatchlings have a mantle length (ML) of approximately 50 mm and can grow to a ML of 45 cm but most adults remain in the range of 20 to 30 cm ML (Boletsky, 1983). Hatchlings closely resemble adults and have only a very brief planktonic phase. Hatchlings rapidly display many of the same body patterns shown in adults (Hanlon and Messenger, 1988), exhibit adult-like predatory behavior (Wells, 1958) and readily distinguish between other hatchlings and prey items (Boletsky, 1983). Many details related to development and learning of adult behaviors in cuttlefish remain vague, as there are significant difficulties studying adult behavior at sea as well as difficulties rearing (and thus observing) hatchlings in the laboratory (Hanlon and Messenger, 1996).

Post-hatching growth in *Sepia* is largely related not only to water temperature, but also to feeding conditions (Boletsky, 1977 and 1979). There is a distinct increase in growth rate at approximately 100 days (juvenile stage) in 25°C water. Male cuttlefish can reach sexual maturity when they are as small as 6-8 cm mantle length (ML). Females may mature at anywhere from 11-25cm ML. Thus, males have earlier maturity and a longer duration of reproductive activity than females (Boletsky, 1983). During maturation many aspects of cuttlefish behavior, such as hunting and aggression and changes in hunting behavior appear to be a direct result of learning (i.e. Boletsky, 1977; Agin et al., 1998; Dickel et al., 2000). As more details of the natural history of *S. officinalis* are clarified, we will undoubtedly understand more about the function of many of their behaviors (including learning).

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Wells (1962) suggested that learning may allow cephalopods to cope with extreme changes in body size during their rapid development thus promoting behavioral plasticity. For instance, young octopuses that outgrow their dens must learn the location of new dens, or young cuttlefish must learn that an organism that was once a predator is now a valuable food resource (Hanlon and Messenger, 1996). Learning is undoubtedly an important factor in several aspects of the natural history of coleoid cephalopods. Examples of cephalopod learning, possible functions of learning, and the underlying neurobiology will be discussed in greater detail in the next two chapters.

Chapter 2

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Because cephalopods' behavioral abilities have been compared to those of lower vertebrates and because they are evolutionarily distant from species more commonly used in learning experiments, cephalopods are worthwhile test species for functional explanations of the evolution of complex nervous systems (Packard, 1972; Budelmann et al., 1997). Thus far, most research on complex learning has focused on vertebrate species or eusocial insects. However, neurobiological models of associative learning are often based on simple molluscan systems (e.g. *Aplysia* and *Hermissenda*). Investigations of learning in cephalopods could provide important insight into the evolution of cognition, by allowing for comparisons of simple and complex behaviors

within a single taxon (Phylum Mollusca). Previous studies of complex learning in cephalopods have focused primarily on learning in octopuses. Octopuses have been shown to exhibit many forms (or levels) of learning: habituation, sensitization, operant conditioning, and associative learning have been demonstrated (Thorpe, 1963). Several extensive reviews of cephalopod learning literature are available (i.e. Sanders, 1975; Mather, 1995; Hanlon and Messenger, 1996).

2.1 Sensitization and Habituation

Simple forms of learning such as habituation (increased threshold to response) and sensitization (decreased threshold to response) have been demonstrated on several occasions in the coleoid cephalopods. For example, when encountering a novel object, octopuses generally pass it under their web (formed b

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(formed by the layer of skin that joins the eight arms) and then to their mouths where they will examine the object. If the same object is presented repeatedly to normal and blind *Octopus vulgaris*, neither group will continue to examine or to pass the object to the mouth after a few trials (Wells and Wells, 1956). Other studies have demonstrated habituation to various additional stimuli in *Octopus* (e.g..,Goldsmith, 1917; Boycott, 1954). Similarly, bay squid (*Loligonuncula brevis*) show a marked reduction in escape jets or defensive body patterns with repeated presentation of a model of a fish (Long et al., 1989). *L. brevis* will dishabituate to this same model after a threat stimulus or a one-hour break (Long et al., 1989). The other form of non-associative learning, sensitization, has also been demonstrated in cephalopods. During sensitization, rewards or punishments that are offered before presentation of a test stimulus will affect subsequent attack behavior for visual, tactile and olfactory stimuli (Wells and Wells 1958, Chase and Wells, 1986, reviewed in Sanders 1975 and Hanlon and Messenger, 1996).

2.2 Associative Learning

Associative learning, involving associations between particular events resulting in long-term changes in behavior, has also been demonstrated in cephalopods (Sanders 1975; Mather, 1995; Hanlon and Messenger, 1996). Associative learning involves more complex processing of stimuli than habituation or sensitization (Thorpe, 1963). For example, octopuses will learn to attack one of a pair of stimuli when the attack is reinforced with either a positive food reward or a negative electric shock (Boycott and Young, 1955). In these

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cases, learning appears quite rapid, though performance is a great deal more erratic than in vertebrates trained under similar conditions (Boycott and Young, 1955). Visual discrimination training, in which separate visual stimuli are presented simultaneously or successively, has also been successfully employed with octopuses, though simultaneous presentation of stimuli yields significantly better performance than successive presentation (Sutherland and Muntz, 1959). This training protocol has been used extensively to examine visual perceptual abilities. Visual discrimination learning has also been demonstrated among decapods as well (i.e. Messenger, 1977; Allen et al., 1985).

Tactile discrimination training has also been successfully demonstrated in octopuses. *O. vulgaris* will readily accept and pass an object to its mouth associated with a positive stimulus while rejecting objects that it learns to associate with negative reinforcement (Wells, 1978; Robertson et al., 1994). Similarly, avoidance learning has been demonstrated in the octopus *Eledone moschata*, which will learn to avoid hermit crabs that would normally be prey when these hermit crabs are carrying particular species of painful sea anemones (von Uexküll, 1905; Boycott, 1954).

Associative learning has also been demonstrated in cuttlefish (Messenger, 1968 and 1977). Through associative conditioning, *Sepia officinalis* will learn to not strike at prey items presented in a glass tube (Sanders and Young, 1940; Messenger, 1968). This inhibition of predatory behavior is categorized as associative learning as striking tentacles against glass is negative reinforcement (painful) for the cuttlefish (Messenger, 1977; Agin et al., 1998). *S. officinalis* with

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tentacles that have been surgically removed show a much slower decrease in response with repeated exposure than do normal cuttlefish (Messenger, 1973).

Reversal learning, which is considered an advanced form of associative learning, has been demonstrated in both octopuses and cuttlefish (e.g.. Boycott and Young, 1958; Wells and Wells, 1957; Mackintosh and Mackintosh, 1964; Karson et al., 2003). Mackintosh and Mackintosh (1964) trained octopuses in a simultaneous visual discrimination task. Once the octopuses attained criterion performance, the task was reversed and the unrewarded object became rewarded. Octopuses more quickly attained criterion level performance across successive reversals and also attained earlier success on later reversals than on earlier reversals. Cuttlefish have also been demonstrated to show improved performance across serial reversals of a simultaneous discrimination problems involving visual and spatial stimuli (Karson et al., 2003). Similar to training octopuses, cuttlefish more quickly attained criterion level performance and reduced numbers of errors across successive reversals. However, significant improvement was only seen across those reversals in which the cuttlefish were trained in the direction contrary to their individual preference.

Another form of complex learning is observational learning, where an individual learns the solution to a problem by watching a conspecific learn the task. Fiorito and Scotto (1992) first suggested that octopuses are capable of social or observational learning. In Fiorito and Scotto's study groups of "demonstrator" octopuses were trained to discriminate between visual stimuli. Once the demonstrator octopuses yielded errorless performance on the task, they were

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they were then tested without a reward while naïve "observer" octopuses were in the field of view. Observers were then tested without reward, and they were shown to attack the correct visual stimulus significantly more than the incorrect visual stimulus. However, in a similar study of cuttlefish, watching predatory behavior of conspecifics did not yield any observational learning effect (Boal et al., 2000). These findings have not been successfully replicated. Moreover, octopuses (unlike squid) are primarily solitary and have no overlap of generations. These two pieces of evidence suggest that Fiorito and Scotto's result may not be a case of imitation brought about by observational learning per se. Instead, perhaps, there was some type of "local enhancement" in which the observer octopuses attention was directed toward the correct stimulus more so than the incorrect stimulus (Thorpe, 1963; Hanlon and Messenger, 1996).

2.3 Spatial Learning

Evidence for spatial learning has also been provided by several field studies using octopuses as subjects. Many octopus species forage away from a home den to which they return repeatedly for shelter (e.g. Ambrose, 1982; Boyle, 1983 and 1988; Hartwick et al., 1984; Mather 1991; Forsythe and Hanlon, 1997). Octopuses may occupy a single den continuously anywhere from one day to five months before moving to a new den (Hartwick et al., 1984). Furthermore, these octopuses are able to relocate their dens from large distances (40 m, out of sight from the den) and after extensive time lapses (Mather 1991; Forsythe and Hanlon, 1997). Octopuses will then change home dens and again use spatial information relocate these sites after foraging (Mather, 1991). Detailed tracking

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data from octopuses in open water is available from only two studies (Mather, 1991; Forsythe and Hanlon, 1997), thus, one cannot draw conclusions concerning spatial learning ability from the field data alone.

Studies of detours and maze learning have supported the hypothesis that octopuses rely on spatial learning (Wells, 1964; Walker et al., 1970; Mather, 1991; Moriyama and Gunji 1997; Boal et al., 2000). Training of *O. maya* in a T-maze with escape as motivation successfully demonstrated rapid learning of the task as well as reversal learning when exits were switched (Walker et al., 1970). Boal et al. (2000) demonstrated that octopuses explore their surrounds and also learn to solve a maze within one day and retain the solution to the spatial problem for at least one week. In this experiment maze escape was used as the sole reward. Moriyama and Gungi (1997) examined spatial learning in octopuses using a wall/obstacle maze but provided food as a reward. In this experiment, the authors cite the fact that octopuses changed actions and significantly reduced their detouring time around the obstacles after training as good evidence for spatial learning. Thus, data from spatial learning experiments indicate that octopuses learn relatively quickly and retain the learned information.

In laboratory experiments, cuttlefish may have also demonstrated spatial learning (Karson et al., 2003; see Appendix C). The cuttlefish in this experiment readily learned to escape from a straight alley maze, which could be considered a very simple form of spatial learning. In more complex mazes it was difficult to ascertain whether cuttlefish learned to follow spatial or visual cues to find the maze exit, as the cues were linked (Karson et al., 2003). However, these experimen

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experiments did not rule out the possibility that cuttlefish can learn to escape a maze based on spatial cues.

While learning to avoid painful stimuli has clear utility for cuttlefish in nature, the utility of other forms of learning (i.e. spatial learning) are not as well understood. For example, unlike octopuses that use home dens for defense, cuttlefish rely primarily on camouflage or burying themselves in the substrate and do not appear to use home dens at all. It is clear, thus, that cuttlefish do not use spatial learning in the same manner as octopuses.

A number of hypotheses could address the utility of spatial learning in cuttlefish. Cuttlefish live in complex, three-dimensional environments, and many species demonstrate particular patterns of spatial use within these habitats (O'Dor et al, 2001). Cuttlefish may use spatial learning to repeatedly negotiate vertical barriers, to relocate particular foraging patches or to relocate mating aggregation areas. For example, *S. apama* often settle on or near particular rocks or ledges, forage away from these rocks and then return to the same location (O'Dor pers. comm., 2002). Spatial learning may be used to relocate particular features of a habitat (Sanders and Young, 1940). Additional hypotheses are drawn from field studies of *S. latimanus,* which gather in breeding aggregations. During these aggregations males position themselves on specific limestone mounds and wait for females (Corner and Moore, 1980). When females arrive, males leave their mound to approach the female. After pursuing the female, a male will return to the mound from which it started the pursuit of the female. Spatial learning may play a role in helping the male

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relocate these mounds. However, the males may use some other cue to locate these mounds as well.

Seasonal onshore/offshore migrations have been documented in *S. officinalis* (Boletsky, 1983) and it has been suggested that spatial learning is involved in locating sites for mating aggregations (Norman, 2000). Because they are short-lived (one-two years) and because *Sepia* is generally semelparous, it is unlikely that learning is involved in these migrations (Karson et al., 2003). It remains clear, however, that by exploring the types of problems that cuttlefish can solve using learning and the cues they use to solve these problems, further functions of learning in the natural history of cuttlefish may become apparent.

Though complex learning studies have focused on octopuses, neurobiological evidence also suggests that cuttlefish, like octopuses, are capable of relatively complex forms learning. Cuttlefish have a larger overall brain to body size ratio than do octopuses (Maddock and Young, 1987). Furthermore, in *S. officinalis* the vertical lobe, a brain region involved in associative learning and memory of tactile and visual stimulii (see Chapter 3) is significant larger. The vertical lobe of *S. officinalis* occupies roughly 24% of the total brain volume, whereas the vertical lobe of *Octopus vulgaris* occupies only about 13% of the total brain volume (Wirz, 1959; Maddock and Young, 1987). In *S. officinalis* specific improvements in learning ability have been directly correlated with increases in the volume of the vertical lobe during post-embryological development (Messenger, 1973; Dickel et al.1998). For example, healthy adult cuttlefish learn to avoid shrimp presented in a glass tube, whereas juveniles a

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juveniles and senescent adults do not (i.e. Chichery and Chichery, 1992; Dickel et al., 2001). Such findings suggest that vertical lobe size in particular could be associated with learning (refer to Chapter 3). Thus, the possibility remains that learning and memory play a significant role in cuttlefish natural history equal to that observed in octopuses.

CHAPTER 3

Cephalopod Brains

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3.1 General Description

Cephalopod brains are the largest and show the most complexity among all invertebrates. They are characterized by large numbers of elaborate sense organs, motor neurons and effectors (Young, 1988). The cephalopod central nervous system is significantly larger and shows significantly more fusion of ganglia than is observed in other molluscs (Young, 1988). In all coleoids, the central nervous system is situated around the esophagus, and optic lobes extend laterally from either side (Figure 1). There are several extensive reviews of literature related to cephalopod nervous systems (e.g., Bullock, 1965; Boyle, 1986).

The central nervous system of coleoid cephalopods (including *Sepia*) is highly centralized between its two eyes and is enclosed in a cartilaginous cranium. Cephalopod brains consist of many fused ganglia, and are organized into numerous lobes connected by complex sets of tracts and commissures (Young, 1971). The esophagus runs through the brain dividing it into a supraoesophageal mass, a suboesophageal mass and the perioesophageal magnocellular lobes. The buccal lobes are anterior to the rest of the brain and are connected to the inferior frontal lobe by large buccal ganglia. Short optic tracts found on both sides of the supraoesophageal mass connect the optic lobes to the central nervous system. Figure 1 shows the anatomical relationships among the major components of the coleoid brain. Each lobe of coleoid brains and their peripheral ganglia consist of a central mass of neuropil containing many axons an

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axons and collaterals, but few cell bodies. Perikaryal layers with both neuronal and glial cell bodies surround the neuropil of the lobes (Boycott 1961).

The suboesophageal mass contains a number of intermediate and lower motor centers, and is thus plays a role similar to the vertebrate spinal cord (Boycott, 1961). In contrast, the supraoesophageal mass contains the higher motor centers, sensory centers and learning centers and is largely controlled by input from the optic lobes (Boycott, 1961). Because of its involvement in learning and memory, the description here will focus on the supraoesophageal mass instead of the suboesophageal mass. Anatomical details related specifically to the brain of *Sepia officinalis* have been described in great detail (Cajal, 1917; Boycott, 1961), but will be briefly outlined in the next section.

3.2 Description of the Supraoesophageal Mass

Figure 2 shows a photograph of a mid-saggittal section of the supraoesophageal lobes of *S. officinalis* with the major recognized lobes labeled. At the top of the mass, there are three distinct lobes, the vertical lobe, inferior frontal lobe and superior frontal lobe (Fig. 2). These higher lobes of the supraoesophageal mass have been associated with motor control as well as learning and memory of visual, chemical and tactile stimuli (Young, 1971). The function of this set of lobes in learning and memory will be described in greater detail in section 3.3. Directly underneath the vertical lobe lies the subvertical lobe and under the subvertical lobe lies the precommisural lobe (Fig 2). The remaining portion of the supraoesophageal mass consists of the anterior basal lobe (divided into anterior and posterior portions), the dorsal and medial basal lobes. whi

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lobes, which form the posterior wall of the supraoesophageal mass, and the lateral basal lobes, which flank either side of the medial basal lobe (Fig. 2). The basal lobe system appears to control motor function including the chromatophores, swimming, fin movements, etc (Boycott, 1961).

This highest portion of the supraoesophageal mass (often referred to as the superior frontal-vertical lobe system) is particularly well developed in *Sepia* (Boycott, 1961; Maddock and Young, 1987). The lobes of this system are connected to one another by many afferents and efferents. Afferent sources of input to the superior frontal lobe include the optic lobes, inferior optic lobe, brachio-cerebral tract, vertical lobe and subvertical lobe (Young, 1979 and 1988). All of these afferents enter through the lateral portion of the superior frontal lobe. The anterior and posterior portions of the superior frontal lobe are distinct suggesting that processing within the superior frontal lobe is divided (Young, 1979). Efferents of the superior frontal lobe from the posterior portion connect to the vertical lobe, while the anterior efferents connect to the subvertical lobes (Young, 1971 and 1979; Agin et al., 2001).

Similarly, the inferior frontal lobe receives afferents from the buccal ganglia, mantle and arms and sends efferents to the superior frontal lobe, buccal ganglia and brachial lobes (Young, 1979). In octopuses, the inferior frontal lobe is highly specialized and plays an important role in chemical/tactile learning and memory (Young, 1971). In cuttlefish, the complexity and function of the inferior lobe is significantly reduced, though it continues to play an important role in the feeding system (Table 1; see also Young, 1979).

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The vertical lobe is well developed in the decapods (Wirz, 1954; Maddock and Young, 1987). There are three distinct classes of cells in the vertical lobe. A mixture of small amacrine cells and "large cells" in the vertical to subvertical lobe tracts (Young, 1979). Medium and large neurons are scattered throughout the neuropil (Young, 1979). Additionally, there are smaller cells in the periphery of the neuropil, most of which are amacrine cells, projecting only within the vertical lobe itself (Young, 1979). The vertical lobe receives a majority of its afferent input from the superior frontal lobe, the subvertical lobe and the optic tract (Young, 1979). A small number of the larger cells sit in the central portion of the neuropil and carry information (efferents) back to the subvertical lobe, superior frontal lobe and the inferior frontal lobe (Young, 1979). According to Young (1988), the frontal/vertical lobe circuit is largely involved in prey selection, receiving input from the tentacles and the optic and buccal lobes, and then regulating attack behavior.

There are several basal lobes (dorsal basal, median basal and anterior basal) and these are located toward the posterior of the brain (Figure 2). The basal lobe system is thought analogous to the vertebrate cerebellum, receiving sensory input from the eyes and statocysts and regulating motor behavior of the eyes and the whole animal (Boycott, 1961; Young, 1977). These lobes have no direct connection with the vertical superior-frontal lobe system (Young, 1977). The basal lobes are the primary processing point for afferent input from the arms and from the buccal mass, but also receive input from the optic tract (Young, 1977). The posterior portions of the dorsal and medial basal lobes connect to the optic lobes

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optic lobes and control eye movements specifically (Boycott and Young, 1956). However, the other lobes in the basal lobe system primarily send their efferent output to motor centers in the suboesophageal mass, such as the chromatophore centers and pedal lobes (Boycott, 1961; Young, 1977).

The optic lobes are the largest lobes of the central nervous system (Young, 1974). In each optic lobe the neuropil and somata are layered consisting of a deep retina surrounded by outer and inner granule cell layers, and a medulla (figure 3; see Boycott, 1961; Young, 1971 and 1974; Budelmann, 1994). The deep retinal layers are composed of distinct regions of tangential and radial fibers and are analogous to the ganglion cell layer of the vertebrate retina (Boycott, 1961, Young, 1974). The medulla functions as both a link to the motor centers and a memory storage site (Boycott, 1961; Young, 1974). The optic lobes receive direct afferent input from the eyes as well as from most of the lobes of the supraoesophageal mass and submit efferent output to the dorsal and medial basal lobes as wells as the subvertical and superior frontal lobes (Boycott 1961).

3.3 Role of the Supraoesophageal Lobes in Learning and Memory

As mentioned in Chapter 2, behavioral experiments with cephalopods have indicated that the vertical lobe and associated regions play an important part in the acquisition and retention phases of learning (Boycott and Young, 1957, Wells and Wells, 1957, Young 1961, Agin et al., 2001). The vertical and subvertical lobes were long known as "silent" areas of the cephalopod brain because electrical stimulation to these areas yielded no specific effect (Bert, 1867). However, surgical removal of these areas yields significantly impaired acquisition and retention of visual memories in both octopuses and cuttlefish (i.e. Sanders and Young, 1940; Boycott and Young, 1950).

Octopuses have two, distinct memory systems thatoverlap to some extent (Young, 1991). One of these memory systems is for chemotactile learning and the other is for visual learning. Each system involves specific connections between cells in several different supraoesophageal lobes (reviewed in Young, 1991). The chemotactile learning and memory system involves the higher lobes of the supraoesophageal region, including the inferior frontal lobe, subfrontal lobe, buccal lobe, superior -frontal, vertical and subvertical lobes (Young, 1991). The chemotactile learning system is either not present or not well developed in cuttlefish, which show significantly reduced inferior frontal lobes as compared with octopuses and rely on chemotactile input for prey handling but not hunting (Young, 1971). Precise details of the chemotactile learning circuit in octopuses are reviewed elsewhere (Young, 1991).

In contrast, the visual learning system of cuttlefish is markedly similar to that of octopuses (Young, 1991). Unfortunately, the complexity of the connections of cells within the optic lobes has made the description of the precise cellular connections and processes underlying visual memory in cephalopods particularly difficult to ascertain (Young, 1974 and 1991). However, a basic description of visual processing has been described. From what is understood, retinal cells send input from the eyes to the cells within the tangential layers of the plexiform region of the optic lobe, which may play a role as feature

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detectors or "classifying cells"(Young, 1971 and 1991). These feature detectors are then thought to send signals to the "memory cells" within the "cell islands" of the optic lobe medulla (Young, 1965). Lending support to this particular hypothesis is evidence from octopuses, which will learn to habituate to visual stimuli with only their suboesophageal mass and optic lobes intact (Boycott, 1954).

The medullary "memory cells" then connect to one of two paired circuits in the central nervous system that involve the superior frontal lobe, vertical lobe, and adjacent subvertical lobe (Young, 1965 and 1991; Fig. 2). Each lobe in each circuit is thought to exert opposing influences on the probability of positive or negative responses to the visual stimuli (Young, 1991). Specifically, in the portion of the circuit comprised of the lateral superior frontal lobe and subvertical lobe circuit, visual signals from the optic lobes are thought to interact with gustatory information from the buccal system, thus promoting attack of prey items (Young, 1991). After removal of the lateral superior frontal lobe octopuses will no longer attack prey items (Boycott and Young, 1955).

In contrast, in the vertical lobe and median superior frontal lobe portion of the circuit, visual information is thought to interact with input from nociceptors, and this circuit mediates learned inhibition of attack behaviors (Young, 1991). Thus, when the vertical-median superior frontal lobe circuit is interrupted, octopuses will continue to attack prey items even when receiving electrical shocks, unless the shocks are given at intervals of five minutes or less (Boycott and Your

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and Young, 1955). Additionally, visual learning is significantly reduced when more than half of the vertical lobe is removed (Boycott and Young, 1957).

It is clear that the two parallel circuits in the system above exert opposite influences on predatory behavior (Young, 1991). The vertical, subvertical and superior frontal regions are not connected directly with any single receptor or any particular motor center (Boycott, 1961). The exclusivity of the connections between these areas and the fact that they are electrically unexcitable provide additional support for the existence of a positive feedback system, such as the one proposed above (Young, 1938; Boycott, 1961).

Boycott and Young's (1955) study also indicated the involvement of the vertical lobe in the transfer from short-term to long-term memory. However, vertical lobe removal does not entirely block the retention of previously learned visual information (Young, 1958). This is because much visual information in long-term memory is stored within the medulla of the optic lobes (Young, 1965). As described above, in octopuses, the vertical lobe plays an additional role in the chemotactile memory system (Young, 1991). The effects of vertical lobe removal during tactile discrimination learning are markedly less than the effects observed in visual discrimination tasks. However, tactile discrimination learning is slower in the absence of the vertical lobe (Wells and Wells, 1957; Wells 1965). Supporting these findings is a study by Robertson (1994), that examined the role of filopodial extension in octopus brains (Robertson, 1994). Cytochalasin D (a compound that breaks down filopodia) was applied to the subfrontal lobes of some octopuses and the vertical lobes of others. The application of cytochalasin

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D to the subfrontal lobes specifically caused blocking of touch learning, whereas application to the vertical lobes did not (Robertson, 1994). This study lends support to the hypotheses that filopodial extension in the subfrontal lobes is involved in tactile learning.

Though octopus spatial learning behavior has been relatively well studied, its neural basis has not yet been studied. It has been suggested that the vertical and superior-frontal lobes may also play an important role in constructing internal spatial representations (Sanders 1975). Sanders further suggests that like the hippocampus of vertebrates, during spatial learning the vertical lobe is probably not involved in memory storage, but is involved in the memory consolidation phase and transfer of information from short to long term memory (see review in Sanders, 1975). To determine the anatomical and cellular networks underlying spatial learning in cephalopods, further neurobiological research remains necessary.

Currently, there are significant gaps in our knowledge related to the neurobiology of learning in cephalopods (Hanlon and Messenger, 1996). Extant studies have focused primarily on electrophysiological recordings and brain ablations in octopuses. Such results are often difficult to interpret in conjunction with complex behaviors like learning because it is often difficult to pinpoint exactly what structure has been interfered with and whether the interference was isolated to this structure alone. Developmental studies presented in the next section further suggest that the circuitry underlying visual learning in *Sepia* is similar to

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similar to that proposed in octopuses; visual and buccal input interact at the vertical, sub-vertical, superior-frontal circuit in a feedback system (Young, 1991).

3.4 Development of Behavior in *Sepia*

During normal development, the cuttlefish vertical lobe nearly doubles in size relative to the superior frontal lobe as well as the rest of the supraoesophageal mass (Dickel et al., 1997). The vertical lobe of *Sepia* comprises about seven percent of the entire brain volume in hatchling cuttlefish, at the juvenile stage this increases to about twelve percent (Wirz, 1959; Frosch, 1971). Behavioral experiments indicate that the development of predatory behavior in young cuttlefish can be directly correlated with development of the vertical, sub-vertical and superior frontal lobes as well as the development of the connections among these lobes (Dickel et al., 1997).

