6-22-2022

Assessing Learning and Memory in the Juvenile Life Stage of the California Two-Spotted Octopus, Octopus Bimaculoides

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NSUWorks Citation

Shaquilla M. Hamlett. 2022. *Assessing Learning and Memory in the Juvenile Life Stage of the California Two-Spotted Octopus, Octopus Bimaculoides.* Master's thesis. Nova Southeastern University. Retrieved from NSUWorks, . (89)
https://nsuworks.nova.edu/hcas_etd_all/89.

Thesis of
Shaquilla M. Hamlett

Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Science
Marine Science

Nova Southeastern University
Halmos College of Arts and Sciences

June 2022

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HALMOS COLLEGE OF ARTS AND SCIENCES

Assessing learning and memory in the juvenile life stage of the California two-spotted octopus, *Octopus bimaculoides*

Presented by

Shaquilla Hamlett

Submitted to the Faculty of
Halmos College of Arts and Sciences
in partial fulfillment of the requirements for
the degree of Marine Science with a specialty in:

Marine Biology and Marine Environmental Science

Nova Southeastern University

June 2022

Abstract

Cephalopods and vertebrates have convergently evolved diverse adaptations such as large brains, problem-solving skills, tool use, and learning and memory abilities. These traits have been observed in cephalopods (e.g., octopus), in which they are able to solve mazes and navigate complex environments. Although there is evidence that all orders within Cephalopoda have an evolved capacity for learning and long and/or short-term memory, some appear to have less advanced cognitive abilities (e.g., nautilus) than others (e.g., octopus). The purpose of this project to determine whether the California two-spotted octopus (*Octopus bimaculoides)* is an example of a cephalopod with higher cognitive function, which may have evolved in response to selective pressures related to finding food and avoiding predators. This species' cognitive ability was assessed in ~4 month old octopus using a food preference test and a learning test (ability to recognize a habitat created from 3D printed rocks and navigate to its hidden food source). Methods for determining associative learning for this species were also developed. Due to possible disinterest in the food sources and premature mortality, neither study yielded substantial insights to cognition or memory in the focal species. The octopus were observed interacting sporadically with experiment objects or hiding, a sharp contrast from the everyday interactions during husbandry where they ate once or twice a day every other day. Histology of octopus' brains from early life stages (i.e., hatchlings and juveniles) from ages three weeks to thirteen weeks did show increased development of the optic, pedal and vertical lobes of the brain, and general overall growth and development of the brain. These results revealed full brain development as young as 11 weeks, suggesting capability for higher learning and memory. Consequently, potential enhancements to *future O. bimaculoides* husbandry and study design are discussed along with the possible outcomes and significance of studies into cephalopod memory and cognition.

Keywords: associative and spatial learning, memory, food preference, neurobiology, marine invertebrate, animal behavior, histology, animal care

Acknowledgements

First, a big thank you to Experiment.com for their Hot Science Summer Grant, and Conchologists of America for the COA grant. Without either, this project would not have been possible. And to the Marine Biological Laboratory for caring and providing the octopus hatchlings, and their veterinarian, Dr. Lisa Abbo, for her expert advice.

I would also like to acknowledge my advisor, Dr. Timothy Swain, my committee members: Dr. Chelsea Bennice, Dr. Eben Gering, Dr. Teresa Iglesias, and Dr. Lauren Nadler and for their expert advice, suggestions, and willingness to hold multiple brainstorming sessions. As well as my undergraduate student assistants: Breanna Calle and Andrew Hartman for their assistance in husbandry and project completion.

Finally, I would like to thank my fellow peers and parents for their support, advice, and listening ear through every bump in the road. Without all of you, this project would have been even more challenging than it was.

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Introduction

The Cephalopod's Environment as a Driver of Behavioral Evolution:

Throughout the Paleozoic era, cephalopods evolved from having shells, and possibly more simplistic brains, to losing their shells and becoming more complex, predatory creatures with problem-solving abilities (Kröger et al., 2005; Sanders, 1975). There are two extant subclasses within the class Cephalopoda: Coleoidea (octopus, squid, and cuttlefish) and Nautiloidea (nautilus). The Nautilus is the only remaining cephalopod that carries an external shell for protection and buoyancy. Coleoids either lack a shell (octopus) or contain an internal one (squid and cuttlefish) that assists in buoyancy and support. As their shells were lost, and these cephalopods became more common in various habitats, they met new challenges related to hunting prey and avoiding predators. Soft-bodied organisms face a higher risk of predation than those with shells, and so evolved adaptations necessary to survive. These additional challenges associated with a soft body led to the hypothesis that cognition evolved to increase survival after the shell was lost. Cognition can be defined differently depending on the focus of the study but is generally used as an index of behavioral flexibility that is adaptive in unpredictable environments (Hanlon et al., 2018; Navarrete et al., 2016; Schnell et al., 2021).