Hatchling cuttlefish show some prey-capturing behaviors that are similar to those observed in adult cuttlefish (Wells, 1958; Boletsky, 1987; Dickel et al., 1997). Thus, a single learning paradigm has been used to examine the relationship between prey capture and learning at different developmental stages (Sanders and Young, 1940; Messenger 1971 and 1973; Dickel et al., 2001). Specifically, cuttlefish are presented a piece of shrimp enclosed in a glass tube. Striking the tube is painful, and the cuttlefish must learn to not strike the visual stimulus of a prey item (associative learning). A hatchling cuttlefish that lacks a well-developed vertical lobe will continue to attack the inaccessible prey, even after many repeated trials (Wells, 1962). Adults with well-developed vertical lobes will learn to associate attacking the shrimp with pain and cease to attack

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the tube-encased prey (Wells, 1962). The reduction in attack behavior is not due to fatigue or temporary inability to strike with tentacles (Messenger, 1973).

Further evidence suggesting the importance of the vertical-superior frontal lobe system in learning stems from the timing of development of these lobes and various behaviors during development. The vertical lobe system of *S. officinalis* takes about four months to develop, and it is not until after this time that learning curves of juveniles appear comparable to those of healthy adults (Messenger 1973 and 1977). Furthermore, environmental enrichment appears positively correlated with the development of learning and memory abilities (Dickel et al., 2000).

Related developmental evidence arises from comparisons of specific hunting behaviors in adult and hatchlings (or juveniles less than four months of age). Adult cuttlefish hunt prey items that move out of their visual field. However, much like learned inhibition of predatory behavior, the hunting response is absent in cuttlefish younger than four months and is also absent in adult cuttlefish thaat have been subjected to vertical lobe lesions (Sanders and Young, 1940). These surgically altered cuttlefish will cease to follow prey items once they exit the visual field (Sanders and Young, 1940). Senescent cuttlefish with degenerating vertical lobe systems also show a marked decrease in hunting behaviors (Chichery and Chichery, 1992). Neurohistological analysis suggests that age-related degeneration of the neuropil in the subvertical, precommissural, superior frontal and inferior frontal lobes is markedly higher than degeneration observed within the vertical lobe (Chichery and Chichery, 1992). Thus, it is the degradatic

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degradation of the entire memory system, not the vertical lobe itself that suppresses normal hunting behavior (Chichery and Chicery, 1992).

3.5 Proposed Functions of the Vertical Lobe

Overall, it appears that the optic and the superior and inferior frontal lobes are initial sites of association between stimuli. However, the exact role of the vertical lobe remains unclear. The vertical lobe may ensure the persistence of the representation of associations made in other supraoesophageal lobes (Boycott and Young, 1955). Another proposed function of the vertical lobe is to keep track of active optic lobe classifying cells until a positive or negative association is made between the stimulus and the outcome (Young, 1965). Young (1965) further proposes that after processing within the vertical lobe and associated circuits, signals are sent back to the optic lobes, where long-term visual memories are stored within the medulla. It has also been suggested that the vertical lobe plays a specific role in consolidating short-term memories into long-term memories (Sanders, 1975). This hypothesis is supported by findings from several ablation studies indicating that the vertical and superior frontal lobes may play an important role in storing memories, and not merely in controlling motor functions during both visual and tactile learning (reviewed in Young, 1964; reviewed in Sanders, 1975).

Numerous transverse fibers from the two optic lobes that cross at the vertical lobe, suggesting that the vertical lobe is a primary site of interocular transfer (Sanders, 1975). Visual discrimination training in one part of the visual field of only one eye is transferred to the optic lobe of the other eye via the vertical lob

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vertical lobe, as inhibiting the optic commissures (and not the vertical lobe pathway) does not entirely block interocular transfer (Muntz, 1961). In this way, the vertical and superior frontal lobes may be similar in function to the vertebrate corpus callosum (Muntz, 1961).

3.6 Comparisons with the Vertebrate Hippocampus

One salient feature of the organization of the cephalopod brain is that the superior-frontal and vertical lobes allow mixing of different input from various different types of receptors such as the eyes, arms, mantle, nociceptors, and chemoreceptors. This organization is quite similar to the vertebrate hippocampus, often considered a vertebrate learning/memory center, which receives input from various association cortices and integrates the input during memory formation (Kandel et al., 2000). In cephalopods, the vertical and superior frontal lobes not only integrate various stimuli, but also enable of the information transfer, for example from the right to the left optic lobe. The complex connections among the various lobes of the cephalopod brain are not nearly as well understood as connections in vertebrates, but it is possible that further investigation of cephalopod brains may illuminate general principles underlying the organization of complex brains.

Chapter 4

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4.1 Associative Learning in *Hermissenda crassicornis*

A variety of molluscan systems have been used as simple models to understand brain function and associative learning at the molecular level. For instance, the marine snail *Hermissenda crassicornis* can be classically conditioned to associate light (the conditioned stimulus) with turbulence (the unconditioned stimulus (Alkon, 1974; Crow and Alkon, 1978). When the body of *Hermissenda* is rotated, the excitation of sensory cells in the vestibular organs excites motor neurons in the foot, causing reflexive contraction of the foot. In contrast, stimulation of the photoreceptors promotes positive phototactic behavior (reviewed in Alkon, et al., 1998). Repeated paired presentation of visual and vestibular stimuli yield a significant reduction in phototactic behavior and an increase in the number of foot contractions in response to light alone (Alkon, 1974; Crow and Alkon, 1978). Intracellular recordings from type B photoreceptors in the eye of conditioned *Hermissenda* show increases in input resistance and a reduction of voltage-dependent potassium currents in response to learning. Both of these effects are dependent on elevated levels of intracellular calcium (Alkon et al., 1998). Thus, associative learning in *Hermissenda* involves a biochemical pathway that includes the long-term molecular regulation of potassium channels by levels of intracellular calcium (reviewed in Alkon et al., 1998; Matzel et al., 1998).

4.2 Protein Kinase C and Associative Learning in Hermissenda

The post-association modification of potassium currents after learning can persist for weeks and appears to result from an increase in intracellular calcium

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inducing the movement of the signaling enzyme protein kinase C (PKC) from the cell cytoplasm to the cell membrane (Alkon and Nelson, 1990; Nelson and Alkon, 1995). At the cell membrane PKC reacts with a signaling protein called calexcitin (Alkon, 1989; Alkon et al 1982, Alkon et al., 1985). In the cytoplasm, PKC acts to increase potassium ion flow thereby decreasing membrane excitability, whereas on the cell membrane PKC reduces potassium ion flow, which, in turn, increases membrane excitability (Nelson et al., 1996; Ascoli et al., 1997).

Protein kinase C has also been identified in vertebrate brain tissue as an important protein regulating memory storage processes (Bank et al., 1998). Imaging studies have demonstrated membrane-specific changes in PKC binding after classical conditioning of the eyeblink response in rabbits and olfactory discrimination and water maze training of the rat (Bank et al., 1988; Olds et al., 1989; Olds et al., 1990). After classical conditioning of the rabbit there is an increase in membrane-associated PKC near hippocampal CA1 cell bodies one day after training (Olds et al., 1989). After three days, PKC migrates from the CA1 cell bodies to the CA1 cell dendrites. This translocation of PKC in CA1 cells can be artificially induced by phorbol ester, which also causes the same potassium ion flow reduction that takes place during associative conditioning (Alkon, 1989). Translocation of PKC to the CA1 membrane by phorbol ester also causes enhanced summation of excitatory post-synaptic potentials (epsps) elicited by the activation of Schaeffer collaterals (reviewed in Golski et al., 1995). This same enhanced epsp summation has been observed in rabbits trained with a classical conditioning procedure (reviewed in Alkon, 1999). Thus, conservation of a molecular sequence for memory storage is implied by parallel cellular events in molluscan and mammalian memory paradigms (Alkon, 1999).

4.3 The Role of Calexcitin

Calexcitin (also called CE or cp20) is a low-molecular-weight (22 kDa), low-abundance G-protein that is stimulated by elevated Calcium levels and the presence of PKC at the cell membrane during associative learning (Nelson et al., 1996). Calexcitin is a high affinity substrate for PKC and has been found in the central nervous system of a variety of species including snails, squid, rabbits, and rats, and in skin fibroblasts of humans (Nelson and Alkon, 1991; Nelson et al., 1994; Kim et al., 1995). In *Hermissenda,* classical conditioning produced by associating visual and vestibular stimuli yields a 2-3 fold phosphorylation-state increase of calexcitin by PKC at the cell membrane (Cavallaro et al., 1997). Thus far, it is unclear whether calexcitin plays a role exclusively in learning and memory (T.J. Nelson, pers. comm., 2001).

The phosphorylation-state increase is followed by calexcitin translocation from the cytosol to three principal types of neuronal membranes: the endoplasmic reticulum membrane, the outer wall membrane and the nuclear membrane (Nelson et al., 1996; Ascoli et al., 1997). At the outer wall membrane, calexcitin inhibits voltage-gated potassium channels and causes increased membrane excitability to further depolarizing stimuli (Nelson and Alkon, 1995). At the nuclear membrane, calexcitin increases the rate of turnover of messenger RNA of several specific proteins (Nelson et al., 1996). Calexcitin elicits Ca^{2+} release from ryanodine receptors on the membrane of the endoplasmic reticulum, resulting in an overall increased amplification of Ca^{2+} signaling (Alkon et al., 1998; Nelson et al., 2001). Therefore, calexcitin appears to serve as a signaling molecule that amplifies calcium elevation in response to learning-associated synaptic transmitters and initiates second messengers. For a summary of the PKC/calexcitin model for associative learning see Figure 4.

Microinjection of CE into *Hermissenda* neurons causes many of the previously described cellular effects of learning, such as increased membrane excitability, reduction of cellular potassium currents, long-lasting depolarization, modified dendrite arborization, and changes in vesicle transport within the axons (Moshiah et al., 1993; Nelson et al., 1996; Alkon et al, 1998). Though a number of other proteins have been implicated in learning, CE is the only protein that reproduces these electrophysiological and morphological effects when it is directly injected into neurons (Ascoli et al., 1997). It has also been discovered that calexcitin undergoes a conformational change in the presence of Ca^{2+}, specifically, a significant increase in presence of alpha helices and a decrease in beta sheets (Ascoli et al., 1997). This same study further suggests that conformational equilibrium of calexcitin may serve as a neuronal trigger for short -term changes in activity after associative learning.

Additionally, it has been discovered that CE binds to both the Ca^{2+} and GTP signaling pathways, and these two pathways are known to interact (Jeng et al., 1987; Sahyoun et al., 1991; De Matteis et al., 1993). Therefore, calexcitin may act to combine two separate signaling pathways in response to temporal association of separate inputs during the acquisition of associative memories (Alkon and Rasmussen, 1988; Alkon, 1995).

As mentioned before, calexcitin has been found in the central nervous system of vertebrates (rabbits and rats) and may play a role in vertebrate, hippocampus-dependent memory processing (Sun et al, 1999). In human Alzheimer's patients, calexcitin immunoreactivity and potassium currents in the CNS are significantly less than in normal human subjects (Kim et al., 1995). These data further imply the role of calexcitin in associative memory. While calexcitin is found in both vertebrates and invertebrates, this does not indicate that it functions identically in both groups as conservation of mechanisms or structures involved in carrying out these mechanisms does not always imply identity of function of the mechanism or structure (Alkon, 1995). To understand the evolution of mechanisms involved in complex learning, it is necessary to first compare mechanisms observed in simple nervous systems to mechanisms involved in complex nervous systems.

4.4 Comparisons with Other Models

One potential problem with generalizing the PKC/calexcitin model of associative learning to other organisms stems from research using a different model based on classical conditioning of the gill withdrawal reflex of *Aplysia* (described in Kandel, et al., 2000*)*. The marine mollusk *Aplysia* can be classically conditioned to a weak tactile stimulus to the siphon (the conditioned stimulus) with an electric shock to the tail (the unconditioned stimulus). Paired presentation of these stimulus results in rapid and sustained withdrawal of the siphon and gill to the conditioned stimulus (Carew et al., 1981). Like the *Hermissenda* model, the cellular cascade that underlies the conditioned and unconditioned stimuli has been identified in *Aplysia*, and this model yields some striking similarities to that observed in the vertebrate hippocampus (Matzel et al., 1998).

In *Aplysia*, presentation of the unconditioned stimulus immediately prior to the conditioned stimulus induces increases in Ca^{2+} in the presynaptic sensory neuron. This increase activates calmodulin, which then binds to adenylyl cyclase, and enhances post-synaptic cAMP activity (Kandel et al., 2000). This mechanism is similar to that found in the vertebrate hippocampal mossy fiber which uses glutamate as a transmitter. In the mossy fiber, glutamate binds to both NMDA and non-NMDA receptors on the CA3 pyramidal cells. It is possible that long-term potentiation (LTP) depends on the influx of Ca^{2+} into the presynaptic cell, and not activation of presynaptic NMDA receptors, or the release of Ca^{2+} into post-synaptic cells. When Ca^{2+} levels increase in the presynaptic cell, adenylyl cyclase is activated. This influx of calcium thus causes an increase in cAMP activity which in turn activates protein kinase A (PKA) (reviewed in Kandel et al., 2000).

Despite their differences, the hippocampal LTP model, the *Aplysia* model and the *Hermissenda* model seem to share several interesting features (Matzel et al., 1998). In each system, intracellular increases of calcium and activation of G-proteins act to modify specific membrane proteins, change membrane excitability and reduce the activation threshold, allowing for modification of action potentials. When comparing the various learning models in this way, the major differences between the models are the class of protein kinase that is activated and the site of modulation relative to the targets of action (Matzel et al., 1998). The specific differences may reflect different solutions to the same types of problems in different species (Matzel et al., 1998).

4.5 Calexcitin in Cephalopods

Calexcitin was previously discovered in and isolated from the optic lobe of squid (Nelson et al., 1994; Nelson et al., 1996). Immunohistochemical labeling demonstrated that CE is found primarily within the plexiform layer of the optic lobe. This region is the initial location of the conjunction of fibers from the retina and optic nerve and also contains a number of amacrine cells (Young, 1974). In the optic lobes, calexcitin is largely localized in axon terminals, consistent with the hypothesized synaptic function of CE affecting axonal transport and neuronal branching (Alkon et al., 1990).

Pilot immunohistochemical and western blotting studies (Karson, 2001, unpublished data) indicated that calexcitin is also present in cuttlefish optic lobes and some regions of the supraoesophageal mass including the vertical and subvertical lobes (Karson, 2001, unpublished data). It is particularly interesting that these regions are thought to be related to associative learning in cephalopods (see previous chapter). Thus, the relationship between calexcitin expression and associative learning in cephalopods may be of significant interest.

Chapter 5

Objectives and Hypotheses

The experiments presented in the following three chapters were designed to gain insight into the possible functions of and mechanisms underlying visual and spatial simultaneous discrimination learning in cuttlefish. For each objective, there are a number of associated hypotheses. The specific objectives and hypotheses that were initially generated are as follows:

1. **To compare the relative rate of simultaneous discrimination learning based on visual versus spatial cues.** Using a two-choice discrimination paradigm, cuttlefish will be trained to escape a maze based on either exit direction (spatial cue) or provided landmarks (visual cue). The following specific *hypotheses* will be tested:

   a. *Visual and spatial cues are both used in simultaneous discrimination learning.* The ability to make visual discriminations has been well documented in cuttlefish, but recent findings suggest they are also able to solve spatial problems. This suggests that visual and spatial cues are both important in problem solving in nature.

   b. *The rate of learning depends on the type of cue used.* A preliminary experiment suggests that cuttlefish use visual landmarks rather than directional cues to find the open exit of a T-maze. This suggests that the learning rate in visual-cue training may be faster than the learning rate observed in spatial-cue training.

2. **To trace the expression of calexcitin (a learning-related protein) in particular brain regions during learning.**
    a. *Calexcitin is located in learning related brain regions in cuttlefish.* Specifically, I predict that trained cuttlefish will yield a greater distribution of calexcitin-stained cell axons in the vertical lobe and optic lobes than motor control animals in the same brain regions.
3. **To correlate calexcitin expression with learning events.**
    a. *Calexcitin is involved in discrimination learning.* Specifically, previous experiments suggest that trained cuttlefish will yield greater protein expression in the vertical lobe and optic lobes than control animals in the same brain regions because these regions are involved in associative learning.
4. **To compare the expression of a learning-related protein among various brain regions of visual cue-trained, spatial cue-trained and control cuttlefish.** Comparisons between groups address the following specific *hypotheses*:
    a. *Faster rates of learning are associated with greater increases of calexcitin.*
    b. *Cuttlefish trained to exit based on spatial cues will yield greater increases in protein in the corresponding optic lobe.* For example, cuttlefish trained to exit the right door may show greater protein activity in the right optic lobe than the left optic lobe.
    c. *Simultaneous discrimination learning is associated with decreases of protein in the cytosolic fractions and increases in protein in microsomal*

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fractions. During associative learning in other molluscs, calexcitin is translocated within the cells. Western blots of various cellular fractions will allow for the determination of whether similar patterns of calexcitin translocation occur during visual or spatial simultaneous discrimination learning in cuttlefish.

Chapter 6

Behavioral Experimentation

6.1 Summary

The objective of the behavioral experiments was to compare the relative rate of simultaneous discrimination learning based on visual versus spatial cues. Four experimental groups (visual, spatial, combined, and control) were tested in a two-choice simultaneous discrimination maze and performance was compared. These experiments supported the initial hypotheses that cuttlefish can learn to solve simultaneous discrimination problem using both spatial and visual cues. The experiments also indicated a significantly decreased number of trials to criterion in the spatial-cue group compared to other groups, and a significantly increased error rate and maze escape time in the combined-cue group when compared to the other experimental groups. Thus, this experiment also supported the second hypothesis that the learning rate differs between experimental groups.

6.2 Materials and Method

Experiments were conducted at the Marine Resources Center (MRC) of the Marine Biological Laboratory in Woods Hole, MA. The tank was 1.8 m wide x 3.04 m long x 0.30 m deep and was divided into quadrants. The total volume of the tank was about 450 gallons. The water supply was drawn from a depth of approximately three meters below the surface of Great Harbor and gravity fed to tanks throughout the MRC building. Salinity ranged from 31 to 33 parts per thousand and the water temperature ranged from 17° to 21° C.

All subjects were laboratory-cultured juvenile *Sepia officinalis* (mantle length 6 -12 cm, 14 males, 10 females) ordered from the National Resource

Center for Cephalopods (MBI, Galveston). Cuttlefish were housed in one of the four tank quadrants, each quadrant holding four to six individuals to minimize the behavioral effects associated with crowding (Boal et al., 1999). Large pieces of PVC pipe and artificial plants were placed in each tank to provide artificial shelter spots. The cuttlefish were fed a mixture of live and frozen fish, squid, shrimp or crabs twice a day, once at 07:00 and once at 17:00. A complete description of cuttlefish mariculture can be found elsewhere (e.g. DeRusha et al., 1989; Hanley et al., 1998).

The maze was designed based on a previous experimental design (Karson et al., 2003). A circular testing arena was constructed from a large, plastic barrel (71x 56 cm diameter, Figure 5). A start tube (16 cm diameter) with doors on both ends was placed through one side of the arena 17 cm below the top edge (12 cm below water surface). Two exit holes (16 cm diameter) were cut on opposite sides of the arena, six cm below and perpendicular to the start tube. Exits were fitted with movable and clear Plexiglas® doors. A clear, Plexiglas® platform was fitted 12 cm above the bottom of arena, creating a "false bottom." *S. officinalis* tend to settle on the floor of tanks and the false bottom prohibited them from reaching the tank floor, thus providing motivation for cuttlefish to escape the maze. A panel of striped fabric (36x36 cm) and a panel of spotted fabric (36x36 cm) were created and fitted with Velcro such that they could surround, and be switched between, either exit (see figure 5).

During a pre-training period, cuttlefish were acclimated to the maze and tested for exit preference. This was accomplished by placing the cuttlefish in the maze wit

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maze with both doors open and switching the visual-cue panels randomly between exits. For each individual, the frequency of exit use in each direction and for each pattern was determined (most frequently used cue during seven successful escapes).

Following pre-training, cuttlefish were randomly placed into one of three groups. First, a visual-cue group was designated and was trained to exit based on the non-preferred panel pattern, spots or stripes, regardless of exit direction and visual-cue panels swapped randomly between doors (n=8). Second, a spatial-cue group was trained based on the non-preferred exit direction, right or left, regardless of panel pattern and visual-cue panels swapped randomly between doors (n=8). Finally, a control group was trained such that each cuttlefish was placed in the maze with both doors always open and the visual-cue panels swapped randomly between the two doors between trials (n=8). The control animals underwent exactly twenty experimental trials, other cuttlefish were trained until they attained the criterion for learning.

For all three groups, individual cuttlefish were placed in the start box and allowed to swim into the testing arena. Once inside the arena, the cuttlefish had seven minutes to escape the maze. The maze exit time was recorded. If a cuttlefish did not escape, it was chased out of the open exit with a net and the escape time was recorded as ten minutes (this value was arbitrary and used for the purpose of statistical analysis). Each cuttlefish received six trials per day with at least a 45-minute inter-trial interval. This continued until six of seven successful, less than one minute, consecutive escapes were achieved. These criter

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criteria were selected on the basis of previous experiments (Karson et al., 2003). Once the cuttlefish reached criterion, a testing trial was run with both doors open to determine whether it continued to use the trained exit. The maze was rotated semi-randomly in the home tank between trials and experimenter hid behind a curtain. This insured that the cuttlefish could not rely on cues from around lab to locate open exit. If the cuttlefish did not use the appropriate exit during the test trial, it was given two additional training trials, and then was re-tested for learning. This happened only one time in one individual which was in the visual cue group. Statistical tests were performed according to methods suggested by Zar (1999) and Siegel and Castellan (1988).

Trained animals were sacrificed within five minutes of testing for learning. The optic and vertical lobes were removed and preserved in 4% paraformaldehyde in 0.1M sodium phosphate buffer for immunohistochemistry (n=13, 4 animals from each group plus 1 naïve animal) or frozen on dry ice for western blots (n=13, 4 animals from each group plus 1 naïve animal).

6.3 Results

The data presented here also include one additional experimental group named "combined-cue." These cuttlefish were trained in a reversal learning experiment (Karson et al., 2003). The cuttlefish in the combined cue group were trained against preference in the same maze, but the striped panel always surrounded the right door and a spotted panel always surrounded the left door. Hence, both the visual and spatial cues were relevant to the exit direction

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(Karson et al., 2003; Appendix C, data included here only refer to Reversal 0 results).

The mantle length on the last day of testing, sex, door preference and group placement of all subjects are reported in Table 2. Including the combined-cue group, 14 females and 23 males were tested. Of the 37 subjects, initial door preferences were distributed randomly between left and right (16 preferred left, 21 preferred right) as well as spotted and striped (21 preferred spotted and 16 preferred striped). In some cases an unequal number of male and female cuttlefish were placed in each experimental group, as it is extremely difficult to sex juvenile cuttlefish using external characteristics.

To directly compare maze performance among animals, trials were divided into blocks of five, and the mean maze escape time and mean error for the first, middle and last block of trials were then calculated and compared between subjects and groups. Because each cuttlefish in the control group was subjected to only 20 trials total, analysis of maze performance for the control subjects included all four blocks of five trials (i.e. all 20 trials). Graphs representing overall group maze escape times across blocks of experimental trials are shown in Figures 6 through 9. Graphs representing overall error over blocks of trials for each experimental group are shown in figures 10 through 13.

Repeated measures analysis of variance (ANOVA) tests indicated that cuttlefish in the visual, spatial and combined groups yielded a significant decrease in escape time, and a significant decrease in the percentage of overall error over the first, middle and last block of trials (results presented in Table 3,

Figure 6). This improvement indicates that the cuttlefishes' maze performance improved with repeated exposure to the simultaneous discrimination problem. Contrarily, the cuttlefish in the control group showed no significant decrease in escape time (Table 3, Figure 6A).

The percentage of overall error was sub-divided into the percentage of error due to not escaping the maze at all (non-escape) and the percentage of error due to not exiting the maze within 60 seconds. exclusive of non-escapes. This was done to determine which factor appeared to contribute more strongly overall errors made, non-escape or non-criterion performance. There was no significant reduction in either type of error for cuttlefish in the control group (Table 3, Figure 7A). There was a reduction in overall error for cuttlefish in the visual group (Table 3, Figure 7B). However, it appears that neither source of error independently significantly affected overall error (Table 3). The decrease in the percentage of errors exclusive of non-escapes more strongly contributed to overall error in the combined cue group, while a decrease in non-escapes more strongly contributed to overall error in the spatial group (Table 3).

The overall mean number of trials to criterion was significantly lower in the spatial cue group than it was in either the visual cue group (one-way ANOVA, F=3.93, P=0.067) or the combined cue group (one-way ANOVA, F=3.35, P=0.079) (Figure 8). The overall mean escape time differed significantly between groups (one-way ANOVA, F=7.93, P<0.0001). A Tukey Test indicated that this significant difference resulted from differences between all pairs except for the visual and spatial groups.

The mean percentage of overall error was significantly less in control cuttlefish than the three experimental groups (one-way ANOVA, F=7.60, P=0.0006) and a Tukey Test indicated that the trained groups did not differ significantly from each other in mean percentage of overall error.

The overall error was again divided in to two types of error, non-escapes and non-criterion performance (exclusive of non-escapes). ANOVA and Tukey tests indicated that the percentage of non- escapes was significantly greater in the combined group than any other group (one-way ANOVA, F=7.99, p<0.0005). The remaining mean error (error not including non-escapes) was significantly different between groups (one-way ANOVA, F=5.575, p<0.005). The Tukey Test indicated that the difference between combined-cue and the visual-cue group most strongly contributed to this significant difference.

Previous work with other cuttlefish has indicated that spatial information may be salient to male cuttlefish than to females (Corner and Moore, 1980). Thus, males and females were compared for the next analyses. Tables 4 and 5 compare the mean escape time and mean error for male and female cuttlefish across blocks in each experimental group. The mean escape times appear to differ between males and females on the first block of trials for the visual, spatial and combined cue groups (Table 4). However, none of these differences were statistically significant (Mann-Whitney U test: visual, $U_{(4,4)}=13$, p>0.10, spatial $U_{(2,6)}=10$, p>0.10, combined, $U_{(5,7)}=22$, p>0.10). The differences between overall mean error for males and females in all groups during the first and second block of trials were not significant (Table 5; Mann-Whitney U Test: visual-cue group, U

$_{(4,4)}$ = 13, p>0.10, spatial-cue group, U $_{(2,6)}$ =10, p>0.10, combined-cue group, U $_{(5,7)}$ =20, p>0.10, control U $_{(2,6)}$=10, p>0.10).

6.4 Discussion

Previous experiments have established that cuttlefish can learn to solve simultaneous discrimination problems (e.g. Messenger, 1977; Karson et al., 2003). This behavioral experiment is the first to demonstrate that cuttlefish are capable of solving both spatial and visual simultaneous discriminations. Though it is often difficult to tease apart spatial and visual memory, as internal representations of space may rely on visual cues such as landmarks, the spatial cues here are independent of visual landmarks (both in the maze and around the lab). The cuttlefishes' ability to solve each type of problem is approximately equal, as there is no significant difference in mean escape time and error, or patterns of improvement over blocks of trials in the visual and spatial groups. This may indicate that cuttlefish rely on both spatial and visual learning in nature. While visual information has clear utility to cuttlefish during hunting and prey selection, perhaps spatial learning has utility in evasion of predators (Karson et al., 2003).

Control cuttlefish showed no improvement in maze performance. This indicates that the improvement in performance seen in the cue-trained groups is not simply due to the cuttlefish learning that there are two exits to the maze, as the control cuttlefish would have had opportunity to learn this as well. Instead, it appears that by reinforcing the selection of a particular exit, the cuttlefish learn something about the particular cues associated with the exits. Furthermore, a decrease in the percentage of over one-minute escapes more greatly contributed to overall error than did a decrease in the number of non-escapes. This may also indicate that cuttlefish did not simply learn that there were two exits, but actually learned to solve the problem using provided cues. Individuals in the control group did not show a significant tendency to use one door or visual cue over the other when exiting, for individuals, responses were split equally between left and right and between spotted and striped exits.

It is interesting that the visual and spatial cue groups yielded significantly better learning performance than the combined cue group from previous experiments. Cuttlefish in the combined cue group were presented with two relevant cues, door direction and pattern, whereas the spatial and visual groups were presented a relevant and irrelevant cue with each trial (either direction relevant, pattern irrelevant or vice versa). In previous reversal learning work with octopuses, improvement in discrimination performance over serial reversals has been attributed not only to increased selection of relevant cue, but decreased attention to the irrelevant cue (for instance cue position or orientation, reviewed in Mather, 1995, and Boal, 1996). In fact, it appears that octopuses presented with both irrelevant and relevant cues learn discriminations better than those presented solely with relevant cues (Sutherland, 1959; Sutherland and Mackintosh, 1971; Messenger and Sanders, 1972). The data presented here are consistent with this interpretation. Such findings may provide powerful evidence for concept-formation in cephalopods, as they not only attend to particular cues, but choose to which cues they will attend, and are thereby sorting out relevant information from irrelevant information (Mather, 1995). Despite these hypotheses, most cephalopod discrimination experiments have employed successive, rather than simultaneous, presentation of cues (Boal, 1996). Thus, such data should be considered more carefully and discrimination experiments should be re-designed.

The findings here further confirm previous observations that there is great individual variation in cephalopod learning abilities (Mather, 1995; Hanlon and Messenger, 1996; Boal, 1996; Boal et al., 2000). Previous authors have suggested that these differences may be due to different temperaments or prior experiences of each individual (Mather and Anderson, 1993). It has further been suggested that the limited behavioral repertoire of paralarval cephalopods as compared with the large range of behaviors in the later benthic adults is a function of increased opportunities for learning in complex benthic habitats (Boletsky, 1992). Different prior learning experiences, then, may account for differences in behavior (Mather and Anderson, 1993). Alternatively, individual preferences for particular door directions may be a function of laterally asymmetrical eye-use. For instance, individual *O. vulgaris* appear to have a "dominant eye" which they use significantly more than the other eye while examining objects in the frontal-visual field (Byrne et al. 2002). The relationship between individual preferences and the behavior of the individual merit further investigation. Also consistent with previous work with cephalopods, is the tendency of cuttlefish to solve the maze problem within a few trials followed by a period of extreme behavioral fluctuation, in which the cuttlefish may not escape

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the maze at all during several trials (J.G. Boal, pers. comm., 2002). Whether this is a function of "boredom" or some other aspect of cephalopod behavior remains to be seen.

Observations that male *Sepia latimanus* may maintain home areas during seasonal mating aggregations (Corner and Moore, 1980) could indicate that spatial information may be salient to male cuttlefish (Karson et al., 2003). However, the data here do not indicate any performance differences between trained juvenile male and female cuttlefish in any cue group. While only two females were present in both the control and spatial cue groups, the visual and combined cue groups contained more females and yielded no sex-specific differences in learning behavior. Thus, a larger sample size may not affect this particular finding. It is possible, however, that differences in maze performance could become apparent as cuttlefish become sexually mature, as males tend to become more aggressive and active with age, whereas females do not. Further studies examining the development of discrimination learning with age could shed light on this particular question. Further discussion of the interpretation of the behavioral results is presented in Chapter 9.

Chapter 7

Immunohistochemistry

7.1 Summary

The immunohistochemical experiments presented here relate to Objectives 2 and 3: To trace the expression of calexcitin in particular brain regions during learning and to correlate expression of a calexcitin in the cuttlefish brain with learning events. Immunohistochemistry was first used to visualize regions of calexcitin expression in the optic lobes and supraoesophageal lobes. Cell counting software was used to count the number of intensely calexcitin immunopositive cells within the optic, vertical, subvertical, and dorsal basal lobes in the visual-cue, spatial-cue and control groups. Calexcitin expression was increased in the optic lobes of visual-cue trained cuttlefish and expression differed between the right and left optic lobes.

7.2 Materials and Methods

Immunohistochemical methods were employed to visualize specific regions of calexcitin immunoreactivity within the supraoesophageal and optic lobes of *Sepia officinalis*. Twelve *Sepia* brains were used for the immunohistochemical studies, four control animals, four visual cue trained animals, and four spatial cue trained animals (Chapter 6). After behavioral experimentation, cuttlefish were rapidly decapitated and the brains (optic lobes and central nervous system) were quickly removed and fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) solution (pH 7.0). Following four days of fixation in paraformaldehyde, the surrounding muscle tissue and cartilage were trimmed, and the brain tissue was immersed in 30% sucrose solution in 0.1M PBS for cryoprotection. Tissue remained in sucrose solution until it ceased to float (approximately 48 hours). Following cryoprotection in sucrose, the tissue was dipped into a beaker of 2-methylbutane resting on dry ice for 10-20 seconds, until the tissue was frozen. Each piece of tissue was then wrapped in foil and stored at -80 °C. Brain tissue pieces were serially sectioned on a cryostat at 30 μm, and slices were stored in a glycerol solution in collecting wells.

Free-floating slices were rinsed in 0.1M phosphate buffered saline (PBS; pH 7.4) three times each for 10 minutes on a rotating platform. Then, slices were pre-incubated for 60 min at room temperature in 10% normal goat serum (Sigma, St. Louis, MO) in PBS with 0.5% Triton-X. Tissue was dip rinsed in PBS and then incubated overnight in CE primary antibody in 0.1% triton-x solution at 4°C. After 16-20 hours of primary antibody incubation, slices were washed in PBS four times for 15 minutes each and then incubated in goat anti-rabbit antibody (Sigma, St. Louis, MO) in PBS (1:300 dilution) for one hour at room temperature. Slices were then rinsed in PBS and then placed in Avidin-Biotin Complex (ABC) solution for 30 minutes (Vectastain® ABC kit, Vector Laboratories, Burlingame, CA). Slices were washed in PBS twice for 10 minutes each wash and placed in 0.1M Tris buffer (pH 7.4) for 10 minutes.

Anti-calexcitin antibody (25U2) was prepared in rabbits from full-length calexcitin protein extracted from squid optic lobes (by Dr. T.J. Nelson, Blanchette Rockefeller Neurosciences Institute, Gaithersberg, MD).

Antibody labeling was visualized using diaminobenzadine (DAB Substrate Kit for Peroxidase, Vector Laboratories, Burlingame, CA). Slices were then mounted sequentially on silanated slides (Sigma Diagnostics, St. Louis, MO) and placed on a slide dryer overnight. Sections were dehydrated in an ascending ethanol series (70%, 90% and 100%), cleared with xylene, mounted with Permount and cover-slipped. All slides were viewed under a Nikon Eclipse E400 microscope and digital images were acquired using a DXM1200 Nikon digital camera.. Optical density measurements and cell counts of these digital images were obtained using NIH Image. Further details of cell counting procedure is presented in sections 7.2 and 7.3.

The negative immunohistochemical control included the substitution of normal goat serum for the primary antibody. This allowed for visualization and comparison of background staining to staining resulting from calexcitin immunoreactivity. All negative controls where normal goat serum had substituted the primary antibody yielded no staining.

7.3 Overall Patterns of Labeling

The calexcitin antibody was made from squid optic lobe calexcitin injected in rabbits, and the patterns of calexcitin staining in the squid and cuttlefish optic lobes are nearly identical (see section 7.5 and Nelson et al.1994 and 1996). Thus, it is reasonable to assume that staining observed here represents true calexcitin immunoreactivity. Calexcitin immunoreactivity was then used as a marker to observe where learning may occur in cuttlefish by correlating the regions of immunoreactivity with previous findings related to location of the processing of associative learning and memory.

The optic lobes of *S. officinalis* consist of the "deep retina" (Cajal, 1917) and the medulla (Figure 15). The deep retina is layered, and includes a striated plexiform region composed of four layers of tangential fibers interspersed with four layers of radial fibers surrounded by distinct inner and outer granule cell layers (Young, 1974; Figure 15, see also Figure 3). The plexiform region is thought to be involved in the visual analysis and classification system and the medulla appears to be a motor center and memory store (Boycott 1961; Young, 1974). The medulla is organized as a series of radial columns that converge toward the center of the tissue interspersed with horizontal dendrites which form a series of layers (Cajal, 1917; Young, 1974). However, the divisions between the layers of the medulla are not as sharp as are the divisions between layers in the deep retina (Young, 1974).

Calexcitin immunopositive cells are evident, and include various-sized centrifugal (approximately 10-15μm diameter) and amacrine cells (approximately 1-10 μm diameter) of the outer granule cell layers of the plexiform zone (Figure 9). There also appears to be staining of amacrine cell axon terminals in the tangential layers of the plexiform region, particularly tangential layers 2 and 3 (Figure 9). The amacrine cells of the inner granule cell layer are also calexcitin immunopositive (Figure 9). A band of large, calexcitin-immunopositive amacrine cells (10-15μm diameter) is located between the inner granule cell layer at the border of the plexiform region and the medulla (Figure 9).

Within the medulla, staining is seen in various sized cells (5-15μm diameter) located within "cell islands" (Cajal, 1917) found in the "zone of radial columns" (Young, 1974). The tracts of fibers (both axons and dendrites) that separate these islands from one another do not show calexcitin immunoreactivity (Figure 10).

The vertical-superior frontal system includes the superior frontal, vertical and sub-vertical lobes. These lobes are thought to play an important role in the consolidation and retrieval of memories (Boycott, 1961; Messenger, 1973, Young, 1940; Chapter 3). The superior frontal lobe exhibits immunoreactivity only in few, small cells (2-7 μm) toward the posterior region of the lobe (Figure 11). This posterior region of the superior frontal lobe sends axons to the vertical lobe (Young, 1979). Calexcitin immunoreactivity is far more evident in the inferior frontal lobe, which shows staining of cells 2-10 μm diameter toward the interior region, which are all thought to send axons to the superior frontal lobe (Figure 12; Young, 1979). There is also staining of large cells (10-15μm) toward the dorsal edge, bordering the superior frontal lobe, and the posterior edge (Figure 12). These large cells send axons to the subvertical lobe as well as to the brachial and buccal lobes found in the suboesophageal mass (Young, 1979).

In the vertical lobe, many of the "large cells" (10-15μm, Young, 1979) surrounding the entire edge of the lobe stain positive for calexcitin (Figure 13). Calexcitin immunoreactivity is particularly evident at the wall of the vertical lobe directly ventral to the subvertical lobe (Figure 14). Several small amacrine cells found throughout the vertical lobe neuropil are also calexcitin immunopositive (Figure 15). Finally, there is strong staining in the axons and the fibers of large cells

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cells (10-15 μm diameter) associated with the vertical-subvertical tracts (Figure 14).

In the subvertical lobe, immunoreactivity is prevalent in medium to large cells (5-15μm) surrounding bundles of fibers in the large anterior region, where the vertical and subvertical lobes connect (Figure 15). Many of the fibers themselves also stain positive for calexcitin. Several large cells scattered irregularly in islands throughout the posterior region of the subvertical lobe also appear to stain positive for calexcitin (Figure 15). A number of larger cells at the ventral portion of this same posterior region (subvertical lobe/precommissural lobe border) also stain positive for calexcitin.

In the anterior basal lobe, a number of medium to large (5-15μm) immunopositive cells are present at the ventral and anterior walls (Figure 16). There is also staining in the smaller neurons (1-5μm) scattered through the tangled neuropil located in both the anterior and posterior portion of the anterior basal lobe (Figure 16).

There is staining of small cells (1-5μm) scattered in irregular islands (Young 1977) throughout the dorsal basal lobe (Figure 17). There are also a number of larger calexcitin immunopositive cells (5-12μm), grouped in clusters within the median basal lobe (Figure 18). There is also distinct calexcitin staining of medium and large cells (5-15μm) at the posterior cell wall which spans both the dorsal basal and medial basal lobes (Figure 18).

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7.4 Comparison of Calexcitin Expression Between Experimental Groups

To examine the affect of maze training on calexcitin expression, the number of strongly-immunopositive cells stained at similar levels was compared in trained and control animals (Chapter 6). The regions compared included the medulla of each optic lobe, deep retina of each optic lobe, vertical lobe, subvertical lobe, and the dorsal basal lobes. These regions were chosen because initial observations indicated they showed calexcitin immunoreactivity and not prone to damage (small tears), which may have affected interpretation of the data.

Using NIH-Image, the gray-scale values of cells stained above background were determined for each slice. The range of values of cells stained above background was then divided into three categories (strongly-stained cells, medium and strongly stained cells and all stained cells). The number of cells in each of these categories was counted in 200μm by 200μm (by 30 μm deep) regions. Nine of these square regions were randomly selected, each from a different layer of tissue; thus, these counts thus reflected calexcitin immunoreactivity throughout the tissue of interest. For the deep retinal regions of the optic lobes, 200μm by 200μm squares were selected such that one encompassed the outer granule cell layer, one the plexiform layers, and one the inner granule cell layer. To eliminate experimenter bias, there was no indication to which individual or group a particular slice of tissue belonged during cell counts.

A Kruskal-Wallis one-way ANOVA was used to compare the mean number of strongly stained cells present in each of the seven brain regions among control, visual cue- trained and spatial cue-trained animals. The mean number of strongly immunopositive cells for each brain region of each experimental group is presented in Table 6. The Kruskall-Wallis test indicated that there was no significant difference between calexcitin expression in the vertical lobe or the posterior region of the dorsal basal lobe between the different experimental groups (Table 7). Calexcitin expression in the subvertical lobe was significantly higher in the control group than in the visually trained group (Tables 6 and 7). However, visually trained cuttlefish show a significantly greater mean number of stained cells in both the medulla and deep retinal region of the right and left optic lobes than either the spatial or control groups. (Table 7).

Calexcitin expression in the left and right optic lobes was compared for each group of cuttlefish using a Wilcoxon signed ranks test (Table 8). Cuttlefish in the control group yielded greater calexcitin expression in the left plexiform layer than in the right plexiform layer (Table 8). Spatially trained animals yielded the same results (Table 8). All other comparisons were statistically insignificant (Table 8).

Because the mean number of cells expressing calexcitin appeared to differ between the right and left optic lobes, it was necessary to determine whether this difference was associated with the initial directional preferences of the individuals (Table 2). Thus, the mean number of strongly stained cells in the right and left plexiform layers and medulla was compared for all animals that initially preferred the right door and for all cuttlefish that initially preferred the left door. For left-preference cuttlefish, the mean number of stained cells did not differ significantly between the medulla of the right optic lobe and the medulla of the left optic lobes (Wilxocon signed ranks test, n=4, C=3, p>0.50). The left plexiform layer appeared to yield greater staining than the right optic lobe plexiform in the left preference group with a borderline-significant p-value (Wilxocon signed ranks test, n=4, C=9, p=0.1250). The right-door preference cuttlefish showed the same pattern of expression. Calexcitin expression in the left plexiform layer was higher than in the right plexiform layer (Wilxocon signed ranks test, n=8, C=30, p=0.0547) and there was no difference in expression between the right and left medulla (Wilxocon signed ranks test, n=4, C=24, p=0.2305).

7.5 Discussion of Overall Patterns of Staining

The cephalopod visual memory system is highly distributed (and redundant) throughout the optic lobes and central nervous system, eventually funneling into a memory storage area (the vertical lobe and optic lobe medulla; Young, 1991; review presented in Chapter 3). The results presented here indicate that calexcitin is distributed within areas thought to be associated with visual learning. Specifically, calexcitin immunoreactivity is prevalent in the tangential layers of the plexiform zone, the granule cell layers and the medulla cell islands, as well as in the vertical, subvertical and superior frontal lobes.

The presence of calexcitin within fibers connecting the vertical and subvertical lobes is interesting in light of studies suggesting that these

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connections in particular play an important role in the aspects of cuttlefish predatory behavior that rely on memory (Dickel et al., 1997). The density of fiber tracts connecting the subvertical and vertical lobes has been shown to increase with age, and this connective development closely correlates with the timing of development of associative learning ability (Dickel et al., 1997). Moreover, cuttlefish at the same developmental stage raised under different temperature conditions yield differences in the density of these tracts; warmer water yields a higher density of tracts (Dickel et al., 1997). Different water temperatures yield quantifiable behavioral differences between the groups including differences in the acquisition of prey pursuit behavior as well as rates of pursuit of prey items (Dickel et al., 1997). Related to this developmental study are data from studies suggesting that hatchling cuttlefish do not follow prey that leaves the visual field ('hunt" it) because they lack a short-term memory system (Messenger, 1977). The presence of calexcitin within the fibers connecting the vertical and subvertical lobes may further suggest the importance of this particular connection as it relates to the formation of associative memories.

Previous studies have proposed that amacrine cells in the vertical and subfrontal lobes evolved from cells of the buccal lobes, which function primarily as simple reflex centers (Young, 1991). The concentration of amacrine cells into distinct lobes may have allowed for associative learning by allowing for prolonged inhibition of particular networks (Young, 1991). When the amacrine cells within the vertical lobe are blocked with drugs, memory is significantly inhibited in octopuses (Young, 1983). In the vertical lobe, calexcitin staining is seen in a nu

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number of small amacrine cells. This finding lends support to the hypothesis that protein expression of vertical lobe amacrine cells is important in associative learning.

Calexcitin expression is visible within the inferior frontal lobe. This may indicate that the inferior frontal lobe plays an important role in the memory system, but not necessarily the visual learning system. In octopuses, the inferior frontal lobes are involved in a tactile memory system that functions separately from the visual memory system (reviewed in Young, 1991). Young (1991) suggests that because of the degree of simplicity of the inferior frontal lobe relative to that of octopuses, decapods have only a limited capacity for tactile discrimination learning. In cuttlefish, the development of the inferior frontal lobe does not correlate with increases in learning prey-catching abilities (Dickel et al., 2001).

Differences between octopuses and cuttlefish in regard to the importance of the inferior frontal lobe may be explained by differences in prey capture behavior. While octopuses rely heavily on tactile information for prey detection, decapods detect their prey primarily by sight and use tentacles for capture (Young, 1988). Thus, in cuttlefish, a tactile memory system would more likely be used in prey handling rather than hunting (Young, 1988).

Calexcitin immunoreactivity is also present in the clusters of large cells in the dorsal basal lobe and the cell wall of the dorsal basal and medial basal lobes but it is unclear how calexcitin expression in these lobes may relate to learning and memory. The function of the dorsal basal lobe is unknown, but may be

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involved in escape and avoidance behavior (Young, 1971). In contrast, it may be that the dorsal basal lobe regulates the tendency to attack visual stimuli (Parriss 1965). It has been difficult to surmise the precise role of the dorsal basal lobe, as the behavioral effects of lesions to this lobe have been difficult to observe and precisely describe (Boycott, 1961). Dorsal basal lobe lesions do not produce gross motor abnormalities, but do produce slight changes in behavior in octopuses (Boycottt, 1961). For example, lesions cause a tendency of laboratory-dwelling octopuses to change their home position more frequently than normal octopuses (Boycott and Young, 1955). The median basal lobe function is also difficult to precisely describe, but is thought to be involved in escape behaviors, as well as swimming and respiration as it controls funnel and mantle movements (Young, 1977).

Overall, the results here are similar to those found by DiCosmo et al. (2000), who used immunohistochemistry to examine Nitric Oxide Synthase-like (NOS) immunoreactivity in the central nervous system of *S. officinalis*. NOS has been suggested as an important molecule underlying associative learning in a variety of organisms (reviewd in DiCosmo et al., 2000). Both calexcitin and NOS are signaling molecules that respond to influxes of calcium. Patterns of NOS expression are remarkably similar to patterns of calexcitin expression seen here (DiCosmo et al., 2000). Specifically, NOS-like immunoreactivity was observed in cells projecting from the subvertical to vertical lobes, the vertical lobe neuropil, and the cell islands of the optic lobe medulla (DiCosmo et al, 2000). Nitric oxide signaling may play an important role in long- term memory. However, the exact

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details of this role are not well understood (Hawkins, 1996). NOS release is activated when there is a calcium influx due to binding of glutamate to NMDA receptors (Garthwaite et al., 1989). NOS inhibition has been demonstrated to block visual and touch learning in octopuses (Robertson et al., 1994 and 1996). It is possible that NOS pathways and the calexcitin pathways interact, or perhaps that different types of learning invoke different molecular cascades in *Sepia officinalis*.

7.6 Discussion of the effects of training on CE histochemistry

My results suggest that intracellular calexcitin expression differs between trained and untrained cuttlefish as well as between spatially trained and visually trained cuttlefish. Specifically, cuttlefish trained to make visual discriminations yield a significantly greater number of intensely calexcitin immunopositive cells in the medulla and deep retina of the optic lobes than in spatially trained and control cuttlefish which had to ignore visual "noise" to solve the maze problem. Visual cue trained cuttlefish showed an increase in intracellular calexcitin relative to the other two groups.

In studies using the marine snail *Hermissenda*, experiments similarly indicated that snails exhibiting memory of positive association yielded a higher concentration of intracellular calexcitin than snails trained with a sensory block (Borley et al., 2002; Kuzirian et al., 2003). Kuzirian et al (2003) hypothesized that learning induced changes should be visible in cells that modulate or control downstream neurons, not downstream neurons themselves. For instance, in *Hermissenda*, learning and memory occurs and is stored in visual cells that interact with a feed-back loop to affect motor neurons. Calexcitin is found in the visual cells, but not the downstream motor cells (reviewed in Kuzirian et al., 2003). Similarly, visual learning and memory in cephalopods occurs in the optic lobes effecting motor neurons via the vertical lobe system. This may explain why calexcitin expression differs between visually trained cuttlefish and other groups in the optic lobes, but not in downstream neurons in the vertical lobe complex. Thus, immunohistochemical results from cuttlefish may further indicate that visual memory consolidation is modulated by intracellular calexcitin concentrations within the visual cells (Borley et al., 2002).

Moreover, the data here lend support to the hypothesis that visual associations are consolidated and stored within the optic lobes, whereas spatial associations are consolidated and stored in other brain regions. Young (1964) proposed that while octopus visual memories are stored in the optic lobes, tactile memories are stored in the posterior buccal or subfrontal region. Previous studies have not examined sites of storage for spatial information, but it is likely that like tactile memories, these too are stored somewhere other than the optic lobes (J.B. Messenger, pers comm., 2003). Perhaps related to this interpretation, is the fact that calexcitin is found primarily within invertebrate visual systems rather than other sensory systems (reviewed in Kuzirian et al., 2003). Calexcitin may be involved exclusively in the visual processing system. Thus, it is quite likely that learning-induced changes in calexcitin may only occur during visual learning (Kuzirian et al., 2003). This possibility is described in more depth in chapter 9.

More difficult to explain is the finding that intracellular calexcitin expression was greater for the control cuttlefish than for the spatial and visual-cue trained cuttlefish in the subvertical lobe. It is possible, perhaps, that the control group learned some association during the twenty trials. It is also possible that the time at which the trained cuttlefish were sacrificed was post-increase or post-release of calexcitin, and a more suitable time period for testing may exist. To test this, an obvious experiment would be to measure learning-induced changes in calexcitin expression at different time intervals following training (five minutes, ten minutes, one hour, 24 hours, etc). Alternatively, calexcitin could be blocked at various time periods pre- and post-association and the affect on learning could be observed. In the next chapter, western blotting techniques are employed to further quantify differences in calexcitin expression between visual, spatial and control goups. Further discussion of possible implications stemming from the immunohistochemical (and western blotting results from Chapter 8) is presented in Chapter 9.

Chapter 8

Calexcitin Expression Quantified Using Western Blots

8.1 Summary

Western blotting was used to further examine Objectives 3 and 4: To correlate expression of a calexcitin in the cuttlefish brain with learning events and to compare the expression of a learning-related protein among various brain regions of visual cue-trained, spatial cue-trained and control cuttlefish. Homogenate, cytosolic and microsomal fractions were prepared from the visual-cue trained, spatial-cue trained and control cuttlefish. Calexcitin was quantified in the optic lobes and supraoesophageal mass of each group. Calexcitin expression was greater in the cytosol and microsomes of optic lobes of visual-cue trained cuttlefish than other regions in other groups. These analyses also indicated differences in expression between the left and right optic lobes. These results were similar to those found in the immunohistochemical experiments (Chapter 7).

8.2 Methods

To prepare tissue for homogenization, cuttlefish were rapidly decapitated and the optic and supraoesophageal lobes were quickly removed and immediately placed in separate microcentrifuge tubes on dry ice. The tubes were then i transferred to a -80 °C freezer. Before preparation of the fractions, each piece of frozen tissue was weighed. The weight of each piece of tissue from each cuttlefish is shown in Table 9.

Fraction Preparation

Homogenate, cytosolic fractions and microsomal fractions for each piece of tissue were prepared using a modification of Gray and Whittaker's method

(1961; modified by T.J. Nelson). A buffer (referred to here as Buffer A) was made from 50.0mM Tris HCl (pH7.5), 10.0mM NaCl, 1.0mM EDTA and 0.5mM EGTA and stored on ice. 30µl of protease inhibition cocktail (PIC) (Sigma-Aldrich) was diluted into 1470 µl Buffer A. Each piece of brain tissue was loosely homogenized (35 strokes) in 1 ml of Buffer A in a chilled Dounce grinder (2 ml capacity).

50 µl of the homogenate were added to 5 µl beta mercapatoethanol in 45µl of NuPage™ 4x LDS sample buffer (Invitrogen Corporation), sonicated for 5 seconds and stored in a freezer at -20°C.

The remaining portion of the homogenate was placed in a 4°C centrifuge and rotated at 10,000 g for 30 minutes. After centrifugation, the pellet was discarded and the supernatant was placed in a 4°C ultracentrifuge and rotated at 100,000 g for 1 hour. The resulting supernatant from this spin was the cytosolic fraction. The cytosolic fraction was added to an equal amount of 10% betamercapetoethanol in LDS sample buffer, sonicated and stored at -20°C.