Cognition, or "intelligence", here collectively refers to skills related to spatial learning, memory, problem solving and tool use (Navarrete et al., 2016; Schnell et al., 2021). There are two main hypotheses concerning this concept. One hypothess suggests that the development of cognitive abilities would have to had to occur long before cephalopods' loss of shells since the time necessary to evolve "intelligence" would have been too great to prevent extinction via predation (Amodio et al., 2019a; Amodio et al., 2019b). The second hypothesis – which is argued here –is that the rate of cognitive skill development increased gradually following the loss of the shell as cephalopods began to use different hunting strategies (e.g., selective vs. opportunistic foraging) (Billard et al., 2020; Hochner & Glanzman, 2016; Ponte et al., 2021). This hypothesis predicts extant species should show differences in their cognitive abilities that can be linked to the ancestral environment and/or life history as cephalopods that are shelled do not have the evolutionary drivers to develop higher cognition due to lower predation pressures. This is seen in how nautilus, have simplistic brains with fewer lobes and neurons than coleoids, with limited ability to spatially learn their environment and retain information (Crook et al., 2009; Crook & Basil, 2013; Koizumi et al., 2016; Ponte et al., 2021; Figure 1). Nautilus also lack a centralized brain that can be differentiated from surrounding tissue unlike that seen in the central nervous system of coleoids, a feature that points strongly towards a greater capacity of learning and memory both in cephalopods and vertebrates (Crook et al., 2009; Koizumi et al., 2016; Ponte et al., 2021). Coleoids' nervous systems contain approximately half a billion neurons, equivalent to those found in dog brains, although in coleoids, majority of their neurons are concentrated in the peripheral nervous system instead of the central nervous system (Hochner, 2008; Hochner, 2012). Octopus also have independent neuronal control in their arms that can function separately from each other and the brain, while still being subject to control by the central nervous system (Gutnick et al., 2020; Hochner, 2012).

The History of Research into Cephalopod Cognition

How intelligence appears in vertebrates:

Key characteristics that humans attribute to organismal intelligence: memory, tool-use, and the ability to learn. Some examples of these traits in vertebrate species include developing tactics and strategies associated with tool use and ability to migrate to their natal breeding ground (Abrahms et al., 2019; Visalberghi et al., 2017). Crows present one vertebrate example of novel tool use, in which they use crosswalks to open nuts by dropping them for cars to crush and then collecting the open pieces when the walking sign turns green (Cory, 2016). They have also been seen bending wire into a hook to access food from a jar (Sugasawa et al., 2017). The New Caledonia Crows are also able to combine objects to construct tools of differing lengths to reach food items placed in a puzzle box (Bayern et al., 2018). Three separate wild bonobo communities are another example where females use leaves as umbrellas during the wettest months (Samuni et al., 2021). More recently, Goffin's cockatoos were shown to be able to use two separate tools (a ball and a stick) to solve the 'Golf Club Task' – a puzzle box with openings to a food reward that can be reached with the ball and stick tools (Osuna-Mascaró et al., 2022). These examples of tool use indicate a high capacity for problem solving, another sign of cognitive prowess (Abrahms et al., 2019; Visalberghi et al., 2017). Extensive studies of intelligence have been done with all types of vertebrates from elephants and primates (e.g. apes and lemurs), to cetaceans (i.e. dolphins and whales; Tsalyuk et al., 2019; Abrahms et al., 2019). Intelligence is known to be limited in some vertebrates such as amphibians and reptiles and was thought to be completely absent in invertebrates until recent studies revealed associative learning in bumblebees, spatial learning in cockroaches and long-term memory in crickets (Matsumoto et al., 2018; Palottini et al., 2018; Pomaville & Lent, 2018; Visalberghi et al., 2017). This serves as evidence that cognition is more common in invertebrates than previously assumed.