To make the microsomal fraction, the pellet leftover from the cytosolic fraction was re-suspended in 200 µl of Buffer A, sonicated for 10 seconds and placed in a 4°C ultracentrifuge and rotated at 100,000 g for 1 hour. The supernatant was discarded, the pellet re-suspended in 100 µl of Buffer A, sonicated for 3 seconds and an equal amount of 10% betamercapetoethanol in LDS sample buffer was added. The microsomal fractions were also stored at -20°C. Thus, in total, each cuttlefish yielded 9 samples; a homogenate, cytosolic and microsomal fraction from each individual's left optic lobe, right optic lobe and supraoesophageal lobes.

Protein Content Determination

The Bradford Method (Bradford, 1976) was used to determine the protein concentration of each sample. First, a standard curve was prepared using bovine serum albumin (BSA) and was used for calibration of a standard curve (Figure 19).

5 μl of each brain tissue sample (homogenate, cytosolic and microsomal fractions) were then diluted in dH_20 to 1:300. 1 ml Coomassie reagent was added to the diluted samples and readings were taken immediately in a spectrophotometer at 595 nm. The actual protein concentration was then determined for each sample using the slope intercept formula from the standard curve (where $(Y-A)/B = X$). Then, the concentration of undiluted sample was found by multiplying the concentration by the dilution factor (310). This number was divided by the number of microliters of sample tested in 100 μl of buffer. This calculation was used to find the concentration of protein in 1 microliter. This was then used to determine the amount of sample needed to load 10 ug/μl of each sample in each gel (the desired protein concentration divided by actual protein concentration equals the amount of sample needed). Each lane holds 19 μl of sample, thus the amount of sample needed subtracted from 15 determined the remaining amount of sample buffer to add to the samples. See Figure 19 for the standard curve and Table 10 for calculations of the crude protein concentration of each sample.

Based on initial results from naïve animals (Karson, 2001, unpublished data), the desired protein concentration chosen was 10 ug/µl. The gels were loaded such that the same type of fraction of the same brain region for different animals was present on each gel.

Electrophoresis

Each sample was denatured by heating the microcentrifuge tubes in 85 °C water for 8 minutes. After the samples were boiled, they were spun on a vortex and chilled on ice for 2-3 minutes. 1 liter of running buffer was prepared using 100 ml of 10X Tris/Glycine/SDS buffer (Invitrogen) in 900 ml dH_2O. NuPAGE® Novex 4-12% Bis-Tris pre-cast gels (15 wells, Invitrogen) were used and gels were run in the Xcell SureLock™ Mini-cell system (Invitrogen). The upper and lower chambers of the mini-cell were filled with running buffer, and the gels were loaded with samples and molecular weight markers (See-Blue Plus, Invitrogen) in three different lanes. Two gels were run simultaneously at 125V for 90 minutes.

Once the gels were run completely, they were removed from the mini-cell and prepared for transfer to membrane. Transfer Buffer was prepared using 50 ml of 20X NuPAGE® transfer buffer (Invitrogen), 1 ml of NuPAGE® antioxidant (Invitrogen), 200ml methanol and 749 ml deionized water. Blotting pads were soaked in700 ml of transfer buffer until saturated. Nitrocellulose membranes (45 µm pore size) were cut to size and soaked in transfer buffer for five minutes. Gels were transferred to membranes at 30V for 1 hour according to instructions provided by Invitrogen.

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Protein detection was performed by staining the membrane directly using the VECTASTAIN® ABC system. To spare antibody, membranes were cut down to include only the regions surrounding 22 kDa (approximate size of calexcitin molecules) by using the marker lanes as cutting reference marks. Because calexcitin was found in the optic and supraoesophageal lobes using immunohistochemistry, it is reasonable to assume that the band on the membrane at 22 kDa is, in fact, calexcitin. The membranes were rinsed for 15 minutes in 4 changes of 0.1% Tween 20 in Tris-buffered saline (TTBS) with evaporated milk to block non-specific binding. 100 μl of goat normal serum (Sigma) was added to 5 ml TTBS and 100 μl of biotinylated goat anti-rabbit antibody (Sigma). The membranes were incubated in this solution with gentle agitation for 30 minutes at room temperature. The membranes were then rinsed with gentle agitation for 15 minutes in TTBS. Membranes were then transferred to vectastain® ABC reagent, again incubated for 30 minutes at room temperature with gentle agitation. The membranes were then transferred to a substrate solution for 15 minutes (DAB substrate kit for peroxidase, Vector Laboratories). Membranes were then rinsed in dH_2O, air dried, wrapped in cellophane and stored at room temperature.

A flatbed scanner (Hewlett Packard Office Jet G85) was used to scan the dyed membranes and create a series of high-resolution digital images. The optical density (including staining intensity and width of the protein signal) was found using NIH Image software. For each membrane, levels were calibrated using the background as a zero value. Images of each membrane containing homogenate, cytosolic and microsomal fractions from each piece of tissue from each of the animals are shown in Figures 20-22.

8.3 Results

Because each sample was run on two different gels (one comparing the same region and fraction for different animals, and one gel comparing different regions in a single animal), the mean optical density for each brain region of each individual was calculated by averaging the two optical density values.

A series of one-tailed Mann-Whitney U test were then used to compare the mean optical density of calexcitin expression on the gels among spatially trained, visually trained and control animals (Table 11). These analyses indicated that calexcitin expression was significantly higher in visual cue trained cuttlefish than spatially trained and control cuttlefish in the left optic lobe homogenate, and the right optic lobe microsomal fractions. However, in the right optic lobe cytosolic fraction, calexcitin expression was significantly lower in the visual and spatial cue groups than in the control group. All other comparisons were not statistically significant (Table 11).

Overall, the amount of calexcitin present in the homogenate of the three brain regions did not differ (Friedman ANOVA by ranks, $Fr=-107.25$, $p>0.50$). The amount of calexcitin present in the left and right optic lobes was significantly greater than in the supraoesophageal lobes for both the cytosolic and microsomal fractions (Friedman ANOVA by ranks, cytosolic $Fr=19.8$, $p<0.01$; $Fr=13.17$, $p<0.01$).

To compare calexcitin expression in the right and left optic lobe within each group, a series of Wilcoxon signed ranks tests were used. Results are shown in Table 12. Calexcitin expression was significantly greater in the left optic lobe homogenate than the right optic lobe homogenate for the visual cue group. No other comparisons yielded significant results.

To determine whether initial door direction preference was related to calexcitin expression in the left and right optic lobes, the data were placed in two groups, left preference and right preference, and mean optical density was compared between the left and right optic lobes. Among left-door preferring cuttlefish, calexcitin expression was slightly higher in the left homogenate fraction than in the right homogenate fraction (Wilxcoxon signs rank test, $n=8$, $C=13$, $p=0.0938$). However, expression did not differ in the cytosolic and microsomal fractions (Wilxcoxon signs rank test, cytosolic, $N=8$, $C=5$, $p>0.50$; microsomal, $n=8$, $C=8$, $p>0.50$). Among right-door preferring cuttlefish, expression did not differ between the left and right optic lobes (Wilxcoxon signs rank test, $n=5$; homogenate, $C=17$, $p>0.50$, cytosolic, $C=6$, $p>0.50$; microsomal, $C=17$, $p>0.50$).

To determine whether initial door preference was related to the weight of the corresponding optic lobe, Wilcoxon signs rank tests were used to compare the weight of the right and left optic lobes among right and left-door preference cuttlefish. This comparison was not statistically significant (right, $n=7$. $C=12.5$, $p>0.50$; left, $n=5$, $C=11$, $p=0.2188$).

8.4 Discussion

The results here are similar to those found using immunohistochemistry. Calexcitin expression appears greater in the optic lobes, specifically, the left optic lobe homogenate and right optic lobe microsomes of visually trained cuttlefish than in the optic lobes of spatially trained and control cuttlefish. Additionally, like the immunohistochemical analysis, among visual cue trained cuttlefish, calexcitin expression was greater in the left optic lobe than the right optic lobe homogenate. Thus, the western blot results may further demonstrate the role of the optic lobes in processing and storing of visual memories.

During associative learning in *Hermissenda*, calexcitin is translocated from the cytosol to the membrane of the endoplasmic reticulum (Nelson and Alkon, 1995; see review in Chapter 4). Thus, it was initially hypothesized that associative learning would be associated with decreases in cytosolic calexcitin and increases in microsomal calexcitin (Chapter 5). The increased levels of microsomal calexcitin in the right optic lobes of visually trained cuttlefish may be associated with the timing of a memory consolidation event. This interpretation may also explain why there was more calexcitin in the cytosol of the right optic lobe of control animals than in the trained groups.

Greater overall levels of calexcitin found in the optic lobes relative to the amount found in the supraoesophageal lobes may indicate that calexcitin signaling is important in learning visual associations. Other associations that involve different brain regions like spatial, tactile, or chemical, associations may in fact rely on molecular cascades exclusive of the PKC/calexcitin system.

Overall, the left optic lobe yielded higher calexcitin expression than the right optic lobe, regardless of the initial preference of the cuttlefish. In the visual-cue-trained cuttlefish, it appears that calexcitin expression is increased in the optic lobe opposite to the direction of the initial preference. In the control and spatial-trained group the opposite trend is observed optic lobes, and, expression is greater in the optic lobe associated with the initial direction preference. Confounding the results is the immunohistochemical analysis which demonstrated more calexcitin in the left optic lobes of all the cuttlefish, regardless of their initial door preference. Again, it may be that cuttlefish prefer one eye over the other, and processing in the non-preferred eye requires a greater amount of neuronal "work." Thus, differences between the optic lobes may relate to internal biases rather than external influences. The preference for spots or stripes appears unrelated to calexcitin expression between optic lobes.

One other possible explanation is that differences between the eyes result from lateralization of eye use. *Octopus vulgaris* uses monocular vision rather than binocular vision to hunt and attack its prey (Muntz, 1961). Furthermore, *O. vulgaris* shows a marked preference for using a particular eye for monocular vision (Byrne et al., 2002). The tendency to use one eye more than the other may result in differential processing ability between the two eyes. In fact, door preference may have resulted from a propensity to use one eye more than the other. However, unlike octopuses, cuttlefish tend to use binocular vision rather than monocular vision (Muntz, 1961). Further experimentation is necessary to determine whether cuttlefish use monocular vision during visual associative learning..

Alternatively, it is possible that different features of visual stimuli are processed by the right and left optic lobes (Byrne et al., 2002). This hypothesis has not yet been tested. This is highly unlikely, as the structure does not appear to differ greatly between the two optic lobes. More likely is that differences between calexcitin in the right and left optic lobes may be associated with the timing of transfer of information between the two lobes. Muntz (1961) demonstrated that octopuses could be trained to make visual discriminations by presenting objects in only one visual field (monocular training). These octopuses then made correct responses to the same discrimination problem presented to the untrained eye. Transfer between the lobes is interrupted when the optic tracts are severed, or when the vertical lobe is removed. It is possible that the training was monocular for some cuttlefish, and that complete transfer of information between the optic lobes had not yet occurred at the time of decapitation. No data exist regarding the timing of interocular transfer of visual associations in cephalopods. While it is possible that greater calexcitin expression in the left optic lobe results from the association of the stimuli first on the left side, further experimentation remains necessary.

Chapter 9

General Discussion

The experiments successfully addressed the initial objectives. First, the relative rates of simultaneous discrimination learning based on visual versus spatial cues were compared. Second, calexcitin expression in the optic and supraoesophageal lobes was successfully traced. Third, calexcitin expression was correlated with visual learning specifically. Finally, calexcitin expression patterns in brain regions of different groups were successfully compared. The results suggest several areas of further investigation, discussed in the following sections of this chapter.

9.1 The Function of Learning in Cephalopods

Sepia officinalis is able to solve a simultaneous discrimination problem using both visual and spatial cues, suggesting that both visual and spatial cues are salient to cuttlefish in nature. However, it is possible that the ability to learn spatial information relates more specifically to general processes underlying flexible foraging strategies (lose-shift, win-stay; Mather, 1991; Hanlon and Messenger, 1996). While the ability to make visual discriminations has been documented in cuttlefish (Sanders and Young, 1940; Wells, 1962; Messenger, 1973), only recently has the ability to solve spatial problems been suggested (Karson et al., 2003; Appendix C). In octopuses, spatial learning has clear utility, for example in locating their home dens (Mather, 1991). Unfortunately, there have been no studies that directly relate to short and long-term movement patterns of *S. officinalis* in nature. Such data are necessary to develop solid hypotheses for the specific function or functions of spatial learning in cuttlefish.

Regardless of the precise function, it seems unlikely that *Sepia* would demonstrate "excess" spatial learning abilities in the laboratory, that is, abilities not used in nature (Hanlon and Messenger, 1996; Karson et al., 2003).

Many hypotheses have addressed general functions of learning in cephalopods. First, it is possible that reliance on learning is imposed by the complexity of the cephalopods' natural habitats (Packard, 1972; Hanlon and Messenger, 1996). For example, coral reefs are highly competitive environments with many predators and prey items. Cephalopods that live in the coral reefs must constantly determine foraging and mating strategies while dealing with many different predators and complex surrounds (Hanlon and Messenger, 1996). Learning may play a key role in the ability of cephalopods to negotiate these environmental complexities. However, it is important to note that learning may not be restricted to those cephalopods living in coral reefs or other complex environments (Hanlon and Messenger, 1996).

It is possible that natural selection acted on traits allowing various species to select appropriate behaviors based on flexible use of stored memories, for instance, complex associations previously encountered in the environment (Day et al., 1999). In this framework, learning in cephalopods may be considered a mechanism related to phenotypic plasticity (Hanlon and Messenger, 1996). Phenotypic plasticity is defined as an "adaptation to environmental variation in time and space" (Dukas, 1998). In changing environments, organisms must often change their responses to particular stimuli, and under such circumstances these species benefit from phenotypic plasticity (Dukas, 1998).

As an example of the importance of plasticity, many cephalopod species change habitats as they age (Hanlon and Messenger, 1996). These habitat changes affect their diets as well as foraging and predator avoidance strategies at different developmental stages and in different habitats. Learning allows animals to change their responses to stimuli when previously experienced associations between stimuli and events are no longer relevant (Dukas, 1998). In cuttlefish, associative learning is commonly used to discriminate between different prey items (Messenger, 1973). This has utility because the size, diet and habitat changes radically during their lifetime (Wells, 1962). Because a cuttlefish must alter its diet and hunting strategy as it develops, the ability to replace old foraging tactics with newer ones would certainly be useful, and this is greatly facilitated by learning. The fact that learning is widespread throughout the coleoids, but not in nautiluses, supports this hypothesis (Wells, 1962). Hard-shelled, slow-moving nautiluses, which do not show drastic changes in lifestyle with growth, might be better off diverting resources elsewhere, as learning would not greatly increase these animals' abilities to forage or avoid predation (Solem, 1974).

Wells (1962) suggests that such developmental shifts in foraging behavior are unlikely innate, as there is great individual variation in diet preference and responses to different types of prey can be easily trained in cephalopods. Learning is an effective strategy only when the organism's environment remains stable to some extent. If the meaning of a particular stimulus changes too frequently, the costs of learning and re-learning probably exceed the very short-lived benefits (Dukas 1998).

Further experiments comparing learning abilities among different species of cuttlefish (as well as between octopuses and cuttlefish) may allow for additional hypotheses related to the function of learning in different cephalopod species. Specifically, observations of *S. pharaonis* and *S. officinalis* in the laboratory indicate striking behavioral dissimilarities (L. Dickel, pers. comm., 2003). For instance, while *S. officinalis* tends to remain partially buried under the substrate, under plants or in corners of laboratory tanks, *S. pharaonis* constantly swims around the tank (pers. obs.). If such differences are present in natural populations, one might expect that these species use spatial information differently. *S. officinalis* may use spatial learning to find adequate "resting spots," or *S. pharaonis* may use spatial information to determine their swimming routes. Or, conceivably, in nature one of these species may not use spatial learning at all. Similarly, one might expect that cephalopods living in dark (deep) habitats rely on visual information and thus learning to a lesser extent than those in shallower regions with more ambient light (Maddock and Young, 1987). Thus, further comparisons among cephalopod species from different marine habitats and comparative observations of field behavior remain necessary.

One final consideration of this topic is related to learning and the short life cycles in cephalopods. Most extant cephalopods, with the exception of *Nautilus*, "live fast and die young" (Hanlon and Messenger, 1996). A short lifespan may be viewed as an adaptation to the environment. Unlike teleosts, cephalopods do not store lipid reserves. It may be advantageous for coleoids to attain maturity and reproduce quickly, rather than risking surviving during a period when energy demands are high but suitable prey is scarce (O'Dor and Webber, 1986). Octopuses and decapods hunt and eat daily (Hanlon and Messenger, 1996). An inadequate foraging period for an adult cephalopod may be a good feeding period for a hatchling that is much smaller and feeding on different food sources. In addition to differences in feeding behaviors in adult and juvenile cephalopods, there are differences in the relative efficiency of locomotion. Locomotion by jet power does not work as efficiently as locomotion by fin movements unless the organism. is relatively small (O'Dor and Webber, 1986). Short life cycles may allow cephalopods spend a proportionally large portion of their life in a developmental stage during which they have adequate food resources to easily accommodate their energetic requirements and more efficient locomotion. This being the case, short life cycles may have evolved in a species that has complex learning ability rather than vice versa.

9.2 Relevant and Irrelevant Cues

The results indicated that the combined-cue group had greater initial difficulty learning the maze than either the visual or spatial cue groups. It would be informative to perform experiments from the same population of cuttlefish to determine the nature of the difference in learning ability between the combined cue and single relevant-cue groups. It may be that learning is enhanced by the simultaneous presentation of irrelevant and relevant cues, whereas linking these cues may "confuse" the cuttlefish (Sanders, 1975). Perhaps cuttlefish are not only learning that one door is correct, but also that the other door is incorrect (Sanders, 1975). To examine this, serial reversal experiments should compare between the combined-cue and the single relevant cue groups (i.e visual cue or spatial cue relevant) and determine whether these cuttlefish learn reversals. An additional extension of the behavioral work would be to set up a conditional learning paradigm where cuttlefish must learn to choose an exit based on a visual cue, but the correct visual cue depends on the context (for example, light on or off). Such experiments are currently underway (Boal and Karson, experiments to begin 6/2003).

9.3 Individual Variation in Behavior

The behavioral results presented here support previous studies demonstrating a tremendous amount of individual variation in learning abilities between individual cephalopod subjects (Mather and Anderson, 1993). Individual variation in behavioral abilities may be a result of particular prior experiences. To test this, one might compare discrimination learning between enriched environment raised and non-enriched environment raised cuttlefish or between wild-caught and laboratory-reared cuttlefish. Previous authors have suggested different "personalities," or temperaments, among individual octopuses (Mather and Anderson, 1993). Such differences may not only result from individual experience, but also from different levels of motivation or agitation. Thus, it may be informative to develop a variety of related learning paradigms and determine whether faster learning rates of particular individuals remain consistent regardless of the paradigm used. Such experiments might also demonstrate a general correlation between particular types of behavioral abilities of cephalopods, as has been observed in several vertebrate models.

9.4 Calexcitin and Visual Associations

While the immunohistochemical data indicated that the local patterns of staining did not appear to differ between the trained and control groups, the intensity of calexcitin staining within particular regions did differ between the groups, indicating higher levels of intracellular calexcitin in some groups. More intensely calexcitin-stained cells were located within the optic lobes of visual-cue trained cuttlefish than spatial or control groups. This finding was supported by the western blot data, which indicated that calexcitin expression was greater in the optic lobe homogenate and microsomal fractions of visual cue-trained cuttlefish than in the homogenate and microsomal fractions of the spatial and control groups.

Failure to demonstrate differences in calexcitin between spatial-cue trained and control cuttlefish may indicate one of several things. First, it is possible that the optic lobe is involved in learning visual but not other types of associations. In octopuses, tactile and visual associations are processed separately, though they do interact in the vertical/superior frontal lobe system (Young, 1991). It may be useful to compare calexcitin expression in visual-learning-related brain areas of octopuses that have learned a visual association to calexcitin expression in the chemotactile-learning-related brain area (the inferior frontal lobe) of octopuses who have learned chemotactile associations. I suggest using *Octopus* because the circuitry underlying visual and chemotactile learning is better understood in octopuses than cuttlefish (see Young, 1991). Spatial learning may occur in yet another set of lobes in the cephalopod brain, perhaps involving basal or suboesophageal regions that are more directly related to the regulation of motor behavior. Although initial hypotheses suggested that the optic lobes process spatial information from the two visual fields, there is no reason to assume that the optic lobes are involved with spatial learning at all (JB Messenger, 2003, pers. comm.).

A second possible explanation for the lack of calexcitin increase during spatial learning is that calexcitin is involved specifically in the molecular cascade underlying visual learning but not spatial learning. This has been proposed in other organisms (e.g. *Hermissenda*) where calexcitin appears exclusively involved with visual systems (Kuzirian et al., 2003). Much like *Hermissenda*, a great deal of the learning and memory processes in cephalopods occur within in the visual processors themselves (Alkon, 1989; Alkon et al., 1990). Kuzirian et al. (2003) conclude that under such circumstances, learning-induced changes in memory would be found in cells that modulate the output of downstream neurons and not the downstream neurons themselves. In other words, differences in intracellular calexcitin levels would be detected within the visual cells rather than the motor neurons these cells regulate. Certainly, the results here indicated changes in visual cells and not within the motor-related brain areas. However, baseline levels of calexcitin were found within motor activity-regulating regions of the supraoesophageal mass and it is important to note that the dorsal and medial basal lobes are connected to the optic tracts. It would be interesting to use immunohistochemistry and western blotting to determine whether calexcitin is also present in the suboesophageal regions that are motor-regulators and are not connected to the visual system. The presence of calexcitin in these regions may suggest that calexcitin does not strictly play a role in visual learning and memory.

The presence of calexcitin in non-visual processing brain areas may indicate that the role is not limited to visual associations or visual learning (Kuzirian et al., 2003). Even when found in brain regions associated with visual learning, in cuttlefish, calexcitin may simply be involved in visual processing and not visual associative learning. In fact, calexcitin may be present in any cells associated with forms of learning and memory or even other functions that are calcium regulated or modulated (Kuzirian et al., 2003). To test whether calexcitin in the optic or supraoesophageal lobes is associated with visual learning specifically, it would be interesting to block calexcitin in these regions using lead or perhaps anti-sense RNA or antisense peptides. By doing so, one could determine whether blocking calexcitin in the manner also blocks visual learning. This would more clearly demonstrate a direct relationship between calexcitin and visual learning, rather than merely between calexcitin expression and neuronal activity. One also might use immunohistochemical studies of calexcitin to observe patterns of expression in the motor-regulatory suboesophageal lobes.

Kuzirian et al. (2003) further surmise that the baseline levels of calexcitin present in naïve snails and in brain regions unaffected by learning suggest that calexcitin remains at a particular homeostatic level and/or is regulated by other sensory inputs. A similar phenomenon may be observed in cuttlefish. To test this hypothesis, one might observe the effects of different types of sensory input exclusive of visual input on intracellular calexcitin levels. This could be accomplished using blinded cephalopods, or cephalopods kept in the dark (Kuzirian, pers comm., 2003). Again, observing tactile learning and calexcitin expression the inferior frontal lobe of octopuses may also help get at this question.

9.5 Time Course of Learning and Calexcitin Expression

It would be informative to observe the time course of visual discrimination learning in this paradigm and compare it to time courses of calexcitin expression in the optic lobes. It may be particularly useful to observe the relationship between calexcitin expression and the visual learning paradigm where cuttlefish are trained to associate the visual stimulus of a prey item with pain (Messenger, 1973). This paradigm is recommended, as it has been particularly well studied and time course has been determined (Messenger, 1973b; Agin et al., 1998). Post-learning calexcitin increases return to baseline levels 24 hours after associative learning in *Hermissenda* (Kuzirian et al., 2003). It would be useful to determine whether such results are found during associative learning in cephalopods. Additionally, it would be interesting to block calexcitin at particular time intervals to determine whether this has any effect on learning. Such experiments are currently being attempted with *Hermissenda* (Kuzirian et al., 2003). One way to block learning may be through the introduction of lead, which has been shown to block learning in *Hermissenda* by blocking calcium channels, thereby inhibiting calexcitin (Kuzirian et al., 1996 and 1998). In these experiments, blocking with lead did not have toxic effects on *Hermissenda* or block normal activity patterns (Kuzirian et al., 1998). This may be an equally useful technique to block visual associative learning in cephalopod molluscs. Antisense RNA and peptides could also be used to block calexcitin and thus perhaps associative learning.

9.6 Interocular Transfer

Perhaps one of the most confusing results of these experiments was the difference in calexcitin expression observed between the left and right optic lobes of the various groups. Not only did calexcitin expression differ between the right and left optic lobes, visual learning yielded greater increases in one of the two optic lobes. Such results may warrant further studies examining the extent to which the left and right optic lobes (or other brain regions) are functionally lateralized. Greater calexcitin expression observed in one optic lobe, may simply imply that a greater number of connections must be established to make associative connections in one optic lobe relative to the other. For instance, because the non-preferred eye is used less frequently it may require a greater threshold of stimulation to initiate activity because there are fewer pre-existing associative connections between cells. It is possible, then, that biases in eye use do directly impact calexcitin expression and result in differences between the right and left optic lobes.

Other lobes may also exhibit lateralization of function that is specific to particular sources of sensory input, or even from the right and left visual fields. However, Wells (1978) asserts that octopus brains are probably functionally symmetrical in terms of the right and left sides of the body and that either side of the brain could control the whole octopus. Because the structure of the right and left optic lobes does not appear to differ, and there is no reason to assume that the right and left optic lobes process different features of the visual field (J.B. Messenger, pers. comm., 2003). Furthermore, hemispheric lateralization is not found in other invertebrates. Further details related to the type of information that is transferred between the optic lobes of cephalopods and the timing of this transfer are yet to be ascertained.

Regardless of whether there is an equivalent to "hemispheric lateralization" in cephalopods, as is observed in vertebrate brains, interocular transfer is an important aspect of visual processing in cephalopods (Muntz, 1962). Interocular transfer in cephalopods occurs via the vertical lobe (Muntz, 1962). Octopuses use monocular vision, thus interocular transfer is quite important. Octopuses trained to use on eye to make a simple visual discrimination show complete transfer to the other side (Muntz, 1961). Transfer is blocked when connections between the vertical and superior frontal lobes are severed (Muntz, 1961). Similarly, touch experiments with octopuses show transfer of information between arms on the left and right side of the body via the supraoesophageal lobes (Wells and Young, 1966). Tactie transfer experiments indicate that if the supraoesophageal lobes are split before training, tactile learning occurs only in the trained side, whereas the untrained and trained sides perform equally if the split is made after training (Wells and Young, 1966). In light of evidence here suggesting molecular differences between the right and left optic lobes in cuttlefish, and other studies suggesting biases in eye use among individual octopuses, such inquiries warrant further exploration (Byrne et al., 2002).