Another invertebrate, the octopus is known for escaping from holding tanks overnight and returning before morning, problem-solving by completing puzzles, and distinguishing between different colored objects (Fiorito & Scotto, 1992; Richter et al., 2016; Stubbs & Stubbs, 2016). The ability of determining color is not something potentially seen in all cephalopods, but more recent studies have found that some species are able to do so (Fiorito & Scotto, 1992; Stubbs & Stubbs, 2016). Octopuses are also known for extensive exploration of new items or areas, and a fascination with human beings. Their exploration, curiosity, and possession of behavioral sophistication has led many scientists to associate them with advanced cognitive abilities. For example, the coconut octopus (*Amphioctopus marginatus*) has been shown to use items such as seashells as tools and shields, carrying the shells with it as it hunts or travels to a new area. If this octopus encounters a predator, they can close themselves up inside these shells as protection (Visalberghi et al., 2017). The common octopus (*Octopus vulgaris*) showed that it is capable of social learning by learning to open a drawer to get a crab food reward after watching another octopus perform this task (Borrelli, et al., 2020). Observations of tool use, memory, and learning by both vertebrates and invertebrates may suggest parallels in the evolution of their cognitive abilities. However, the cognitive abilities of cephalopod invertebrates are still being discovered, especially among different age groups. Consequently, further studies into cephalopod behavior promise insight to general questions about animal behavior and its evolution.

Biodiversity within Cephalopods as a Potential Driver of Cognitive Evolution:

Octopus, Squid, & Cuttlefish:

Octopus are the only cephalopod that lacks all aspects of a shell; the cuttlefish has an internal structure made of aragonite called the cuttlebone that extends throughout the mantle (head region of cephalopods), while the squid possesses a hard/rigid structure called the pen that extends the entire length of its mantle (Figure 1; Hanlon & Messenger, 2018). All three groups contain species with an ink sac, chromatophores and papillae (pigment-filled and texture changing skin organs, respectively) for camouflage, defense, and communication (Hanlon & Messenger, 2018). Those species without shells have developed large brains and sense organs, such as complex eyes with a lens and an iris (similar to the structure of eyes in vertebrates), but all coleids, except nautilus, have very small olfactory organs since they are chemo-tactile hunters that also use visual cues to find prey (Hanlon & Messenger, 2018). The octopus lacks tentacles and instead has eight arms covered in suckers (Hanlon & Messenger, 2018). The squid and cuttlefish both have eight arms with two retractable tentacles (suckers only present on distal end), but the tentacles are much longer than the arms in squid (Hanlon & Messenger, 2018). These tentacles are used for attacking prey while arms are used for multiple purposes including manipulation of objects/food items and movement (Hanlon & Messenger, 2018). Cephalopods grow very quickly, but only live one to two years, with a rare few living up to four to five years. The majority are semelparous, only reproducing once in their lifetime (Hanlon & Messenger, 2018).

The brain of cephalopods has 40 interconnecting lobes (Hochner 2012; Shigeno et al. 2001). The two optic lobes are the largest and are responsible for visual detection, learning, and motor functions (Brown & Piscopo, 2013; Hochner 2012; Figure 2). The suboesophageal lobes is used primarily for motor functions while the supraoesopagal lobes is used for learning and memory along with the vertical lobes (Brown & Piscopo, 2013; Hochner 2012; Shigeno et al. 2001; Figure 2). Growth speed of these lobes varies between cephalopod classes with cuttlefish potentially developing the vertical lobe quicker than some squid (Shigeno et al. 2001) Squid lack the subfrontal or medial inferior frontal lobe of the brain indicating a limited capacity for learning (Young 1991). The small suboesophagel lobes indicate limited memory capabilities (Young 1991).

Nautilus:

Out of all the cephalopods, nautilus is the only one that still possesses an external chambered shell (Figure 1C; Hanlon & Messenger, 2018). This shell is used for protection and controlling buoyancy. It contains a series of chambers that are connected by the siphuncle (a tube-like structure), which alters the gas-water mixture in each chamber to achieve neutral buoyancy (Hanlon & Messenger, 2018). Evidence suggests that the nautilus relies more on olfactory than visual abilities as the nautilus' eyes are simple, resembling a pinhole camera and lacking a lens, which limits the eyes' sensitivity and resolution of images (Hanlon & Messenger, 2018). Their appendages are composed of around 90 thin tentacles without suckers, but they are still adhesive due to the cells lining them containing mucopolysaccharide (Hanlon & Messenger, 2018; Muntz & Wentworth, 1995; von Byern et al., 2012). Nautilus are slow growing cephalopods that live over 20 years (Hanlon & Messenger, 2018). They are iteroparous, reproducing more than once in their lifetime (Hanlon & Messenger, 2018).