9.7 Future Directions

It would be interesting to compare the vertebrate-like learning capabilities and the underlying neural correlates of the abilites of cephalopods to that of vertebrates. It would be equally interesting to compare the neural correlates underlying learning in cephalopods to those observed in simple molluscan systems. Comparisons between cephalopods and vertebrates may further illuminate general principles underlying complex forms of learning. Whereas comparisons between cephalopods and simple molluscan systems could inform us, for example, to what extent cephalopods use complex learning and to what extent they solve problems using more ancient molluscan strategies. Eventually, comparisons of complex learning in vertebrates and cephalopods will aid in our understanding of the principles underlying the organization and evolution of complex brains and behaviors.

APPENDIX A

Tables

Table 1: Brain lobes associated with the supraoesophageal mass *Sepia officinalis* and presumed functions as identified in the squid *Loligo* (Young, 1974, and 1977).

Region	Function Controlled
Perioesophageal mass	Regulation of jet propulsion and escape behavior
Subpendunculate lobe	Regulation of ocular pressure
Precommisural lobe	Unknown
Anterior basal lobes	Steering (dipping/rolling)
Posterior basal lobes (median and dorsal)	Steering (yawing), jet propulsion, tentacle movement and chromatophores
Inferior frontal lobe	Tactile learning and memory
Superior frontal lobe	Visual learning and memory, control of attack behavior,
Vertical lobe	Tactile and visual learning and memory
Subvertical lobe	Learning and Memory
Optic lobes	Vision

Table 2: Initial descriptions of the cuttlefish trained in the behavioral experiments, including name, sex, group, mantle length at beginning of experiment, door direction preference and visual-cue preference.

Name	Sex	Group	Mantle Length (cm)	Directional preference	Visual-cue preference
Bruiser	male	control	10.5	left	spot
Freckle	female	control	6.3	right	spot
Isis	male	control	7.4	right	spot
Pokey	female	control	8	right	spot
Nougat	male	control	7.5	right	stripe
Raoul	male	control	8.8	right	stripe
Willow	male	control	7.3	left	spot
Stohlio	male	control	7.2	right	stripe
Howard	male	spatial	9.7	left	stripe
Kidd	male	spatial	7.2	right	spot
Satine	female	spatial	7.6	right	spot
Snookums	male	spatial	9	left	spot
Bindi	female	spatial	8.1	right	spot
Marilyn	male	spatial	8.2	right	spot
Prince	male	spatial	7.1	left	stripe
Nestle	male	spatial	8.5	right	spot
Guy	male	visual	9.1	right	spot
Ipanema	female	visual	7.6	left	stripe
Mustang	female	visual	7.6	left	spot
Orion	male	visual	6.2	right	stripe
Scratch	male	visual	7	left	stripe
7&7	female	visual	8.4	left	stripe
Stone	female	visual	7.9	right	spot
Bilbo	male	visual	6.4	left	spot
Lola	female	visual	7	right	stripe
Matey	male	combined	6.8	right	stripe
Chip	male	combined	7.3	right	stripe
WW	female	combined	7.5	right	stripe
Fleck	male	combined	7.4	left	spot
Dottie	male	combined	7.8	left	spot
Mrs. Dash	female	combined	7.7	right	stripe
Pan	male	combined	9.7	left	spot
Maximus	male	combined	10.1	right	stripe
Biggie	male	combined	9.6	right	stripe
Little Suzy	female	combined	6.4	left	spot
Fubar	female	combined	6.2	left	spot
Lena	female	combined	7.1	left	spot

Table 3: Repeated measures ANOVA results of cuttlefish maze performance across the first, middle and last block of experimental trials (or all four blocks of trials for control cuttlefish) for all groups. Analyses include decrease in mean maze escape time, decrease in mean overall error, decrease in percentage of error exclusive of non-escapes and decrease in non-escapes. Significant decreases demonstrate learning and are indicated by a p-value listed in boldface.

Analysis	n	Group	F	p-value
Decrease in escape time between the first, middle and last block of trials	8	Control	$2.094_{(3,21)}$	p>0.25
	8	Visual	$9.075_{(2,14)}$	**p<0.005**
	8	Spatial	$8.929_{(2,14)}$	**p<0.005**
	12	Combined	$14.311_{(2,22)}$	**p<0.0005**
Decrease in overall error between the first, middle and last block	8	Control	$2.000_{(3,21)}$	p>0.10
	8	Visual	$11.53_{(2,14)}$	**p<0.001**
	8	Spatial	$12.631_{(2,14)}$	**p<0.001**
	12	Combined	$28.774_{(2,,22)}$	**p<0.0005**
Decrease in percentage of error (exclusive of non-escapes)	8	Control	$1.000_{(3,21)}$	p>0.25
	8	Visual	$1.663_{(2,14)}$	p >0.25
	8	Spatial	$5.810_{(2,14)}$	**p<0.025**
	12	Combined	$26.143_{(2,22)}$	**p<0.0005**
Decrease in percentage of non-escapes	8	Control	$2.492_{(3,21)}$	p>0.25
	8	Visual	$2.726_{(2,14)}$	p>0.1
	8	Spatial	$14.212_{(2,14)}$	**p<0.0005**
	12	Combined	$4.224_{(2,22)}$	**p<0.05**

Table 4: Mean ± standard error of maze escape times (seconds) for the first, middle and last block of five trials during training for male and female cuttlefish in the control, visual, spatial and combined cue groups. Control group includes the 2nd block of five trials as "middle block." None of these differences were statistically significant (Mann-Whitney U test: visual, $U_{(4,4)}$ =13, p>0.10, spatial U $_{(2,6)}$ =10, p>0.10, combined, $U_{(5,7)}$ =22, p>0.10).

	First Block		**Middle Block**		**Last Block**	
	Male	Female	Male	Female	Male	Female
Control	109.23 ± 38.69	114.80 ± 55.40	81.37 ± 61.64	120.40 ± 77.20	32.13 ± 14.57	26.70 ± 1.10
Visual	338.70 ± 90.90	190.00 ± 44.77	235.55 ± 57.26	144.70 ± 67.30	36.55 ± 15.94	31.00 ± 7.57
Spatial	238.96 ± 79.18	381.80 ± 38.2	135.03 ± 34.02	141.70 ± 76.7	27.76 ± 6.65	15.00 ± 4.6
Combined	406.26 ± 60.63	514.72 ± 37.28	384.31 ± 59.43	387.12 ± 60.70	49.43 ± 17.52	46.8 ± 13.07

Table 5: Mean ± standard error of overall percentage of error for the first, middle and last block of five trials during training for male and female cuttlefish in the control, visual, spatial and combined cue groups. Control group includes the 2nd block of five trials as "middle block." The differences between males and females were insignificant (Mann-Whitney U Test: visual-cue group, $U_{(4,4)} = 13$, $p>0.10$, spatial-cue group, $U_{(2,6)} = 10$, $p>0.10$, combined-cue group, $U_{(5,7)} = 20$, $p>0.10$, control $U_{(2,6)} = 10$, $p>0.10$).

	First Block		**Middle Block**		**Last Block**	
	Male	Female	Male	Female	Male	Female
Control	30.00% ± 8.56%	40.00% ± 0.00%	13.33% ± 13.33%	50.00% ± 10.00%	10.00% ± 6.83%	20.00% ± 0.00%
Visual	85.00% ± 9.57%	60.00% ± 11.55%	55.00% ± 15.00%	50.00% ± 20.82%	10.00% ± 5.77%	15.00% ± 5.00%
Spatial	57.67% ± 14.06%	100% ± 0%	43.33% ± 12.02%	50.00% ± 10.00%	6.67% ± 4.21%	0% ± 0%
Combined	80.00% ± 11.55%	92.00% ± 4.90%	88.57% ± 23.04%	84.00% ± 7.48%	0% ± 0%	8.00% ± 8.00%

Table 6: Mean number of intensely stained calexcitin cells in various 200μm by 200μm by 30μm cuttlefish brain regions post-training. Kruskall-Wallis one-way ANOVA was then used to determine whether there were significant differences between the groups. Regions printed in italic indicate statistically significant differences between the groups (Refer to Table 7). Counts displayed in boldface indicate values significantly larger than the other groups.

Region	Control	Visual	Spatial
Vertical Lobe	2.875±0.25	2.8±0.17	2.6±0.12
Subvertical Lobe	**7.415±1.02**	5.1±0.833	6.555±0.64
Dorsal Basal Lobe	7.135±0.39	6.92±0.09	7.03±0.9
Right Optic Lobe (Deep Retina)	3.67±0.25	**6.18±1.09**	3.76±0.29
Right Optic Lobe (Medulla)	3.2±0.46	**5.49±0.30**	4.75±0.25
Left Optic Lobe (Deep Retina)	4.78±0.19	**6.6±0.56**	5.00±0.41
Left Optic Lobe (Medulla)	3.775±0.81	**6.36±0.81**	5.00±0.41

Table 7: Kruskall-Wallis one way ANOVA results for differences in the mean number of strongly calexcitin stained calls in various brain regions (refer also to Table 6). Significant differences between the groups are indicated by a boldface p-value.

Region		**p-value**
Vertical Lobe	N=12 $_{(4,4,4)}$, KW=3.16	p>0.10
Subvertical Lobe	N=12 $_{(4,4,4)}$, KW=6.31	**p<0.05**
Dorsal Basal Lobe	N=12 $_{(4,4,4)}$, KW=0.77	p>0.10
Right Optic Lobe (Deep Retina)	N=12 $_{(4,4,4)}$, KW=10.86	**p<0.001**
Right Optic Lobe (Medulla)	N=12 $_{(4,4,4)}$, KW=7.30	**P<0.05**
Left Optic Lobe (Deep Retina)	N=12 $_{(4,4,4)}$, KW=7.431	**P<0.05**
Left Optic Lobe (Medulla)	N=12 $_{(4,4,4)}$, KW=4.813	**P<0.10**

Table 8: Wilcoxon signed ranks test results, comparing the mean number of strongly calexcitin stained cells in the left and right optic lobe for each experimental group. Significant results indicated in boldface (note, small sample size precluded p-values less than 0.0625).

	Left optic lobe plexiform versus right optic lobe plexiform	Left optic lobe medulla versus right optic lobe medulla
Control (n=4)	T^+=10, **p=0.0625**	T^-=6, p=0.4375
Visual (n=4)	T^+=5.5, p=.5000	T^+=7, p=0.3125
Spatial (n=4)	T^+=10, **p=0.0625**	T^+=7, p=0.3125

Table 9: Data on the initial tissue weight of the pieces of tissue used for western blot analysis. Initial direction preference and group data also included. See Table 2 for additional information.

Cuttlefish	Group	Brain region	Weight (g)	Direction preference
Stone	Visual	Left Optic Lobe	0.29	Right
		Right Optic Lobe	0.21	
		Supraeosophageal Lobes	0.67	
Lola	Visual	Left Optic Lobe	0.17	Right
		Right Optic Lobe	0.2	
		Supraeosophageal Lobes	0.67	
Guy	Visual	Left Optic Lobe	0.22	Right
		Right Optic Lobe	0.24	
		Supraeosophageal Lobes	0.67	
Scratch	Visual	Left Optic Lobe	0.25	Left
		Right Optic Lobe	0.22	
		Supraeosophageal Lobes	0.44	
Howard	Spatial	Left Optic Lobe	0.33	Left
		Right Optic Lobe	0.36	
		Supraeosophageal Lobes	0.54	
Snookums	Spatial	Left Optic Lobe	0.23	Left
		Right Optic Lobe	0.24	
		Supraeosophageal Lobes	0.44	
Marilyn	Spatial	Left Optic Lobe	0.14	Right
		Right Optic Lobe	0.18	
		Supraeosophageal Lobes	0.44	
Isis	Control	Left Optic Lobe	0.53	Right
		Right Optic Lobe	0.44	
		Supraeosophageal Lobes	1.15	
Nougat	Control	Left Optic Lobe	0.27	Right
		Right Optic Lobe	0.22	
		Supraeosophageal Lobes	0.65	
Willow	Control	Left Optic Lobe	0.28	Left
		Right Optic Lobe	0.24	
		Supraeosophageal Lobes	0.72	
Bilbo	Visual	Left Optic Lobe	0.38	Left
		Right Optic Lobe	0.35	
		Supraeosophageal Lobes	0.86	
Freckle	Control	Left Optic Lobe	0.35	Right
		Right Optic Lobe	0.44	
		Supraeosophageal Lobes	1.24	

Table 10: Bradford method results used to determine the protein concentration for each fraction (homogenate, cytosolic and microsomal fractions of the left optic lobe (LOL), right optic lobe (ROL), and supraoesophageal lobes (CNS) of each cuttlefish. Columns A and B indicate the identity of each cuttlefish, the group they belonged to and the fraction of interest. Column C shows the absorption reading of each fraction read in a spectrophotometer at 595 nm. Column D shows the amount of protein in each diluted fraction, found using the slope intercept equation Y=-0.005631 + 0.00386119X (see Figure 26). Column E shows the amount of protein in undiluted samples (Column D multiplied by final dilution factor of 310). Column F shows the amount of sample loaded to yield a protein concentration of 10 μg/μl. Column G shows the amount of additional buffer required to load the sample (19 μl total) into each lane.

A	B	C	D	E	F	G
	Fraction	**Absorption Value (Y)**	**Diluted Protein ug/ml**	**final protein ug/ul**	**sample loaded ul**	**Buffer loadedl ul**
Willow	LOL HOM	0.001	1.7174	0.53239	**18.78**	*0.22*
	ROL HOM	0.006	3.0123	0.93382	**10.71**	*8.29*
control	CNS HOM	0.12	32.537	10.0864	**0.99**	*18.01*
	LOL CYT	0.047	13.631	4.22555	**2.37**	*16.63*
	ROL CYT	0.056	15.962	4.94812	**2.02**	*16.98*
	CNS CYT	0.112	30.465	9.44414	**1.06**	*17.94*
	LOL MIC	0.08	22.177	6.87498	**1.45**	*17.55*
	ROL MIC	0.129	34.868	10.809	**0.93**	*18.07*
	CNS MIC	0.152	40.824	12.6556	**0.79**	*18.21*
Lola	LOL HOM	0.368	96.766	29.9974	**0.33**	*18.67*
	ROL HOM	0.001	1.7174	0.53239	**18.78**	*0.22*
visual	CNS HOM	0.073	20.364	6.31298	**1.58**	*17.42*
	LOL CYT	0.047	13.631	4.22555	**2.37**	*16.63*
	ROL CYT	0.21	55.846	17.3122	**0.58**	*18.42*
	CNS CYT	0.205	54.551	16.9107	**0.59**	*18.41*
	LOL MIC	0.024	7.6741	2.37897	**4.20**	*14.80*
	ROL MIC	0.016	5.6022	1.73668	**5.76**	*13.24*
	CNS MIC	0.058	16.48	5.10869	**1.96**	*17.04*
Howard	LOL HOM	0.097	26.58	8.23985	**1.21**	*17.79*
	ROL HOM	0.316	83.298	25.8225	**0.39**	*18.61*
spatial	CNS HOM	0.182	48.594	15.0642	**0.66**	*18.34*
	LOL CYT	0.048	13.89	4.30583	**2.32**	*16.68*

Table 10 Cont'd	ROL CYT	0.057	16.221	5.02841	**1.99**	*17.01*
	CNS CYT	0.342	90.032	27.9099	**0.36**	*18.64*
	LOL MIC	0.032	9.746	3.02125	**3.31**	*15.69*
	ROL MIC	0.024	7.6741	2.37897	**4.20**	*14.80*
	CNS MIC	0.151	40.565	12.5753	**0.80**	*18.20*
Snookums spatial	LOL HOM	0.005	2.7533	0.85353	**11.72**	*7.28*
	ROL HOM	0.054	15.444	4.78755	**2.09**	*16.91*
	CNS HOM	0.052	14.926	4.62698	**2.16**	*16.84*
	LOL CYT	0.214	56.882	17.6333	**0.57**	*18.43*
	ROL CYT	0.201	53.515	16.5896	**0.60**	*18.40*
	CNS CYT	0.068	19.07	5.91155	**1.69**	*17.31*
	LOL MIC	0.269	71.126	22.049	**0.45**	*18.55*
	ROL MIC	0.229	60.766	18.8376	**0.53**	*18.47*
	CNS MIC	0.024	7.6741	2.37897	**4.20**	*14.80*
Guy visual	LOL HOM	0.152	40.824	12.6556	**0.79**	*18.21*
	ROL HOM	0.158	42.378	13.1373	**0.76**	*18.24*
	CNS HOM	0.087	23.99	7.43699	**1.34**	*17.66*
	LOL CYT	0.096	26.321	8.15956	**1.23**	*17.77*
	ROL CYT	0.114	30.983	9.60471	**1.04**	*17.96*
	CNS CYT	0.753	196.48	60.9075	**0.16**	*18.84*
	LOL MIC	0.037	11.041	3.42268	**2.92**	*16.08*
	ROL MIC	0.116	31.501	9.76528	**1.02**	*17.98*
	CNS MIC	0.068	19.07	5.91155	**1.69**	*17.31*
Scratch visual	LOL HOM	0.101	27.616	8.56099	**1.17**	*17.83*
	ROL HOM	0.211	56.105	17.3925	**0.57**	*18.43*
	CNS HOM	0.03	9.228	2.86068	**3.50**	*15.50*
	LOL CYT	0.073	20.364	6.31298	**1.58**	*17.42*
	ROL CYT	0.378	99.356	30.8002	**0.32**	*18.68*
	CNS CYT	0.175	46.781	14.5022	**0.69**	*18.31*
	LOL MIC	0.203	54.033	16.7502	**0.60**	*18.40*
	ROL MIC	0.014	5.0842	1.57611	**6.34**	*12.66*
	CNS MIC	0.08	22.177	6.87498	**1.45**	*17.55*
Isis control	LOL HOM	0.066	18.552	5.75098	**1.74**	*17.26*
	ROL HOM	0.00001	1.461	0.4529	**22.08**	*-3.08*
	CNS HOM	0.051	14.667	4.54669	**2.20**	*16.80*
	LOL CYT	0.069	19.329	5.99184	**1.67**	*17.33*

Table 10 Cont'd	ROL CYT	0.139	37.458	11.6119	**0.86**	*18.14*
	CNS CYT	0.068	19.07	5.91155	**1.69**	*17.31*
	LOL MIC	0.103	28.134	8.72156	**1.15**	*17.85*
	ROL MIC	0.112	30.465	9.44414	**1.06**	*17.94*
	CNS MIC	0.17	45.486	14.1007	**0.71**	*18.29*
Marilyn spatial	LOL HOM	0.025	7.9331	2.45925	**4.07**	*14.93*
	ROL HOM	0.048	13.89	4.30583	**2.32**	*16.68*
	CNS HOM	0.082	22.695	7.03556	**1.42**	*17.58*
	LOL CYT	0.001	1.7174	0.53239	**18.78**	*0.22*
	ROL CYT	0.001	1.7174	0.53239	**18.78**	*0.22*
	CNS CYT	0.052	14.926	4.62698	**2.16**	*16.84*
	LOL MIC	0.002	1.9764	0.61267	**16.32**	*2.68*
	ROL MIC	0.001	1.7174	0.53239	**18.78**	*0.22*
	CNS MIC	0.09	24.767	7.67785	**1.30**	*17.70*
Nougat control	LOL HOM	0.172	46.004	14.2613	**0.70**	*18.30*
	ROL HOM	0.051	14.667	4.54669	**2.20**	*16.80*
	CNS HOM	0.001	1.7174	0.53239	**18.78**	*0.22*
	LOL CYT	0.05	14.408	4.4664	**2.24**	*16.76*
	ROL CYT	0.059	16.739	5.18898	**1.93**	*17.07*
	CNS CYT	0.03	9.228	2.86068	**3.50**	*15.50*
	LOL MIC	0.001	1.7174	0.53239	**18.78**	*0.22*
	ROL MIC	0.133	35.904	11.1301	**0.90**	*18.10*
	CNS MIC	0.057	16.221	5.02841	**1.99**	*17.01*
Stone visual	LOL HOM	0.075	20.882	6.47355	**1.54**	*17.46*
	ROL HOM	0.062	17.516	5.42984	**1.84**	*17.16*
	CNS HOM	0.072	20.105	6.2327	**1.60**	*17.40*
	LOL CYT	0.059	16.739	5.18898	**1.93**	*17.07*
	ROL CYT	0.031	9.487	2.94097	**3.40**	*15.60*
	CNS CYT	0.053	15.185	4.70726	**2.12**	*16.88*
	LOL MIC	0.051	14.667	4.54669	**2.20**	*16.80*
	ROL MIC	0.237	62.838	19.4799	**0.51**	*18.49*
	CNS MIC	0.022	7.1561	2.21839	**4.51**	*14.49*
Freckle control	LOL HOM	0.001	1.7174	0.53239	**18.78**	*0.22*
	ROL HOM	0.034	10.264	3.18183	**3.14**	*15.86*
	CNS HOM	0.081	22.436	6.95527	**1.44**	*17.56*
	LOL CYT	0.052	14.926	4.62698	**2.16**	*16.84*

Table 10	ROL CYT	0.06	16.998	5.26926	**1.90**	*17.10*
Cont'd	CNS CYT	0.346	91.068	28.2311	**0.35**	*18.65*
	LOL MIC	0.068	19.07	5.91155	**1.69**	*17.31*
	ROL MIC	0.184	49.112	15.2247	**0.66**	*18.34*
	CNS MIC	0.172	46.004	14.2613	**0.70**	*18.30*
Bilbo	LOL HOM	0.102	27.875	8.64128	**1.16**	*17.84*
	ROL HOM	0.037	11.041	3.42268	**2.92**	*16.08*
visual	CNS HOM	0.211	56.105	17.3925	**0.57**	*18.43*
	LOL CYT	0.077	21.4	6.63413	**1.51**	*17.49*
	ROL CYT	0.124	33.573	10.4076	**0.96**	*18.04*
	CNS CYT	0.147	39.53	12.2541	**0.82**	*18.18*
	LOL MIC	0.147	39.53	12.2541	**0.82**	*18.18*
	ROL MIC	0.121	32.796	10.1667	**0.98**	*18.02*
	CNS MIC	0.189	50.407	15.6262	**0.64**	*18.36*

Table 11: Mann Whitney U test results comparing mean calexcitin expression on western blot membranes among the homogenate, cytosolic and microsomal fractions of the left optic lobe, right optic lobe and supraoesophageal lobes between each experimental group (visual-cue, spatial-cue and motor control). A boldfaced p-value indicates significant differences between groups, and the significantly higher group is also denoted in boldface..

Region	Comparison	U	P
Left Optic Lobe Homogenate	Spatial vs. **Visual**	$14_{3,5}$	**=0.10**
	Control vs. **Visual**	$16_{4,5}$	**=0.10**
	Spatial vs. Control	$4_{3,4}$	>0.10
Right Optic Lobe Homogenate	Spatial vs. Visual	$12_{3,5}$	>0.10
	Control vs. Visual	$15_{4,5}$	>0.10
	Spatial vs. Control	$7_{3,4}$	>0.10
Supraoesophageal Lobe Homogenate	Spatial vs. Visual	$3_{3,5}$	>0.10
	Control vs. Visual	$8_{4,5}$	>0.10
	Spatial vs. Control	$3_{3,4}$	>0.10
Left Optic Lobe Cytosolic	Spatial vs. Visual	$9_{3,5}$	>0.10
	Control vs. Visual	$12_{4,5}$	>0.10
	Spatial vs. Control	$7_{3,4}$	>0.10
Right Optic Lobe Cytosolic	Spatial vs. Visual	$12_{3,5}$	>0.10
	Control vs. Visual	$20_{4,5}$	**=0.001**
	Spatial vs. **Control**	$12_{3,4}$	**=0.05**
Supraoesophageal Lobe Cytosolic	Spatial vs. Visual	$7_{3,5}$	>0.10
	Control vs. Visual	$8_{4,5}$	>0.10
	Spatial vs. Control	$9_{3,4}$	>0.10
Left Optic Lobe Microsomal	Spatial vs. **Visual**	$9_{3,5}$	**<0.05**
	Control vs. Visual	$8_{4,5}$	>0.10
	Spatial vs. Control	$9_{3,4}$	>0.10
Right Optic Lobe Microsomal	Spatial vs. **Visual**	$13_{3,5}$	**=0.10**
	Control vs. **Visual**	$18_{4,5}$	**=0.05**
	Spatial vs. Control	$8_{3,4}$	>0.10
Supraoesophageal Lobe Microsomal	Spatial vs. Visual	$10_{3,5}$	>0.10
	Control vs. Visual	$5_{4,5}$	>0.10
	Spatial vs. Control	$5_{3,4}$	>0.10

Table 12: Wilcoxon signed ranks test comparing mean calexcitin expression on western blot membranes in the left and right optic lobe for each experimental group. Significant differences between the lobes are indicated in boldface.

	Left optic lobe homogenate versus right optic lobe homogenate	Left optic lobe cytosolic versus right optic lobe cytosolic	Left optic lobe microsomal versus right optic lobe microsomal
Control (N=4)	T^-=7, p=0.3125	T^+=7, p=0.3125	T^+=6, p=0.4375
Visual (N=5)	T^+=15, **p=0.0313**	T^+=12.,=0.1563	T^+=7, p=0.500
Spatial (N=3)	T^+=3, p=0.6250	T^+=3, P=0.6250	T^-=5, p=0.2500

APPENDIX B

Figures

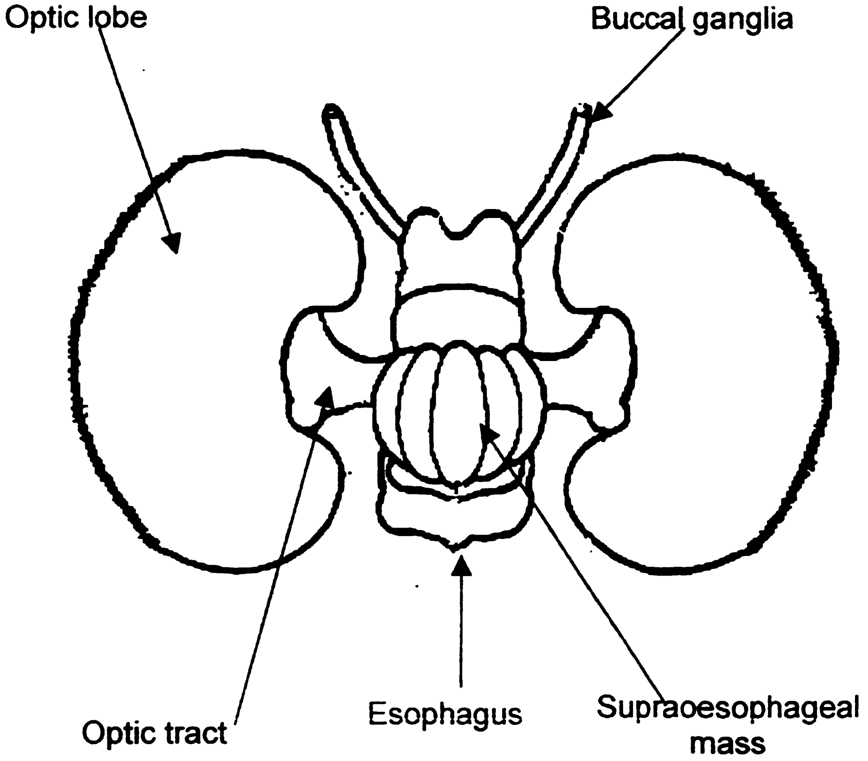

Figure 1: A diagram of the brain of an octopus as seen from above showing the basic organization of the major components of the cephalopod brain (after Young, 1971; Sanders, 1975). Anterior end of the octopus located at top of diagram, posterior at bottom.