The brain of nautilus has fewer lobes and neurons than the coleoids and are missing some of the key components for extensive learning and memory. For example, the optic lobes are much smaller (Crook & Basil, 2008; Crook & Hanlon, 2009; Young 1965)

Trends in Ecology & Evolution

Figure 1:

Examples of the four distinct groups of cephalopods discussed in this study. (A) *Sepia officinalis* – common cuttlefish, (B) *Sepioteuthis sepioidea* - Caribbean reef squids, (C *Nautilus pompilius*) - Nautilus, and (D) *Octopus vulgaris* -common octopus (Amodio et al., 2019).

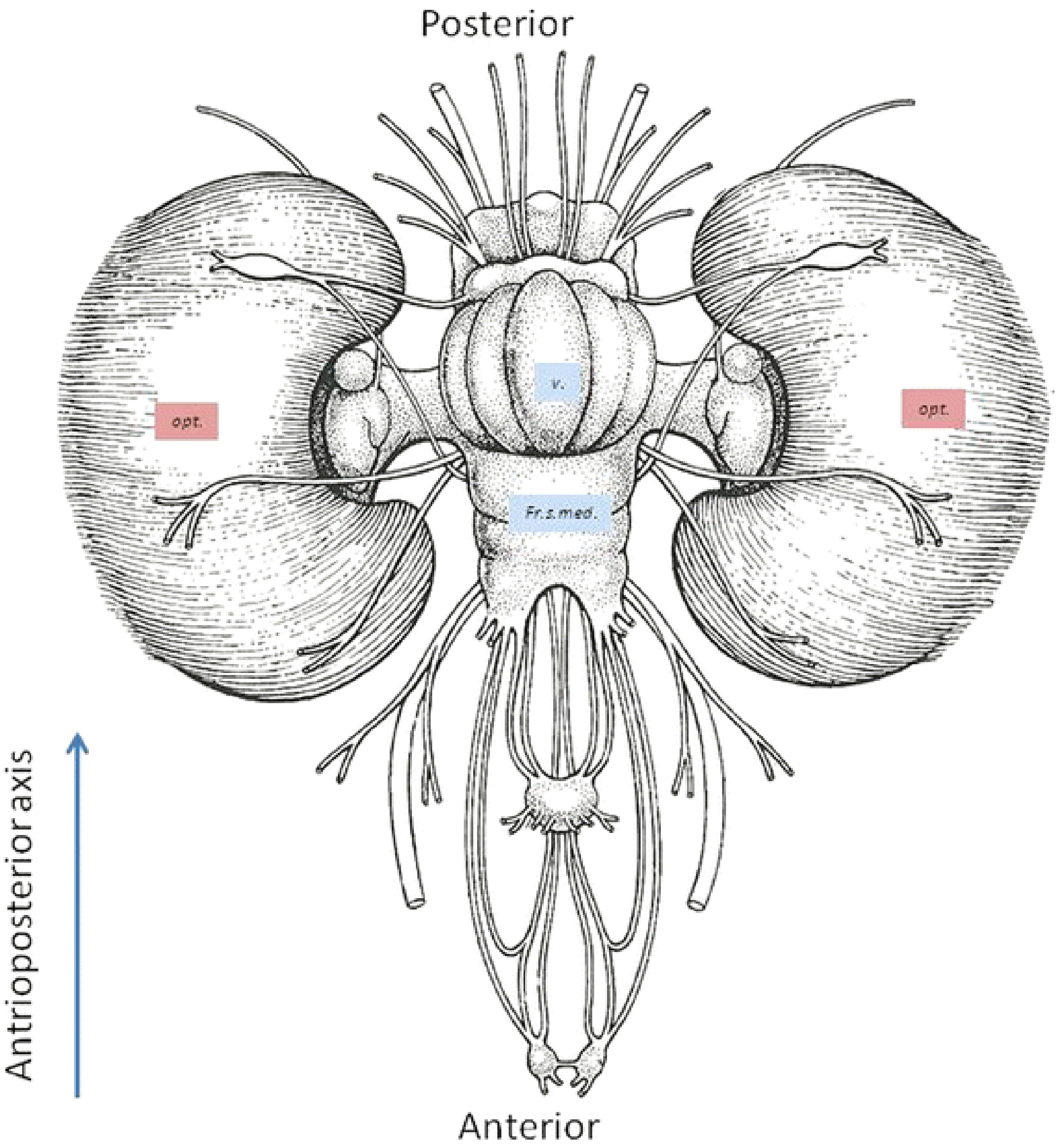


Figure 2:

Depiction of the dorsal view of the central nervous system of Octopus vulgaris major brain lobes. V is the (vertical lobe), opt. (optic lobe), Fr s.med (medial superior frontal lobe) (Young 1991).