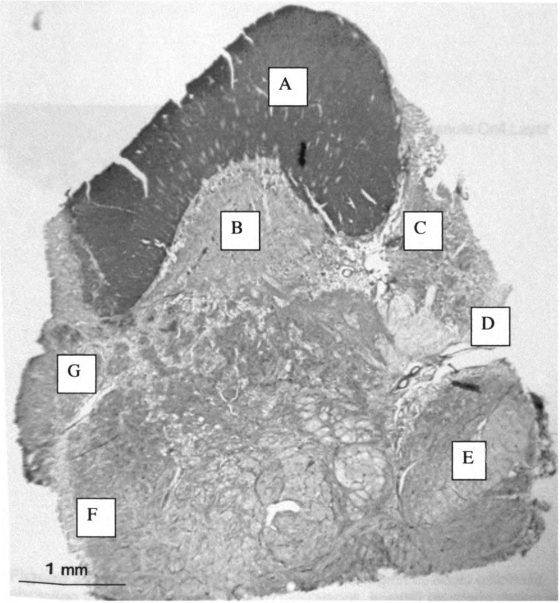

Figure 2: Saggital Section of the supraoesophageal mass of *Sepia officinalis*. Regions are labelled as follows: A) vertical lobe, B) subvertical lobe, C) superior frontal lobe, D) inferior frontal lobe, E) anterior basal lobe, F) medial basal lobe, G) dorsal basal lobe. Anterior of cuttlefish=right, dorsal=up.

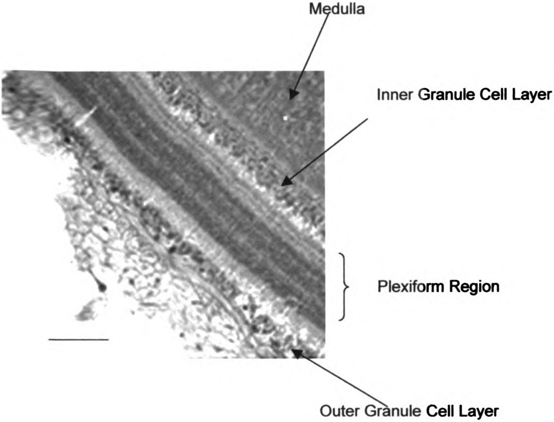

Figure 3: Transverse section of the deep retinal region of *Sepia officinalis*, showing the inner and outer granule cell regions, layered plexiform region and medulla (Magnification=20X). Black bar indicates distance of 0.1mm.

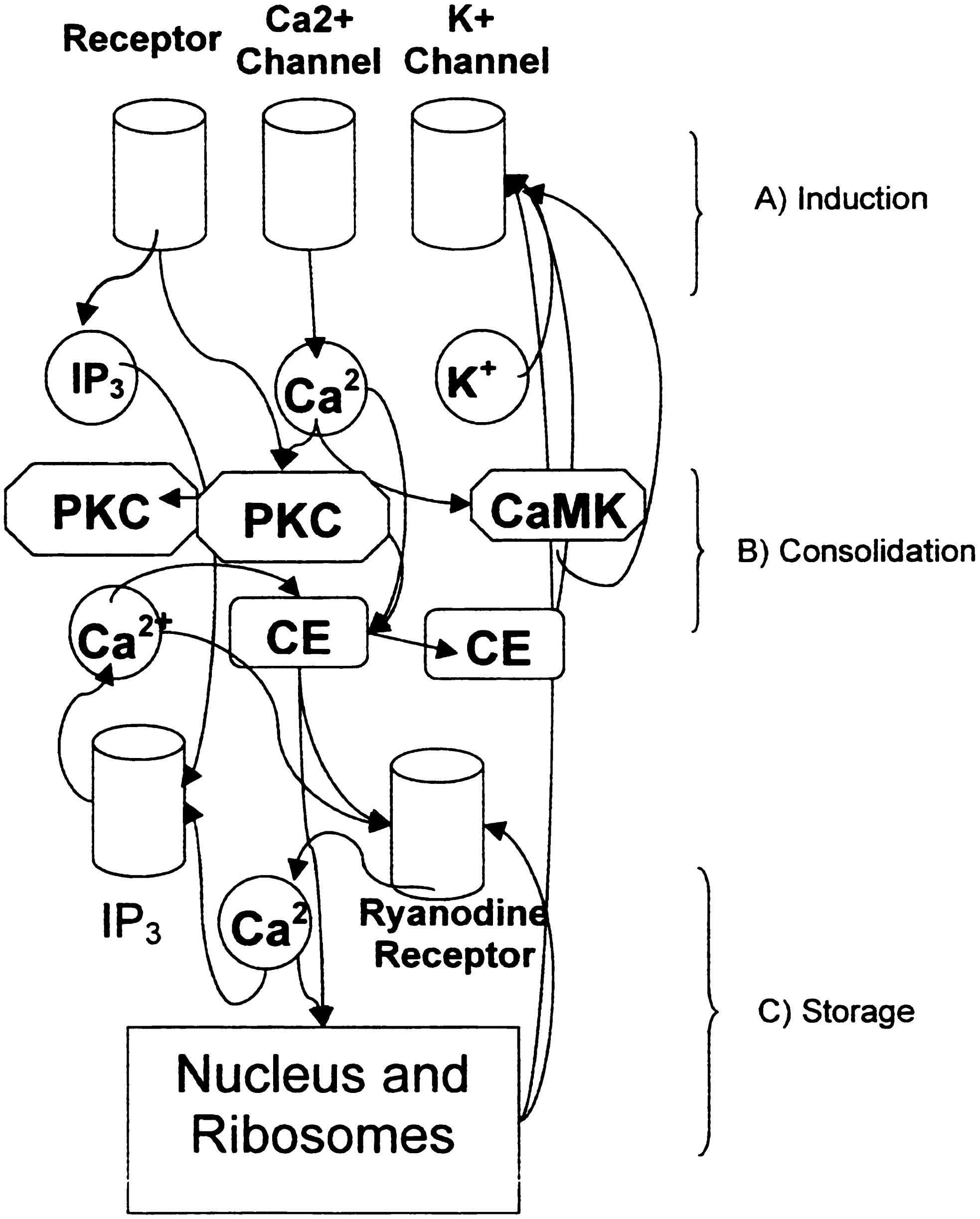

Figure 4 : Diagram illustrating the major cellular components underlying associative memory in *Hermissenda* (adapted from Alkon, 1998). A) Induction involves an influx of calcium, phosphorylation of PKC and subsequent translocation from cytoplasm to the cell membrane, phosphorylation of Calexcitin (CE) and translocation to the cell membrane and inhibition of voltage-gated potassium channels. B) Consolidation, CE is translocated to the ER membrane and nuclear membrane thus activating the ryanodine receptor and further inhibiting potassium. C) Storage, involving re-uptake of excess calcium as well as DNA and protein synthesis.

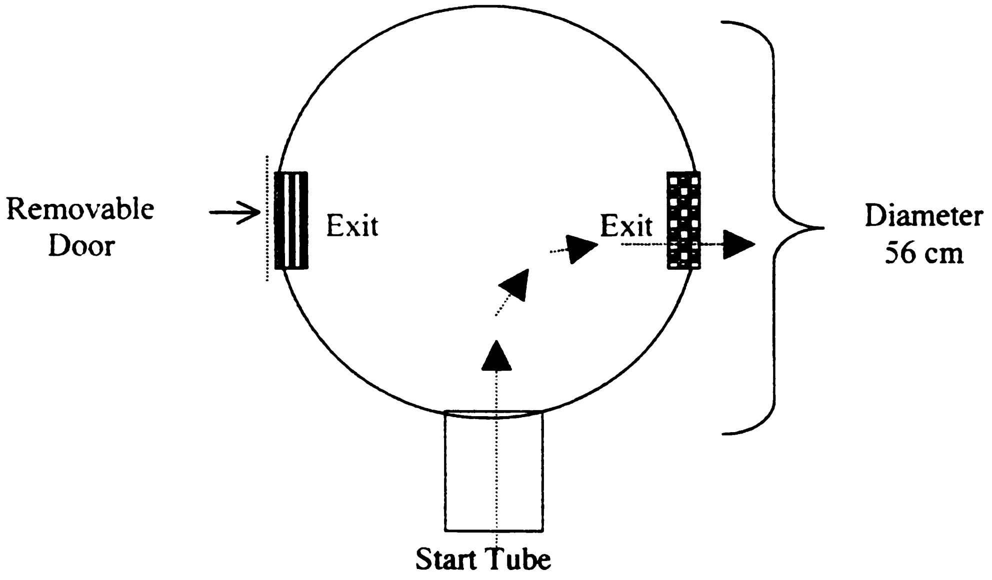

Figure 5: Schematic drawing of the simultaneous discrimination maze as viewed from above. Important maze features include the start tube and two exit holes cut through the walls of the arena, patterned panels (visual cues) that can be switched randomly between doors, clear plexiglas doors, and a clear, plexiglas "false bottom" fitted in the maze. An example escape route is depicted with dashed arrows.

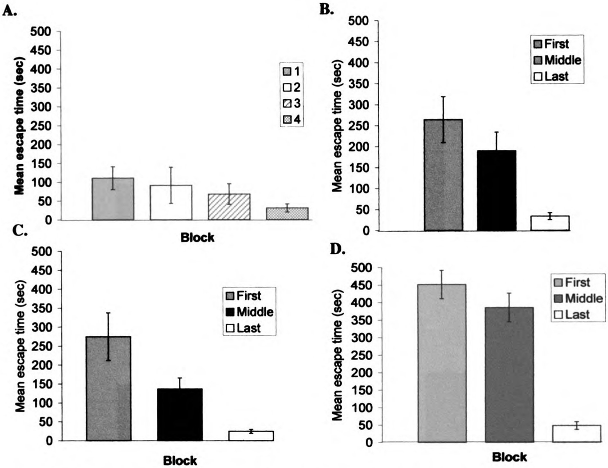

Figure 6: Mean escape time ± standard error over the first, middle and last block of five trials for cuttlefish in each experimental group. A) Control group, which only received 20 experimental trials, shows results for all four blocks of five trials, the apparent decrease was insignificant (repeated measures ANOVA, n=8, F=2.094($_{3,21}$), p>0.25). B) Visual-cue group showed a statistically significant decrease (Repeated Measures ANOVA, F=9.075 $_{(2,14)}$, p<0.005). C) The spatial-cue group also showed a statistically significant decrease (Repeated Measures ANOVA, F=8.929 $_{(2,14)}$, p<0.005). Finally, the combined-cue group showed a statistically significant decrease as well (Repeated Measures ANOVA, F=14.311 $_{(2, 22)}$, p<0.0005).

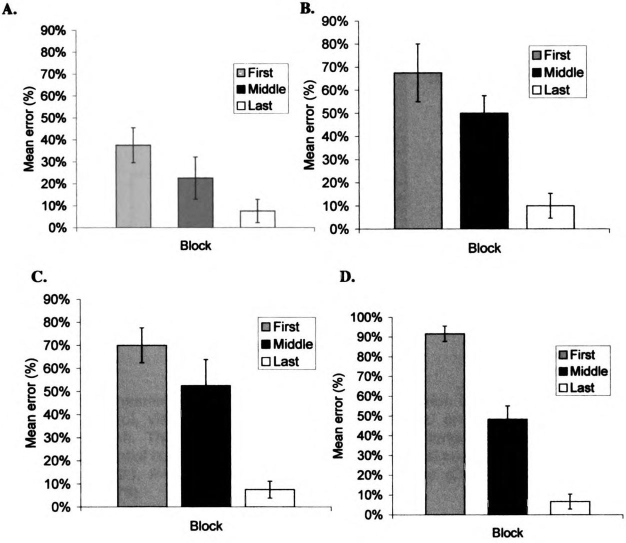

Figure 7: Mean overall error time ± standard error over the first, middle and last block of five trials for cuttlefish in each experimental group. A) Control group did not show a signifcant decrease in error (repeated measures ANOVA, n=8, $F=2.000_{(3,21)}$, $p>0.10$). B) The visual-cue group showed a statistically significant decrease in error (Repeated Measures ANOVA, $F=11.53_{(2,14)}$, $p<0.001$). C) The spatial-cue group also showed a statistically significant decrease (Repeated Measures ANOVA, $F=12.631_{(2,14)}$, $p<0.001$). Finally, the combined-cue group showed a statistically significant decrease as well (Repeated Measures ANOVA, $F=28.774_{(2, 22)}$, $p<0.0005$).

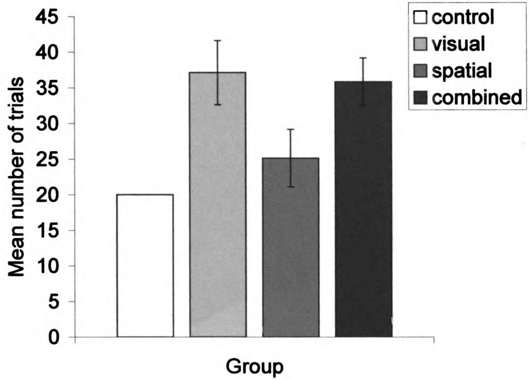

Figure 8: Comparison of mean number of trials to criterion level performance between control, visual cue-trained, spatial cue-trained and combined cue-trained cuttlefish. The overall mean number of trials to criterion was significantly lower in the spatial cue group than it was in either the visual cue group (One-Way ANOVA, F=3.93, P=0.067) or the combined cue group (One-Way ANOVA, F=3.35, P=0.079).

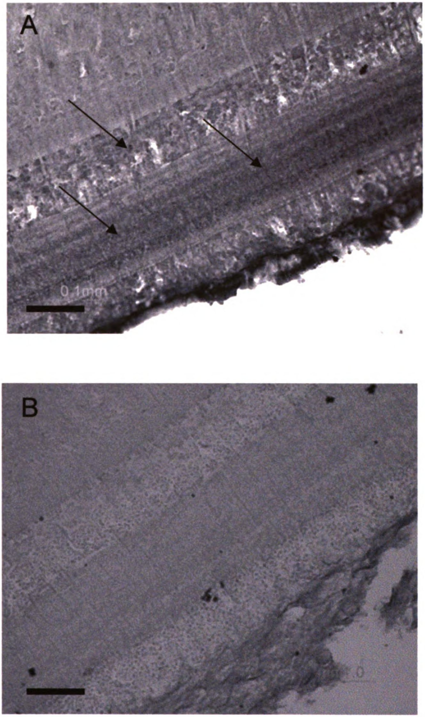

Figure 9: Horizontal sections of the plexiform layer of the optic lobe of *S. officinalis*. Black arrows demonstrate regions of intense staining. Black bar indicates distance of 0.1 mm. A: Calexcitin immunopositive cells of different sizes are evident throughout the granule cell layers, and tangential layers of the plexiform region. B: Same region with calexcitin staining absent because the primary antibody is replaced with goat normal serum.

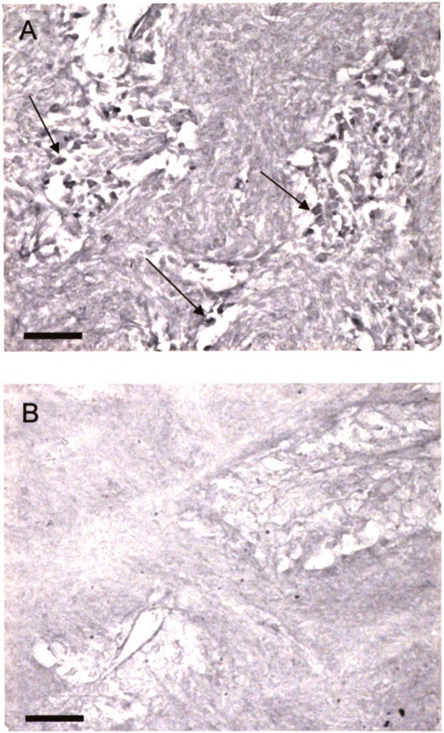

Figure 10: Horizontal sections of the medulla of the optic lobe of *S. officinalis*. Black arrows demonstrate regions of intense staining. Black bar indicates distance of 0.1 mm. A: Calexcitin immunopositive cells are present throughout the "cell islands" of the medulla. B: Same region with staining absent because primary antibody replaced with goat normal serum.

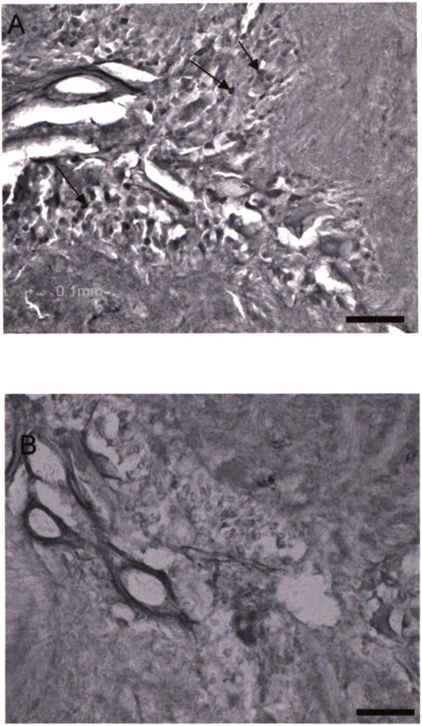

Figure 11: Horizontal sections of the superior frontal lobe of *S. officinalis* . Black arrows demonstrate regions of intense staining. Black bar indicates distance of 0.1 mm. A: Calexcitin immunopositive cells are present in the most posterior portion of the lobe. B: Same region with staining absent because primary antibody is replaced with goat normal serum.

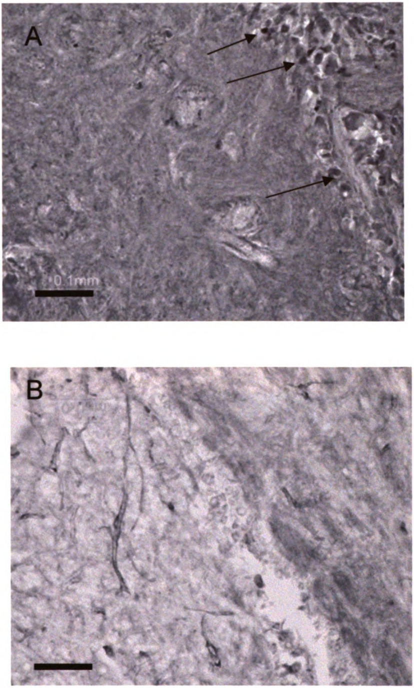

Figure12: Horizontal sections of the posterior inferior frontal lobe of *S. officinalis*. Black arrows demonstrate regions of intense staining. Black bar indicates distance of 0.1 mm. A: Large calexcitin immunopositive cells are located toward the dorsal/posterior edges of the inferior frontal lobe. B: Same region with staining absent because primary antibody is replaced with goat normal serum.

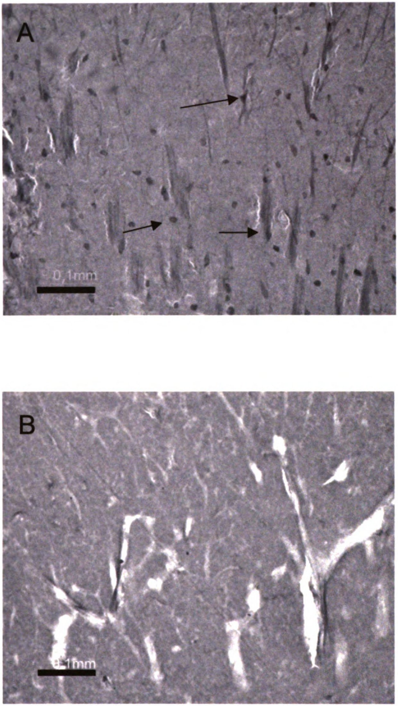

Figure 13: Horizontal sections of the vertical lobe of *S. officinalis.* Black arrows demonstrate regions of intense staining. Black bar indicates distance of 0.1 mm. A: Calexcitin immunopositive amacrine cells are evident throughout the neuropil. B: Same region with staining absent because primary antibody is replaced with goat normal serum.

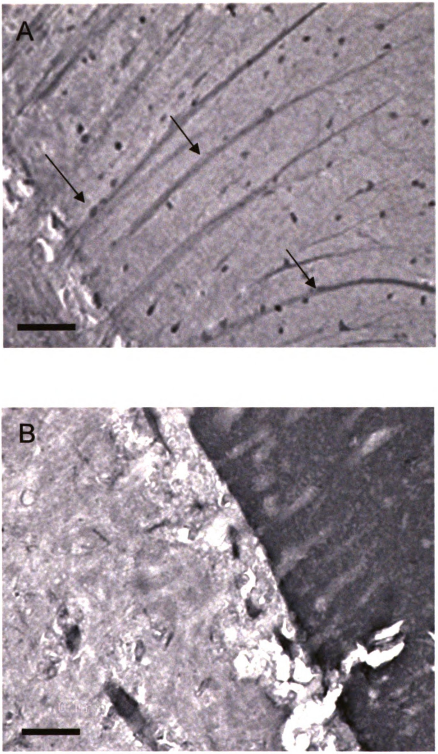

Figure 14: Horizontal sections of the vertical/subvertical lobes of *S. officinalis*. Black arrows demonstrate regions of intense staining. Black bar indicates distance of 0.1 mm. A: Calexcitin immunopositive tracts connect the vertical and subvertical lobes. B: Same region with staining absent because primary antibody is replaced with goat normal serum.

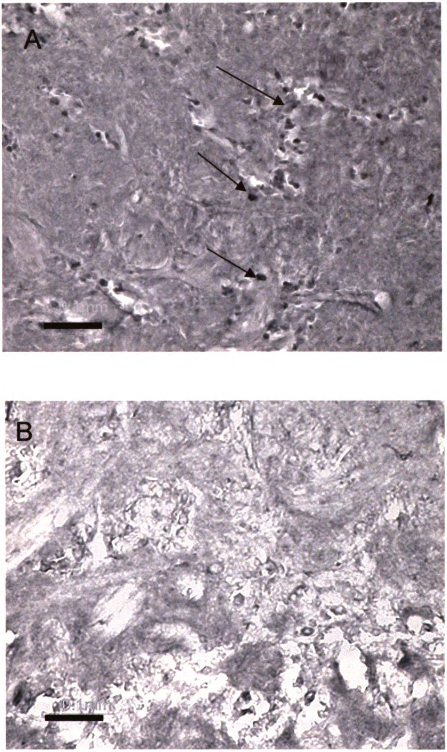

Figure 15: Horizontal sections of the subvertical lobe of *S. officinalis*. Black arrows demonstrate regions of intense staining. Black bar indicates distance of 0.1 mm. A: Calexcitin immunopositive cells are located throughout islands scattered in the neuropil. B: Same region with staining absent because primary antibody is replaced with goat normal serum.

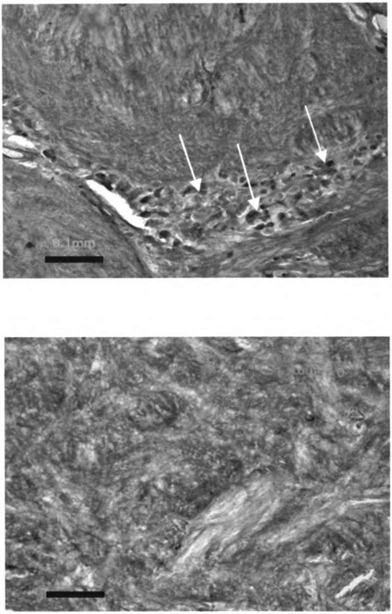

Figure 16: Horizontal sections of the anterior basal lobe of *S. officinalis*. Black bar indicates distance of 0.1 mm. A: Calexcitin immunopositive cells of different size (white arrows) in the cell layer of the lateral region of the lobe. B: Same region with staining absent because primary antibody is replaced with goat normal serum.

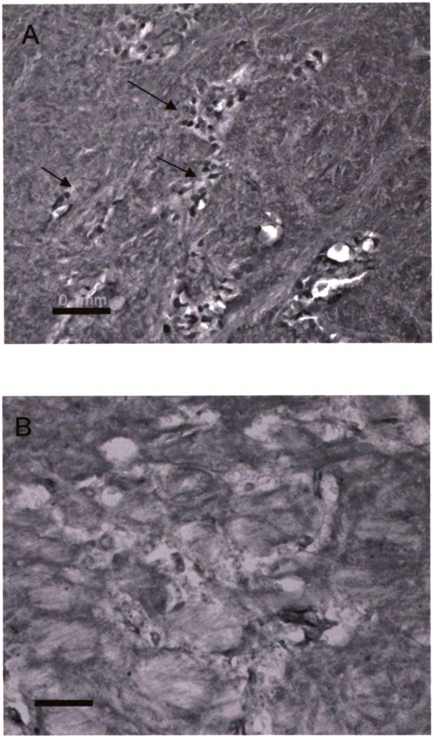

Figure 17: Horizontal sections of the median basal lobe of *S. officinalis*. Black arrows demonstrate regions of intense staining. Black bar indicates distance of 0.1 mm. A: Calexcitin immunopositive cells of different size throughout clusters of cells in the neuropil. B: Same region with staining absent because primary antibody is replaced with goat normal serum.

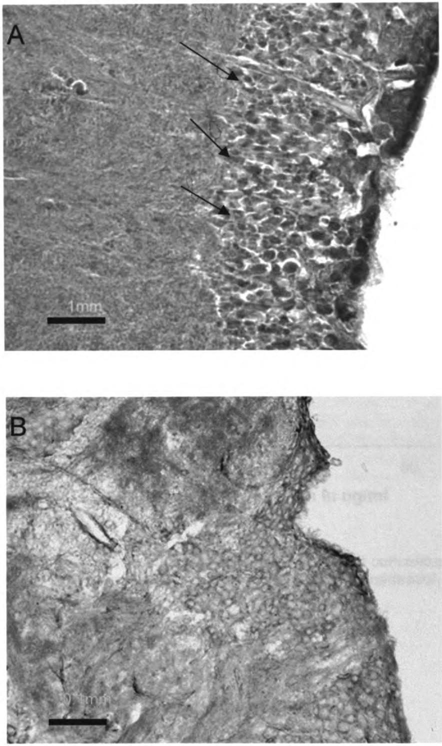

Figure 18: Horizontal sections of the posterior wall of the dorsal basal lobe of *S. officinalis*. Black arrows demonstrate regions of intense staining. Black bar indicates distance of 0.1 mm. A: calexcitin immunopositive cells at the posterior wall. B: Same region with staining absent because primary antibody is replaced with goat normal serum.

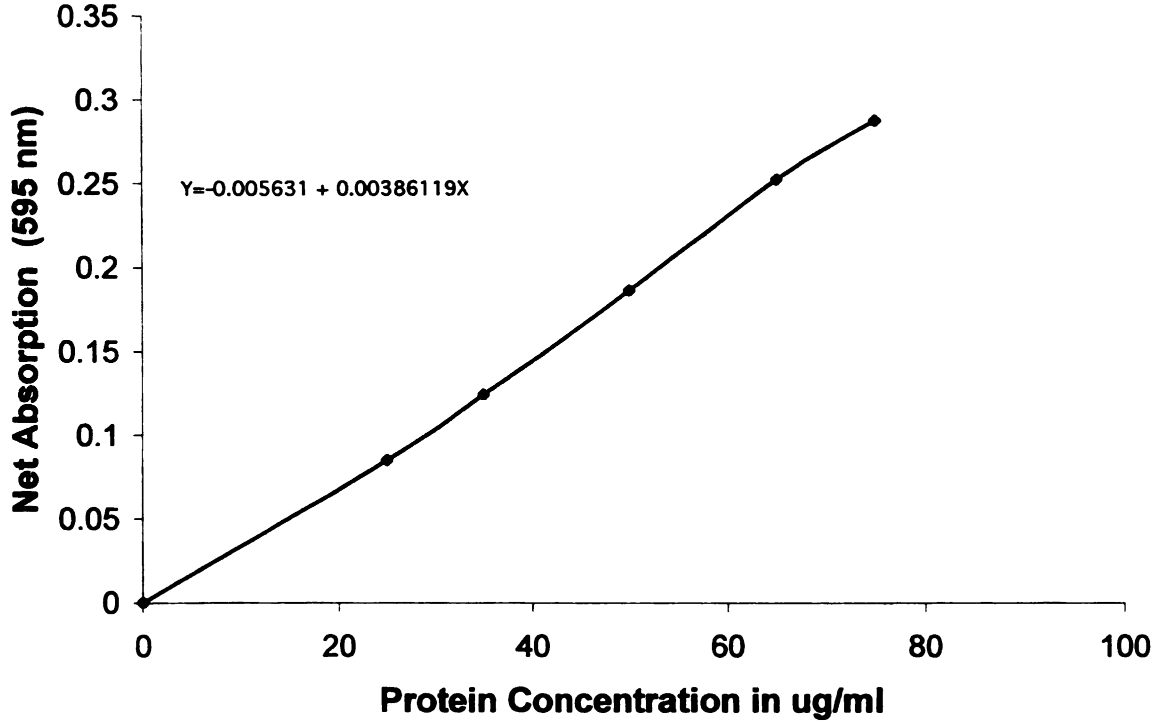

Figure 19: Protein concentration curve of BSA at different concentrations read at 595 nanometers used to determine the protein concentration of samples for western blotting.

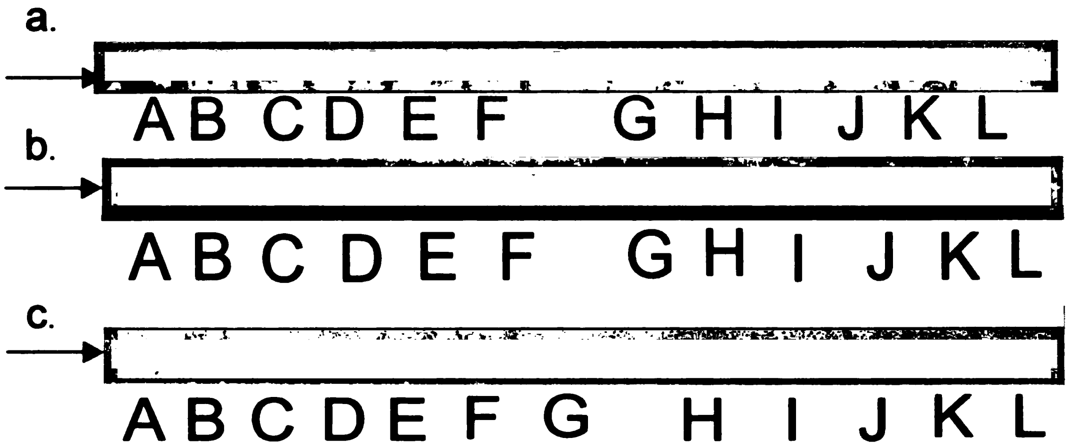

Figure 20: Nitrocellulose membranes loaded with homogenate and immunostained for calexcitin. Arrow indicates approximate location of 23 kDa on the membrane. Cuttlefish identities are as follows: A-Willow, B-Lola, C-Howard, D-Snookums, E-Guy, F-Scratch, G-Isis, H-Marilyn, I-Nougat, J-Stone, K-Freckle, and L-Bilbo. Blank spaces are location of the molecular weight marker on the gels. a.) left optic lobe homogenate, b.) right optic lobe homogenate, and c.) supraoesophageal region homogenate.

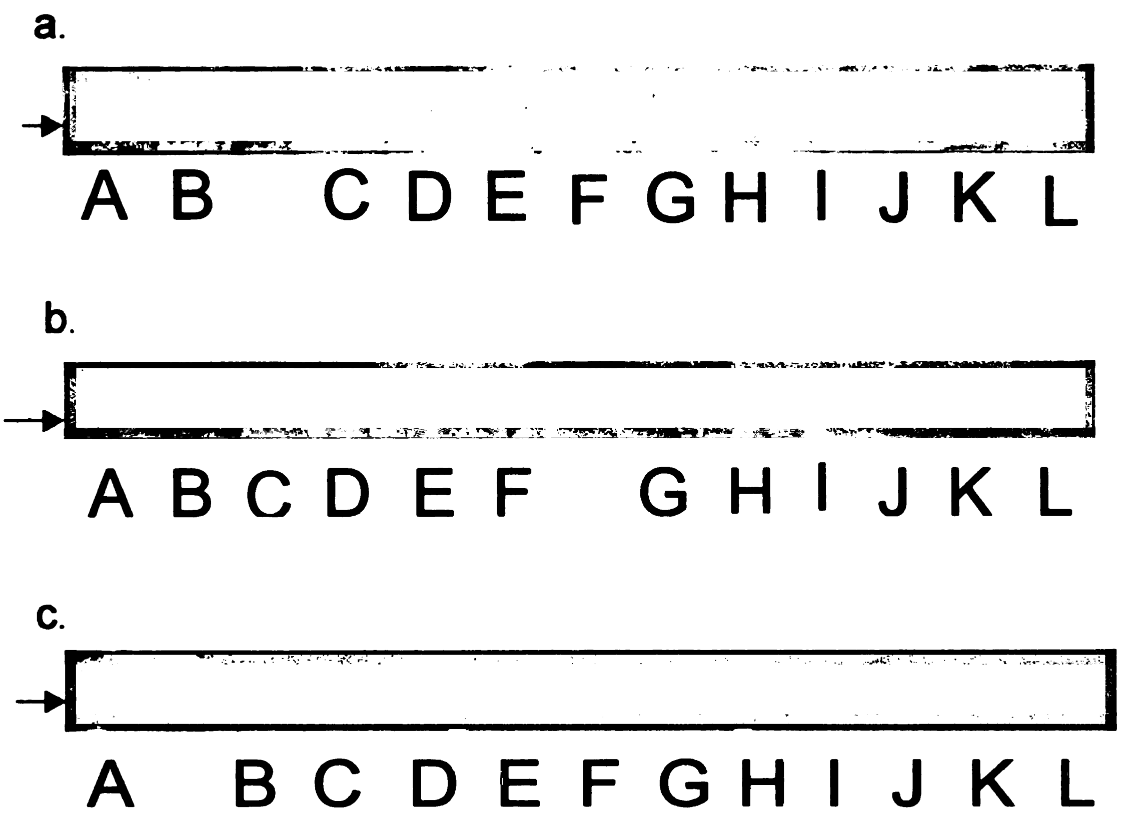

Figure 21: Nitrocellulose membranes loaded with microsomal fractions and immunostained for calexcitin. Arrow indicates approximate location of 23 k Da on the membrane. Cuttlefish identities are as follows: A-Willow, B-Lola, C-Howard, D-Snookums, E-Guy, F-Scratch, G-Isis, H-Marilyn, I-Nougat, J-Stone, K-Freckle, and L-Bilbo. Blank spaces are the location of the molecular weight marker on the gels. a.) left optic lobe cytosolic fraction, b.) right optic lobe cytosolic fraction, and c.) supraoesophageal region cytosolic fraction.

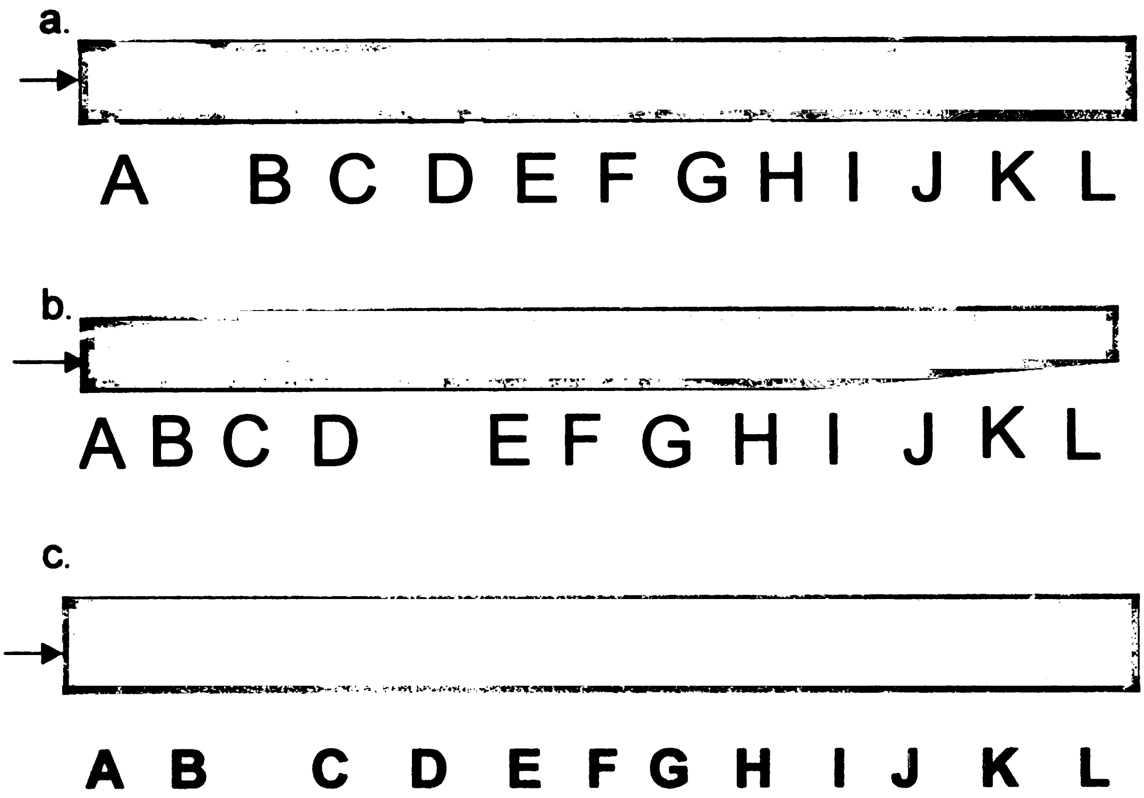

Figure 22: Nitrocellulose membranes loaded with cytosolic fractions and immunostained for calexcitin. Arrow indicates approximate location of 23 kDa on the membrane. Cuttlefish identities are as follows: A-Willow, B-Lola, C-Howard, D-Snookums, E-Guy, F-Scratch, G-Isis, H-Marilyn, I-Nougat, J-Stone, K-Freckle, and L-Bilbo. Blank spaces are the location of the molecular weight marker on the gels. a.) left optic lobe microsomal fraction, b.) right optic lobe microsomal fraction, and c.) supraoesophageal region microsomal fraction.

APPENDIX C

Reprint

Karson, MA, Boal, JG and RT Hanlon. 2003. Experimental Evidence for Spatial Learning in Cuttlefish (*Sepia officinalis*). Journal of Comparative Psychology, *in press*.

Laboratory mazes were used to study spatial-learning capabilities in cuttlefish (*Sepia officinalis*), using escape for reinforcement. In preliminary observations, cuttlefish in an artificial pond moved actively around the environment and appeared to learn about features of their environment. In laboratory experiments, cuttlefish exited a simple alley maze more quickly with experience and retained the learned information. Similar improvement was not found in open-field mazes or T-mazes, perhaps because of motor problems. Cuttlefish learned to exit a maze that required them to find openings in a vertical wall. The wall maze was modified to an arena, and simultaneous discrimination learning and reversal learning were demonstrated. These experiments indicate that cuttlefish improve performance over serial reversals of a simultaneous, visual–spatial discrimination problem.

Cephalopod mollusks have complex nervous systems and highly diverse behaviors. Sensitization, habituation, associative learning, and spatial learning have all been demonstrated (for reviews, see Bitterman, 1975; Hanlon & Messenger, 1996; Mather, 1995; G. D. Sanders, 1975). Because cephalopods' behavioral abilities have been compared with those of lower vertebrates and because they are evolutionarily distant from species more commonly used in learning experiments, cephalopods are worthwhile test species for functional explanations of the evolution of complex nervous systems (Budelmann, Bullock, & Williamson, 1997; Packard, 1972).

Many octopus species forage away from a home den that they return to repeatedly for shelter (e.g., Ambrose, 1982; Boyle, 1983, 1988; Forsythe & Hanlon, 1997; Hartwick, Ambrose, & Robinson, 1984; Mather 1991). Studies of detours and maze learning have supported field observations suggesting spatial learning (Boal, Dunham, Williams, & Hanlon, 2000; Mather, 1991; Moriyama & Gunji, 1997; Wells, 1964). Other complex learning behaviors observed in octopuses include avoidance learning (Boycott, 1954), discrimination learning (Sutherland & Muntz, 1959), and reversal learning Mackintosh & Mackintosh, 1964). Octopuses learn relatively quickly and retain the learned information. It is clear that learning is an important aspect of the natural history of octopuses.

Neurobiological evidence suggests that cuttlefish, like octopuses, could be capable of complex learning. Cuttlefish have a larger overall brain to body size ratio than do octopuses (Maddock & Young, 1987). Furthermore, the vertical lobe in the cuttlefish, a brain area thought to be involved in learning and memory (Young 1960 and 1965), occupies roughly 24% of the total brain volume, whereas the vertical lobe of the octopus occupies about 13% of the total brain volume (Maddock & Young, 1987; Wirz, 1959). In cuttlefish, an improvement in learning has been correlated with increases in the volume of the vertical lobe during post-embryological development (Dickel, Chichery, & Chichery, 1998; Messenger, 1973). This finding suggests that vertical lobe size could be associated with learning. Thus, learning and memory could play an important role in cuttlefish natural history.

Cuttlefish are clearly capable of associative learning. Through negative reinforcement, cuttlefish can be trained to not strike at prey items (e.g., Agin, et al,, 1998; Chichery & Chichery, 1992; Dickel et al., 1998; Messenger, 1968 and 1977).

There are no published studies of spatial learning in cuttlefish; however, the natural history of cuttlefish provides little evidence for good spatial-learning ability (Boletsky, 1983; Hanlon & Messenger, 1988). Unlike octopuses, cuttlefish do not take shelter in home dens but instead rely primarily on crypsis for defense (Boletsky, 1983). Seasonal onshore–offshore migrations have been documented in cuttlefish (Boletsky, 1983), and there is evidence that Australian cuttlefish return seasonally to a specific breeding site (Hall & Hanlon, 2002). However, because of their short lifespan (1–2 years) and typical semelparity, it is likely that learning is not involved in these migrations. Cuttlefish in nature frequently swim in and around vertical barriers, and tagging experiments indicate that Australian cuttlefish forage away from and then return to particular rocks (O'Dor, personal communication, 2002). Spatial learning may be useful in negotiating these obstacles (F. K. Sanders & Young, 1940). There is still much to be discovered about the role of spatial learning in the natural history of cuttlefish.

Preliminary observations of 7 cuttlefish placed individually in a large, outdoor artificial pond (13.4 m by 13.7 m; see Figure 1A) indicated that cuttlefish moved around a new environment in a manner consistent with an interpretation of exploration. There was a barrier within the experimental pond with a small opening that allowed the cuttlefish to travel from one side of the pond to the other (see Figure 1A). Cuttlefish appeared to remember the location of this hole and returned to the site of the hole when it was blocked off (the open hole was approached an average of 14.3 times/hr; the closed hole was approached an average of 25.8 times/hr). The cuttlefish's repeated use of the hole in the barrier and frequent return to this hole suggest that the cuttlefish learned about features of the artificial pond and retained the information from one day to another. Thus, it seemed that cuttlefish could be used in laboratory experiments designed to evaluate spatial learning.

We attempted to provide cuttlefish with maze problems that were comparable with a natural spatial-learning problem, using escape as motivation. In Experiment 1, we asked whether cuttlefish would learn the simple task of exiting a straight alley when escape was the sole motivation. Experiments 2, 3, and 4 were initial attempts to design appropriate mazes that allowed for choice and to assess spatial learning in cuttlefish. Finally, in Experiment 5, we asked whether cuttlefish would show improvement over serial reversals of a two-choice, spatial–visual discrimination problem.

General Method

Experiments were conducted at the Marine Biomedical Institute (MBI) of the University of Texas Medical Branch, Galveston, Texas, and the Marine Resources Center (MRC) of the Marine Biological Laboratory, Woods Hole, Massachusetts. At the MBI, all experimental tanks were interconnected on the same 13,000-L recirculating sea water system, dedicated to holding cephalopods and their live food. Water was a mixture of natural seawater from the Gulf of

Mexico and artificial seawater made from Instant Ocean brand salts (Aquarium Systems, Mentor, OH). Salinity ranged from 31 parts per thousand (ppt) to 35 ppt, and water temperature ranged from 16 °C to 18 °C. In this closed system, water exiting each tank passed through mechanical, chemical, and biological filters and was treated with ultraviolet light to kill pathogens. Water flow was continuous at all times, including during experimental trials. At the MRC, the water supply was drawn from a depth of approximately 3 meters below the surface of Great Harbor and gravity fed to tanks throughout the MRC building. Salinity ranged from 31 ppt to 33 ppt, and the water temperature ranged from 15 °C to 21 °C.

All subjects were laboratory-cultured cuttlefish (*Sepia officinalis*) from the National Resource Center for Cephalopods (MBI, Galveston, TX). Cuttlefish were housed in small groups (4–7 individuals) in large experimental tanks (MBI, 122 cm long by 183 cm wide by 80 cm deep; MRC, 366 cm diameter by 90 cm deep). Gravel, large pieces of polyvinyl chloride pipe, and artificial plants were placed in each tank. Unless otherwise specified, experiments were conducted in the home tanks. The cuttlefish were fed a mixture of live and frozen fish, shrimp, and crabs twice a day in the morning and early evening. A complete description of cuttlefish mariculture can be found elsewhere (e.g., DeRusha, Forsythe, DiMarco, & Hanlon, 1989; Hanley et al., 1998).

Data were log transformed where noted and analyzed using parametric and nonparametric statistics as specified (Siegel & Castellan, 1988).

Experiment 1

Method

This experiment tested the feasibility of using a simple escape maze with cuttlefish. Seven sub-adult cuttlefish (9–15-cm mantle length [ML]) were used. An alley (150 cm long by 20 cm wide by 20 cm deep) was constructed with wooden sides and a clear, Plexiglas bottom (see Figure 1A). The alley was suspended over and submerged in a home tank, such that the water depth in the alley was 10 cm. The last 20 cm of the alley had no bottom so the cuttlefish could escape by swimming down into the tank below.

Cuttlefish were placed at the closed end of the alley and allowed to swim to the open end to escape into the home tank below. Cuttlefish are highly visual and like to settle (and bury) on the bottom in a large, open space. They did not settle on the clear bottom of the alley but swam until they found their way out.

There were four stages to the experiment. In Trials 1–15, the open end of the maze faced north. In Trials 16–20, the open end faced south. In Trials 21–25, the open end again faced north. During Trials 26–30, the open end was reversed on each trial.

Results

The performances of all cuttlefish are plotted in Figure 2. The cuttlefish showed significant overall reduction in escape time from a mean of 123 s in Trial 1 to a mean of 20 s in Trial 30: repeated measures analysis of variance (ANOVA), six blocks of five trials, $F(5, 30) = 6.74$, $p < 0.001$. Differences between individuals were not significant: repeated measures ANOVA, six blocks of five trials, $F(5, 30) = 1.10$, $p > 0.25$. There was no detectable effect on maze-escape performances from reversing the maze orientation.

Discussion

The cuttlefish quickly learned the simple task of exiting a straight alley with escape as the only motivation. Results were similar to those obtained by Walker, Longo, and Bitterman (1970) using octopuses in an alley with a food reward at the end of the maze. Whereas octopuses required extensive shaping before readily pursuing the food, cuttlefish required no pre-training in this escape maze. The increase in escape time observed between Trials 21 and 25 was probably due to a water quality problem resulting from a blower failure.

Overall, this experiment suggests that learning in cuttlefish can be evaluated using a simple escape maze. Additionally, the lack of effect of maze reversal (Trial 25) suggests that the cuttlefish did not rely on visual features around the laboratory to solve this simple maze problem.

Experiment 2

Method

An open-field maze was constructed of a round tank (100 cmdiameter by 7 cm deep) with a clear Plexiglas bottom and opaque, blue sides (see Figure 1B). A hole (20 cm diameter) was cut into the bottom of the maze 11 cm from one side. This hole allowed the cuttlefish to swim out of the bottom of the maze into the home tank below. For Group 1, a landmark was provided (6-cm by 6-cm by 2-cm piece of Styrofoam tethered and floating directly above the maze). No specific landmarks were provided for Group 2.

Individual sub-adult cuttlefish (*N* = 18; 7–9-cm ML) were placed in the maze and allowed to swim until escaping into the home tank (no time limit). Trials were repeated twice daily for 20 days. For the first 25 trials, training proceeded as described above. For Trials 26–30, the location of the opening was rotated 180°. In Trials 31–40, the location of the maze exit was assigned randomly to one of the four cardinal directions, with the constraint that each direction was presented at least twice.

Results

No significant improvement in maze escape time was found: repeated measures ANOVA, $F(4, 52) = 2.35$, $p = 0.67$.

Discussion

In this experiment, problems arose from the peculiar way that cuttlefish swim (see Figure 3). Cuttlefish swim slowly forward and backward by using their fins and swim quickly backward by jetting with their funnels. Fine directional control is not possible when jetting, and jetting cuttlefish move haphazardly around the mazes. A number of forward-swimming cuttlefish turned around once they found the maze exit and attempted to jet backward through the exit hole. At times, they missed the hole and had to turn around and again search for the exit. Other times, the cuttlefish would find the escape hole on a forward approach, but on contacting the hole, they became excited or agitated and jetted backwards. Some of the cuttlefish repeatedly forward approached, touched, and jetted backward, appearing "frustrated" with their inability to get through the maze. These various motor constraints probably interfered with maze learning.

Experiment 3

Method

A T maze (100 cm long by 20 cm wide by 10 cm deep) was constructed with black Plexiglas sides and a clear Plexiglas bottom (see Figure 1C). Exits (20 cm by 20 cm) were cut through the bottom at the end of each arm of the T and fitted with removable clear Plexiglas doors. These holes allowed the cuttlefish to swim down into the home tank below. White panels on the walls of the right arm of the T and green panels on the left arm were provided as directional cues (i.e., bright vs. dark).

Sub-adult cuttlefish (*N* = 15; 8–13cm ML) were first pre-trained with 12 trials in an alley maze (the central alley of the T maze; see also Experiment 1). After runway training, the cuttlefish were then pre-trained with 12 trials with the open arm of the maze varied (forced turn; six right and six left turns in semi-random order; Fellows, 1967). The cuttlefish were then divided into three groups and given 24 training trials as follows: For Group 1, the exit was on the right (white) arm of the T maze. For Group 2, the exit was on the left (green) arm of the T maze. For Group 3, the exit was located on either the right (white) or left (green) arm of the maze, varied semirandomly from trial to trial.

Trials were conducted twice a day with a 6-hr inter-trial interval. Immediately after training, learning was tested in two ways. First, cuttlefish received one trial with both exits open (Test 1). This tested the possibility that cuttlefish could detect the location of the open exit using an unintended cue (e.g., water current). Second, the cuttlefish received one trial with the exits to both maze arms open and the wall panels reversed (right arm green, left arm white; Test 2). This tested the relative importance of turning direction versus wall panel cues.

Results

No improvement in exit time was found: arcsine transformed proportions, repeated measure ANOVA, for group, $F(2, 11) = 0.55$, $p > 0.50$; for trial, $F(3, 33) = 0.28$, $p > 0.80$; for interaction, $F(6, 33) = 1.33$, $p > 0.25$. Individual performances were examined for evidence of learning. Three cuttlefish from Group 2 that chose the left–green arm in their initial choice and in Test 1 chose the right–green arm in Test 2.

Discussion

Motor constraints may have again interfered with learning. The cuttlefish often swam down the runway portion of the maze, but when they got to the top of the T, they often rested on the bottom and did not move much thereafter. At this point, they needed to make a 90° turn right or left in a small space, interrupting momentum toward finding the exit in the maze. A Y-shape maze could work better.

The 3 cuttlefish that chose the green arm in both Test 1 and Test 2 may have chosen the maze arm on the basis of visual landmarks rather than directional cues. However, this result is uncertain because no evidence for learning was found.

Experiment 4

Method

Observations from the pond suggested that cuttlefish could learn to use openings in vertical barriers. Consequently, a wall maze was constructed by placing a vertical, opaque Plexiglas wall 50 cm from the short end of a rectangular experimental tank, creating a small testing arena and a larger home tank (see Figure 1D). The side of the wall facing the testing arena was covered with material: artificial sea grass on the left and camouflage mesh on the right. Two holes (20 cm diameter) were cut into the wall: one on the left and one on the right. The hole on the left side was 10 cm above the bottom of the tank, and the hole on the right side was 60 cm above the bottom of the tank. Both exit holes remained open throughout the experiment. A piece of opaque, plastic sheeting the exact width of the tank was angled at 45° from the base of the wall to the water surface on the opposite side of the testing arena (see Figure 1D). Thus, within the testing arena, there was no horizontal surface on which the cuttlefish could settle, providing increased motivation for escape. A gravel substrate was provided in the home tank.