Examples of Cognitive Abilities Observed Across Cephalopods

Cognition collectively refers to skills related to tool use, problem solving, memory, and spatial learning (Navarrete et al., 2016; Schnell et al., 2021). This section gives examples of cognitive abilities seen in cephalopods to provide a basis for further behavioral studies focused specifically on learning and memory as an indicator of cognition in early life stages.

Problem solving:

Plentiful evidence illustrates the octopus' ability to solve complex problems. A recent study showed that this octopus species could solve increasingly complex puzzles at five levels with a remarkable level of behavioral flexibility as the puzzle difficulty increased (Richter et al., 2016). Similarly, another study found that this species could open sealed translucent glass jars to capture the live crab within. The time and number of errors decreased with each successive trial, while exploration remained approximately consistent (Fiorito et al., 1990).

In another octopus species, *Enteroctopus dofleini* (giant Pacific octopus), the octopus was able to learn how to open a child-proof bottle, reducing the time to access the herring food item by10 times over 26 training events. (Anderson & Blustein, 2006).

Mazes:

Octopus are centrally placed foragers, meaning their foraging and mating activities are focused on the area surrounding their home den, although this hasn't been studied for juveniles. During foraging events, *O. cyanea* typically traveled approximately 40- 60 meters from their den to forage during events that lasted 1-3 hours. They varied their routes and foraging locations using a different route to return to their den and avoiding visiting the same foraging location on consecutive days most likely to allow for prey accumulation (Forsythe & Hanlon, 1997; Hvorecny et al., 2007). The ability to vary routes and locations illustrates the octopus' spatial orientation and learning abilities, as they require individuals to recognize landmarks and remember directions (Healy, 1998; Hvorecny et al., 2007; Golledge, 1999; Shettleworth, 1998). These abilities are vital for a lifestyle incorporating exploratory foraging and long-distance travel. Behavioral assays with mazes have been designed to study these abilities in cephalopods and other animals in a controlled, laboratory setting. Individuals were trained for two maze configurations and were required to discriminate one escape route (Hvorecny et al., 2007). Complex spatial learning following training was determined by whether the organisms chose the correct escape pathway for each maze, which most individuals were able to complex after approximately 18-43 trials between experimental Tasks 1-3 (Hvorecny et al., 2007). Although studies on this topic have been completed in the adult stage for a number of cephalopod species, whether the juveniles stage is also a centrally placed forager remains unknown.

Individual recognition

Individual recognition (IR) can be important for social behaviors such as territorial defense or mate choice. This skill requires skills in discrimination among sensory cues. Only organisms that have cognitive adaptations or complex nervous systems have developed IR (Tricarico et al., 2011). Traditionally, only birds, mammals, and some fish were thought to be capable of IR (Tibbetts & Dale, 2007; Tricarico et al., 2011). However, studies in several invertebrate species such as insects (D'Ettorre & Heinze, 2005; Tibbetts, 2002; Tricarico et al., 2011) and decapod crustaceans (i.e., fiddler and hermit crabs) (Atema & Steinbach, 2007; Aquiloni & Gherardi, 2010; Deeto et al., 2006; Gherardi & Tricarico, 2007; Tricarico et al., 2011; Van der Velden et al., 2008), indicate that this trait is also found in these taxa.

A study examining IR in *O. vulgaris* found that octopus can distinguish between familiar and unfamiliar conspecifics (individuals of the same species) and recall previously encountered individuals for at least one day (Tricarico et al., 2011). This was determined by use of visual and physical interactions between the octopus (Tricarico et al., 2011). While not all cephalopod species have been seen with this ability, evidence of IR in any cephalopod species could provide evidence for that species' cognitive advancement (Boal, 1996; Tricarico et al., 2011). Field observations of mimic octopuses provide an example of complex communication, in which individuals are able to detect conspecifics visually, approach one another and briefly touch arms to exchange chemosensory information potentially to identify and remember animal's species or sex (Hanlon et al., 2007; Dr. Bennice's personal observations).

Tool Use:

Tool use, defined here as the use of other objects to serve a specific purpose, is seen in multiple vertebrate species such as sea otters that create make-shift anvils to open mollusks (Riedman & Estes, 1990); bottlenose dolphins that use sponges to protect their rostra while digging for burrowed prey on the seafloor (Patterson &Mann, 2011); and rays and skates fan water to reveal benthic prey (Carrier et al., 2012; Mann & Patterson, 2013). Similar tool use is present in cephalopods as well. The common cuttlefish squirts water to unearth prey and build burrows (Mather, 1995; Von Boletzky & Von Boletzky, 1970), while the bobtail squid uses water jets to create burrows (Mann & Patterson, 2013; Von Boletzky & Von Boletzky, 1970). In all three cases where water is used, the water itself it being used as the tool (Carrier et al., 2012; Dill, 1977; Jearld & Miller, 1983; Mather 1995; Pitman & Durban, 2012) .

Associative Learning:

Associative learning is defined the ability to connect a positive or negative stimulus to a specific reward (Balsam et al., 2010; Mitchell et al., 2009; Wasserman & Miller, 1997). These associations are usually made the with use of sound, food, or light stimuli to signal a reward or punishment is coming or to generate desired behaviors in exchange for a reward; often, once expected, a behavior may even occur before it can be signaled for (Mitchell et al., 2009; Ridgway et al., 2014; Wasserman & Miller, 1997). This type of learning may lead to flexible planning, where the individual would ignore one stimulus when a better reward would be given at a later time. This was seen in chimpanzees that would switch from accepting lesser desired food to waiting for different, more favorable option (Beran et al., 2016; Lind, 2018). Although originally assumed to only occur in higher vertebrates like primates and humans, associative learning has been observed bats that associate sensory stimuli with a food source, an endoparasitic wasp that was able to associate food with the color orange versus yellow, and in rats which were able to associate a specific food flavor to a specific location in a maze (Lucchetta et al., 2008; Morris & Day, 2003; Page et al., 2012). One of the most famous examples were with Pavlov’s dogs, where dogs were trained to start salivating (unconditional response) at the sound of a bell (conditional stimulus) that signaled that a food reward (unconditional stimulus) was imminent, instead of simply for the food item itself (Mclead, 2018; Wang et al., 2016).

Although associative learning is less studied in cephalopods, the few studies to date indicate that they possess this type of cognition. For example, *Eupymna scolopes* (Hawaiian bobtail squid) quickly learned not to strike at a beaker that contained shrimp, to wait for the accessible food option, and even began to ignore the inaccessible food all together within three days (Zepeda, et al., 2017). This type of foraging-based learning could either be governed by cognitive abilities such as future planning or through standard associative mechanisms such as incentive learning (Schnell et al., 2021).