Individual subadult cuttlefish ($N = 18$; 9–13-cm ML) were placed in the testing arena midway between the openings and facing the back wall. The time to exit the arena (maximum 10 min) and the exit used were recorded. If a cuttlefish did not escape, the escape time was recorded as 1,000 s.

Results

Cuttlefish demonstrated a significant decrease in exit time within 10 trials: repeated measures ANOVA, $F(9, 135) = 7.47$, $p < .001$ (see Figure 4). The mean escape time in the 1st trial was 15.6 min (only 3 of 18 cuttlefish escaped), whereas the mean escape time in the 10th trial was 4.0 min (15 of 16 escaped). This was not a result of an improvement in maze escape time (mean first escape time = 154.0 s, *SEM* = 33.2; mean last escape time = 154.0 s, *SEM* =40.3). Thirteen of 18 cuttlefish preferred the left–lower hole, and cuttlefish exited through their preferred hole an average of 78.00% of the time (range = 50.00%–100.00%, *SD* = 16.17).

Discussion

Cuttlefish learned to escape from this two-choice maze. They did not escape more quickly; time to escape the small arena was simply the time it took the cuttlefish to turn around and swim out. Initially, those that did not exit swam rapidly around the testing arena, repeatedly approaching the corners. At the end, those that did not escape wedged themselves between the plastic sheeting and the wall and remained still for the full 10 min.

Results from this experiment indicate that the problem with the open field and T-mazes lies not with the cuttlefish learning abilities but instead with the maze design and cuttlefish motor behavior. In both the wall maze and the alley maze, cuttlefish exited while swimming slowly forward, and thus, motor constraints did not interfere with learning.

Cuttlefish are generally benthic; thus, the greater preference for the left–lower hole may have resulted because cuttlefish remained near the bottom of the testing arena and the lower hole was easier to locate.

Experiment 5

Method

The purpose of this experiment was to further evaluate learning of a simultaneous, visual–spatial discrimination task. Subsequently, reversal learning of this problem was tested. Twenty cuttlefish were used in this experiment: 13 adults at the MRC (15–25-cm ML) and 8 sub-adults at the MBI (10–15-cm ML). A circular testing arena was constructed from a large, plastic barrel (71 cm deep_ 56 cm diameter; see Figure 1E). A start tube (16 cm diameter) with doors on both ends was placed through one side of the arena, 17 cm below the top edge (12 cm below the water surface). Two exit holes (16 cm diameter) were cut on opposite sides of the arena, 6 cm below and perpendicular to the start tube. A panel of striped fabric (36 cm by 36 cm) surrounded the exit located on the right side of the start tube, and a spotted panel of fabric (36 cm by 36 cm) surrounded the exit located on the left side of the start tube. Exits were fitted with movable, clear Plexiglas doors. The bottom of the arena was fitted with a plastic mesh cone (point up, 12 cm high) such that, like the wall maze, there was no horizontal surface on which the cuttlefish could settle. The maze was placed within the home tank at the beginning of each set of trials.

In each trial, 1 cuttlefish was herded into the start tube. After 1 min, the door to the testing arena was opened. The cuttlefish was given 3 min to exit the tube and enter the arena. If the cuttlefish did not enter the arena, the experimenter chased it into the maze using a net. Once the cuttlefish was inside the arena, the start tube was blocked off. The cuttlefish was given 7 min to escape the arena before the experimenter chased it out of the open exit with a net. The escape time for each trial was recorded. Any trial in which the cuttlefish failed to escape was assigned an escape time of 10 min. In each trial, the maze was positioned in a random direction within the home tank to control for the possibility that cuttlefish used cues around the laboratory to locate the open exit.

There were four stages to this experiment: pre-training, preference testing, training, and reversal. Individual cuttlefish (*N* = 21) were pre-trained with both exit doors open. Trials were repeated until the cuttlefish independently exited the maze once through each exit. After pre-training, exit preference was determined by observing the exit most frequently used in five further escapes. After pre-training and preference testing, experimental trials began. In the first set of training trials (Reversal 0), the cuttlefish's preferred exit remained closed. Each cuttlefish received six trials per day (at least 45-min intertrial interval) until six of seven consecutive escapes were achieved in less than 1 min. Once this criterion had been reached, the cuttlefish were given a probe trial with both exits open. This probe tested the possibility that cuttlefish could detect the location of the open exit using an unintended cue (e.g., water current). If the cuttlefish did not escape through the trained exit, they were given two more training trials and then another probe trial; this occurred only three times, in 3 different cuttlefish. Once the cuttlefish was trained, the open door was closed, the opposite door was opened, and the training procedure repeated. Training and door reversal continued as time permitted.

Results

All cuttlefish completed pre-training successfully (range = 5–16 trials). There were no notable performance differences between cuttlefish tested at MBL and MBI or between female and male cuttlefish. Nine cuttlefish preferred the striped (right) exit and 12 cuttlefish preferred the spotted (left) exit. Fourteen cuttlefish had strong preferences (4 or 5 out of 5 trials). These strong preferences were split evenly between the two exits (8 cuttlefish preferred the striped exit, and 6 preferred the spotted exit).

For the 20 cuttlefish that completed Reversal 0 (training against preference), the mean number of trials was 36 (range = 13–72), the mean escape time was 5 min 8 s (range = 2 min 50 s–6 min 24 s), and the mean percentage of escapes in less than 1 min was 36.2% (range = 13.9%–64.7%). One cuttlefish died during the course of training for Reversal 0.

There was a marked improvement in performance for cuttlefish that completed subsequent reversals. For cuttlefish completing Reversal 2, the mean number of trials during Reversal 2 was 17 (range = 6–28), the mean escape time was 2 min 48 s (range = 1 min 4 s–4 min 6 s), and the mean percentage of escapes in less than 1 min was 61.9% (range = 33.3%–76.9%).

Four cuttlefish completed six reversals and showed a significant improvement in maze performance. Improvement was indicated by a significant decrease in the number of trials per reversal, a significant decrease in the percentage of errors per reversal, and a significant increase in the number of escapes in less than 1 min (see Table 1 and Figures 5 and 6). The apparent decrease in average escape time across all reversals was not significant (see Table 1).

For further analyses, performances on Reversals 0, 2, 4, and 6 (against original preference) were considered separately from performances on Reversals 1, 3, and 5 (consistent with original preference). For all variables, there was significant improvement when trained against original preference but not when trained with original preference (see Table 1).

Two of the cuttlefish that completed Reversal 6 showed performances suggestive of one- to two-trial learning in later reversals: One cuttlefish completed Reversals 3 and 6 in just eight trials per reversal, and the other cuttlefish completed Reversals 5, 6, and 9 in eight trials per reversal. Because criterion level performance was six of seven consecutive escapes in less than 1 min, the absolute minimum possible number of trials in a reversal was seven.

Discussion

Previous authors have established that cuttlefish can learn a simultaneous discrimination task (Messenger, 1977). This experiment is the first to establish that cuttlefish improve over serial reversals of a simultaneous discrimination problem. Results are consistent with those found in octopuses (Mackintosh & Mackintosh, 1964; Young, 1962) but not successively (Mackintosh, 1962). Improvement over serial reversals may indicate that cuttlefish are "learning to learn" (Harlow, 1949). This ability allows animals to update or relearn solutions to problems when previous solutions are no longer relevant.

Significant improvement in escape performance was observed across reversals in which the cuttlefish were trained against their original door preference; only minor (statistically insignificant) improvement was observed across reversals in which the cuttlefish were trained consistent with their original door preference. This is similar to findings from octopus discrimination-learning experiments (Boal, 1996).

The performances of 2 cuttlefish were indicative of two-trial learning. Mackintosh and Mackintosh (1964) also observed an octopus that showed one-trial learning. One-trial learning has been attributed only to vertebrates (see reviews in Bitterman, 1989; Mackintosh, Wilson, & Boakes, 1985). The few published studies on reversal learning in marine invertebrates (e.g., crabs: Datta, Milstein, & Bitterman, 1960; isopods: Thompson, 1957) did not show one-trial learning, but anecdotes with octopuses suggest that very rapid learning of some tasks should be investigated more thoroughly.

It remains unclear which cue the cuttlefish used to locate the open exit: direction relative to the start box or panel pattern surrounding the exit. Further experimentation to distinguish between these alternatives is currently in progress.

General Discussion

This is the first set of experiments to examine spatial and reversal learning in cuttlefish. Specifically, this set of experiments demonstrated that cuttlefish (a) can solve an experimental maze problem with escape as the sole motivation, (b) have particular motor needs that can confound the results of spatial-learning experiments, and (c) show spatial learning and improvement across serial reversals when these confounders are overcome.

It is unclear how cuttlefish may use such learned spatial information in the natural environment. Unlike many octopuses, which probably use learning to relocate home shelters, cuttlefish rely primarily on crypsis for defense. Cuttlefish constantly move in and around vertical obstacles in the natural environment, and spatial learning may help them negotiate these obstacles (Sanders & Young, 1940). Alternatively, perhaps cuttlefish use spatial learning to relocate good foraging patches or return to a prominent landmark between forages (O'Dor, personal communication, 2002). Unfortunately, we have no field data that address these particular hypotheses.

Spatial learning is widespread among animals. Spatial-learning studies have focused on mammals and birds but have also been successful in reptiles, amphibians, and fish as well as in invertebrate groups such as arthropods and octopuses (for reviews, see Capaldi, Robinson, & Fahrbach, 1999; Golledge, 1999; Sherry, 1998; Wehner, 1981). Spatial problems typically encountered by different animals, such as returning to home or finding food sources, are remarkably constant across different species. Locating food resources, shelter, and escape routes are each facilitated by spatial learning. It remains unclear which aspects of cephalopod life history might have caused their great cognitive divergence from other mollusks. These mazes could provide a tool for future studies addressing this question more precisely.

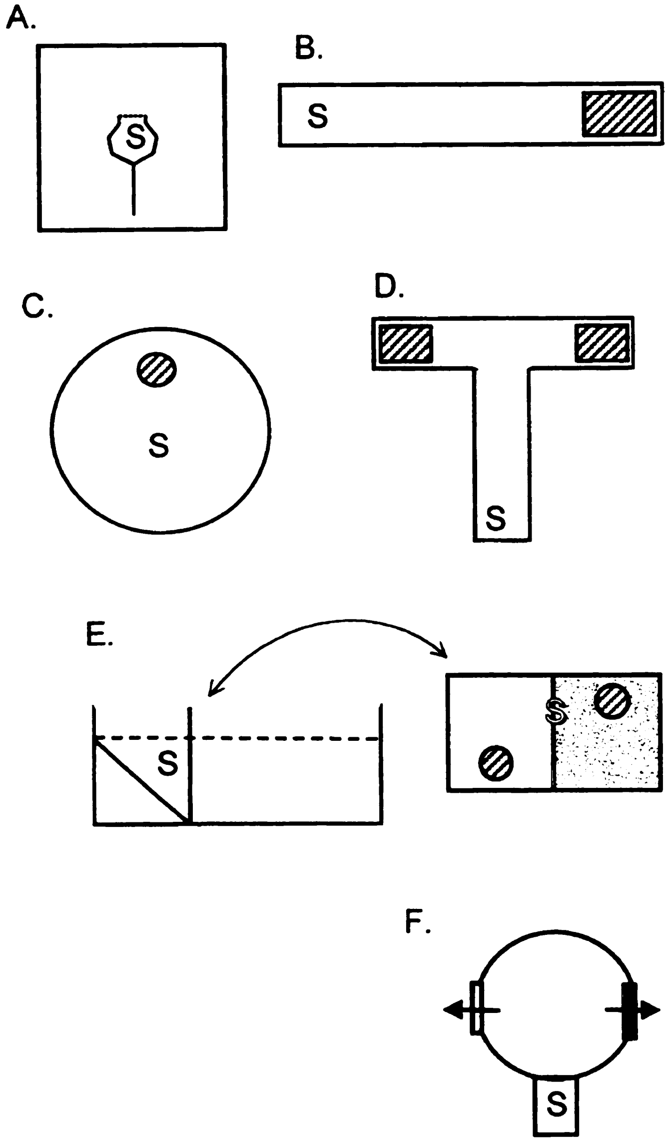

Figure 1. Illustrations of the mazes tested with cuttlefish as seen from above (except for E). Starting points are indicated with an S, and exit locations are shaded with diagonal lines. A: Artificial pond used for preliminary observations. B: Alley maze used in Experiment 1. C: Open-field maze used in Experiment 2. D: T maze used in Experiment 3. E: Wall maze used in Experiment 4 (left: side view) with detailed view of the wall and two exit doors (right). F: Two-choice discrimination maze used in Experiment 5.

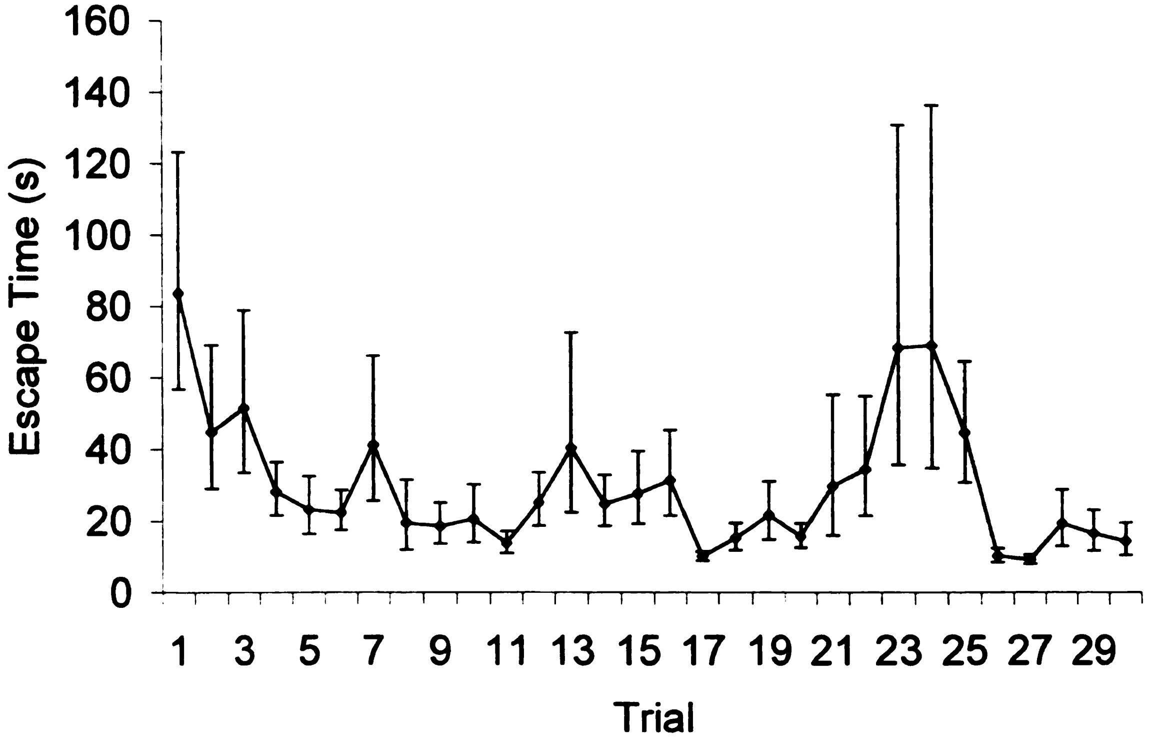

Figure 2. Mean escape time for cuttlefish in the runway maze (Experiment 1; *N* = 7). The maze orientation was reversed at Trial 16, Trial 21, and Trials 26–30. Error bars represent standard errors

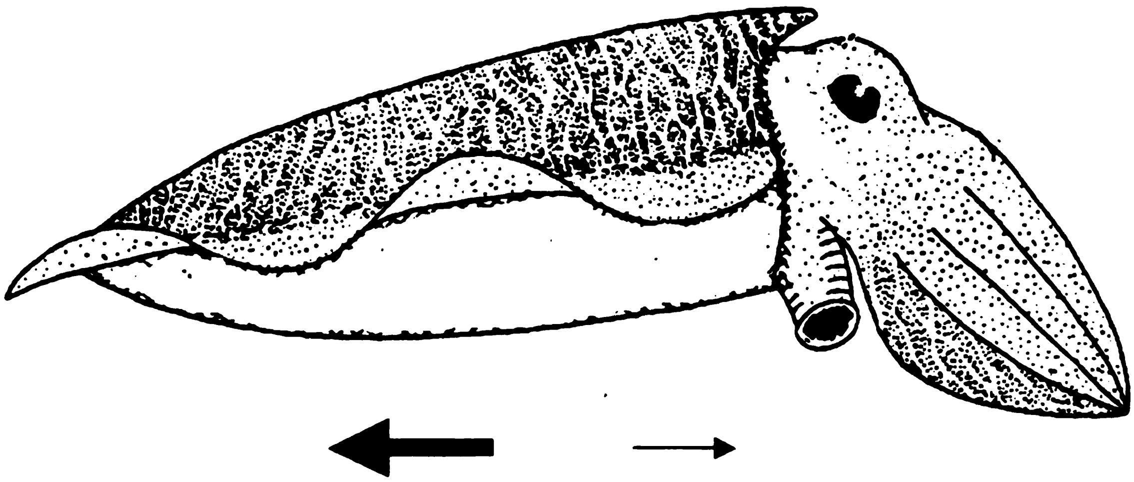

Figure 3. Cuttlefish use fins for slow, forward movement (small arrow) and jet propulsion for rapid, backward swimming (large arrow). Adapted from Tompsett, 1939.

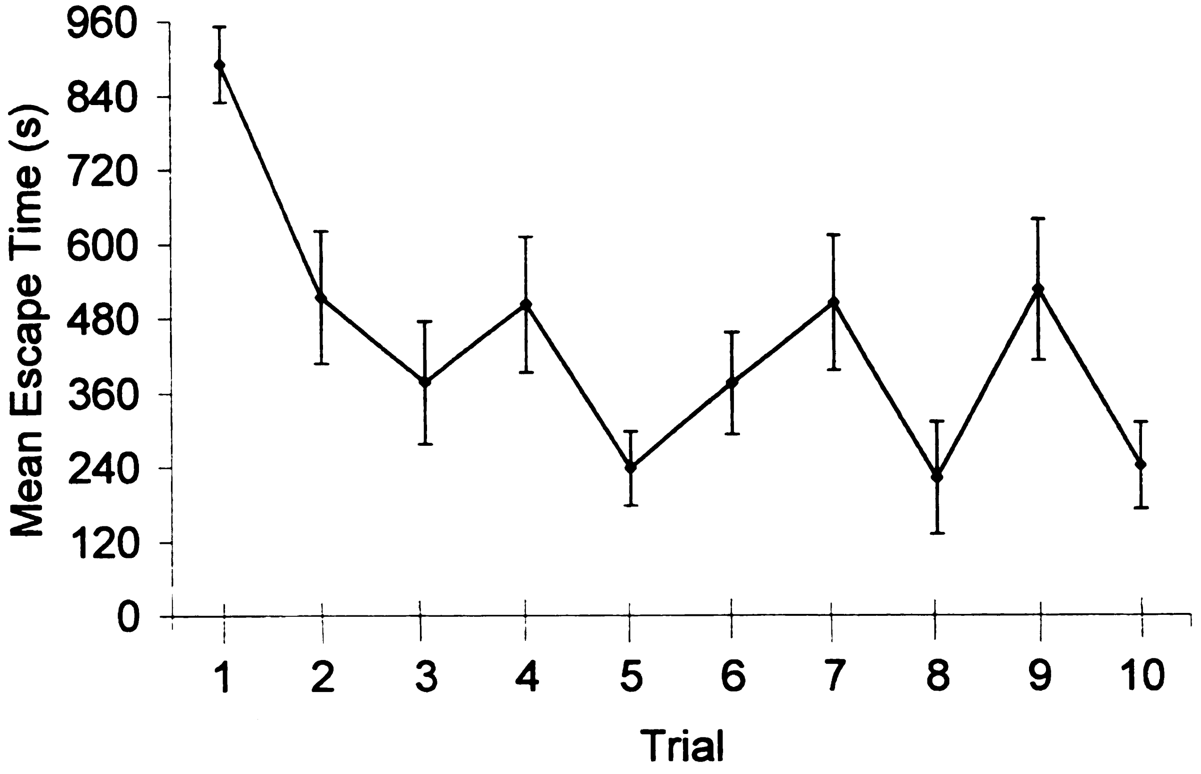

Figure 4. Mean escape time for cuttlefish in the wall maze (Experiment 4; *N* = 18). Cuttlefish demonstrated a significant decrease in exit time within eight trials. Error bars represent standard errors.

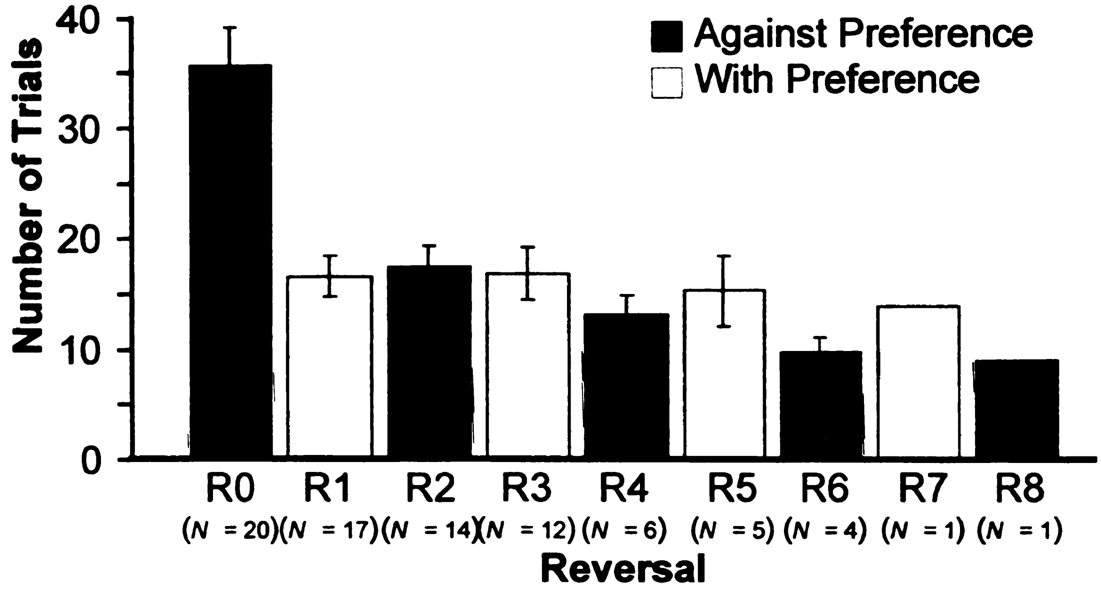

Figure 5. Mean number of trials until reversal (criterion-level performance; Experiment 5; *N* = 20). Cuttlefish showed a significant decrease in the number of trials per reversal when trained against original preference (solid bars) but not when trained in the direction consistent with original preference (open bars). Error bars represent standard errors. R = Reversal.

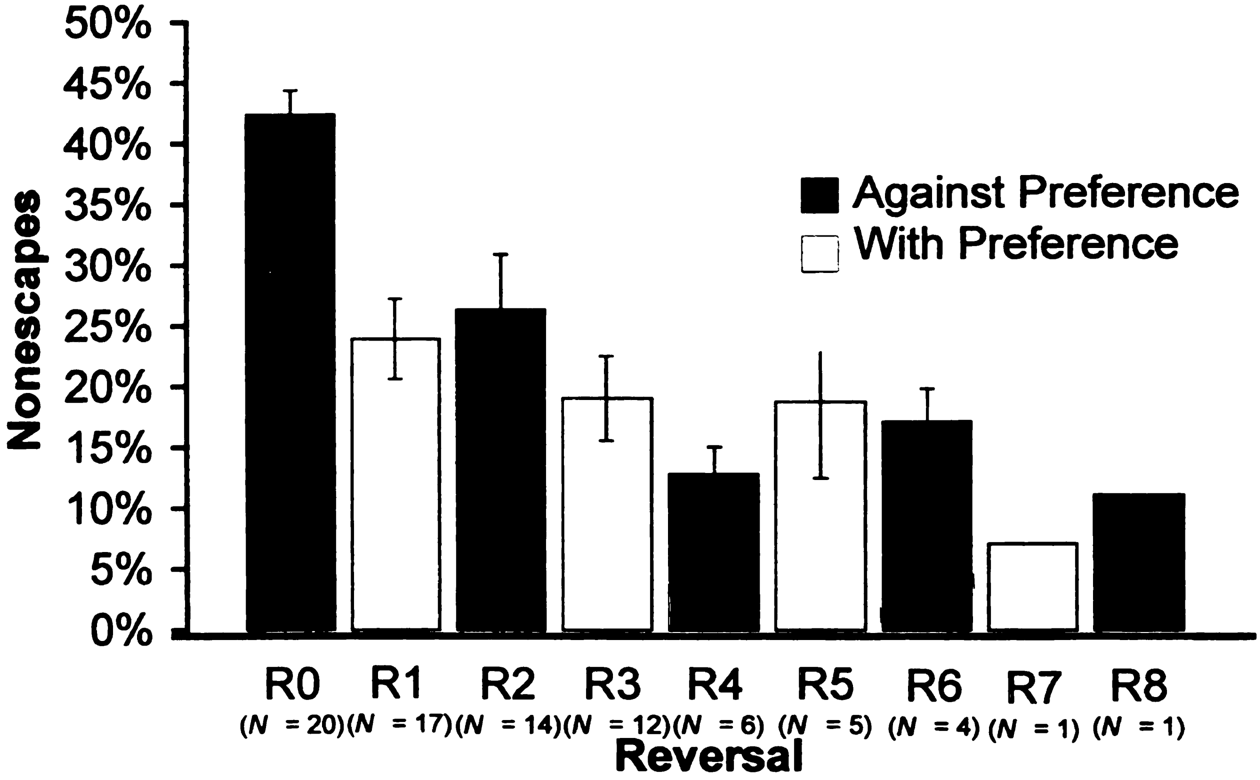

Figure 6. Mean percentage of errors (nonescapes) per reversal (Experiment 5; *N* = 20). Cuttlefish showed a significant decrease in the percentage of errors per reversal when trained against original preference (solid bars) but not when trained in the direction consistent with original preference (open bars). Error bars represent standard errors. R = Reversal.

Table 1. Results of analyses of the effects of serial reversals on learning (Page Test for Ordered Alternatives). Significant results indicated in boldface.

Variable	N	k	L	p
Number of trials per reversal				
-overall	4	7	505	**<0.01**
-against preference	4	4	115	**<0.01**
-with preference	4	3	50	>0.05
Percentage of error				
-overall	4	7	521	**<0.001**
-against preference	4	4	114	**=0.01**
-with preference	4	3	53	>0.05
Number of escapes < 1 min				
-overall	4	7	497	**<0.025**
-against preference	4	4	112	**<0.05**
-with preference	4	3	46	>0.05
Mean escape time				
-overall	4	7	480	>0.10

LITERATURE CITED

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