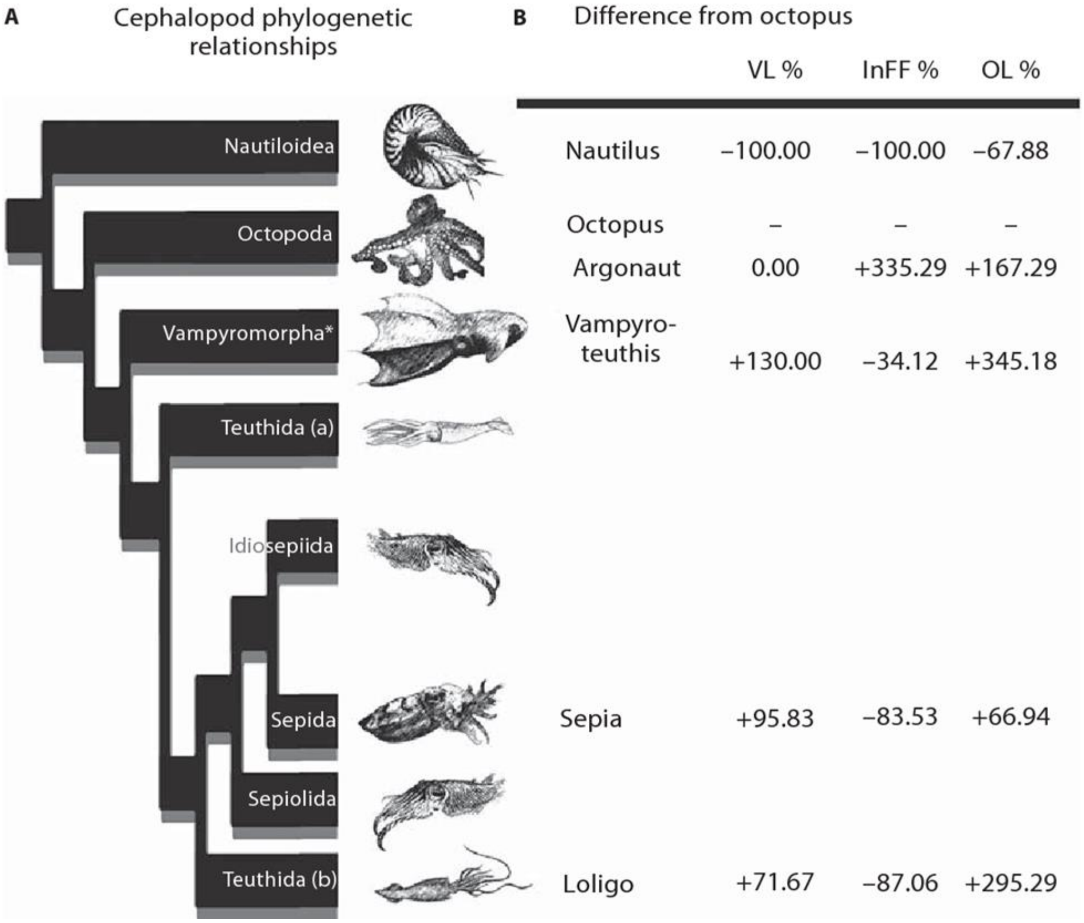
Existing Evidence of Learning and/or Memory in Cephalopods

Nautilus

As the last shelled cephalopod, nautilus is an important group to study spatial learning ability to understand the evolution of cognition in cephalopods with and without shells. These cephalopods lack the coleoid-like areas in the brain for learning and memory seen in other cephalopods, as mentioned previously, and so have limited memory storage capacity (Catalini, 2008; Crook et al., 2009; Figure 3). Two studies examined nautilus' ability to navigate through a simple maze to a beacon at the exit. Both studies found that although it was able to do so with increasing accuracy with time and recalled information for about fourteen days, it was dependent on visual cues. This lack of sensory redundancy indicates that nautilus use a specific cue (i.e. visual) in the wild in order to navigate an environment. Thus, it may not actually be able to navigate the area without the cue, which can be interpreted as a of limitation in cognitive abilities in nautilus (Crook et al., 2009; Crook & Basil 2013).

Octopus

Octopus have been tested with various methodologies to determine if they possessed long-term vs short-term memory. One well known study looked at an octopus's ability to open a jar; although always successful the time to solve this puzzle decreased with practice, indicating learning (Catalina, 2008; Fiorito & Scott, 1992). In some species, such as the *Enteroctopus dofleini* (giant Pacific octopus), individuals can travel during hunting periods as far as seven hours from its den while still recalling its den's location (Boal et al., 2000; Hanlon et al., 2018; Scatà et al., 2016). To avoid over-hunting in the same foraging grounds, multiple octopus species (e.g., *O. vulgaris* and *O. cyanea*) have even been seen to avoid areas where they had recently hunted, showing they can remember where those spots were (Boal et al., 2000; Forsythe & Hanlon, 1997). In another study, *O. vulgaris* and *Octopus briareus* (Caribbean reef octopus) were seen to be able to recognize a 90˚contrast pattern produced by polarization when offered shrimp on a specific target, and learned to still recognize the pattern even as the contrast was changed even slightly in as little at 20˚ (Shashar & Cronin, 1996).



	VL %	InFF %	OL %
Nautilus	−100.00	−100.00	−67.88
Octopus	–	–	–
Argonaut	0.00	+335.29	+167.29
Vampyro-teuthis	+130.00	−34.12	+345.18
Sepia	+95.83	−83.53	+66.94
Loligo	+71.67	−87.06	+295.29

Figure 3:

This shows a cladistic analysis of the extant forms of major cephalopod groups.

(A) Relationship amongst the cephalopod phylogeny. (B) Sizes of the vertical lobe complex (VL), inferior frontal complex (InFF) and Optic Lobe (OL) for different species of the main cephalopod groups. This data compares volume of the brain regions to the total volume of the central brain (not including the optic lobes) (Grasso & Basil, 2009).

Squid

Although few laboratory studies have focused on squid cognition since they are difficult to maintain in a laboratory setting, this group has also shown evidence of heightened cognitive skills. In a maze study, six out of twelve *Sepitoteuthis lessoniana* (bigfin reef squid) showed conditional discrimination and heightened learning abilities over several trials as it increasingly was able to choose the correct exit of a maze based on visual cues that indicated which exit was open versus closed (Spady & Watson, 2020). The time to complete each trial decreased as well (Spady & Watson, 2020). During the prawn-in-the-tube trials of one study, *E. scolopes* was able to learn to inhibit striking behavior at tubes until accessible food was offered within ten minutes and could recall this learned behavior for twelve days (Zepeda, et al., 2017).

Cuttlefish

Cuttlefish have been found to have a larger brain-to-body ratio than octopus, which suggests they may possess more advanced cognitive abilities than other cephalopods. To date, however, most studies of cuttlefish have only focused on one species, *Sepia officinalis* (the common cuttlefish). One study examined if *S. officinalis* could recall vertical or horizontal visual cues in more complex mazes (i.e., whether the walls were at a diagonal or flat across), similar to navigation through their naturally three-dimensional world. It showed that in simple environments, the cuttlefish doesn’t need vertical cues (i.e., walls at a diagonal) to navigate, but as complexity increased, they must use more of their senses to maneuver. (Karson et al., 2003; Scatà et al., 2016). Juvenile *Sepia bandenis* (dwarf cuttlefish) showed their capacity for short-term memory even as young as eight days old when they showed that they were able to distinguish between instances where striking was beneficial (i.e., prey captured) during training sessions and when it would not (Bowers et al., 2020). At the adult stage, common cuttlefish quickly learned to associate a blue flashing light with feeder fish (i.e., live goldfish) (Cole & Adamo, 2005). Self-control (i.e., delayed gratification) is a sign of cognition in animals such as primates. This trait has also been seen in other common cuttlefish which delayed attacking prey for 50-130 seconds, showing that it could associate which pattern provided the best reward for its patience (Schnell et al., 2021).

Background Information of Chosen Research Species: *Octopus bimaculoides*

Current and past research have focused primarily on studying the learning capabilities of three species: *O. vulgaris* (Borrelli & Fiorito, 2008), *S. officinalis* (Scatà et al., 2016), *and A. marginatus* (Finn et al., 2009). The species used for this study was the California two-spotted octopus (*O. bimaculoides*), which is one of two octopuses commonly used in research due to their successful breeding and husbandry in a laboratory setting. Despite a broad research program in this species, there are few studies that use them to research cognition and memory. To strengthen our knowledge of spatial learning and memory among cephalopods, more examples of different species at various age ranges are necessary. In *O. bimaculoides*, we provide a more diverse look at octopus cognition focusing on the of juvenile life stages specifically (Borrelli & Fiorito, 2008). This species typical mantle length ranges from 17.5-58cm and is light brown or mottled in color with two distinct ocelli (false eye-spot, with one under each eye (Hamilton et al., 2014; King, 2019). Their geographic range spans from northern California, to Baja California, and the west coast of Mexico, where they are found in intertidal or littoral areas with rocky reefs, canyon ledges, caverns, and/or man-made pipes. They are mainly nocturnal, spending diurnal hours sheltered in burrows when not hunting for mollusks and crustaceans (Hamilton et al., 2014; Hofmeister et al., 2016).

This study's overall aim was to examine the spatial learning and memory abilities of juvenile O. bimaculoides to better understand the cognitive abilities for early life stage ocotpus.

Hypotheses:

(1) *O. bimaculoides* will be capable of learning and memory as juveniles.
(2) *O. bimaculoides* will demonstrate increased learning following repeated training sessions.
(3) Following training session, *O. bimaculoides'* pathways to the food source will become more direct (i.e., shorter), and the time to find food will decrease – indicative of memory and spatial learning.
(4) Time to learn will increase directly with habitat complexity.

(5) There will be early development of the vertical lobe of the brain, indicating the capability for memory and learning.

Individual Husbandry:

Permits:

The protocol was reviewed and approved by the NSU Institutional Animal Care and Use Committee (IACUC), as of July 6th, 2021 with protocol number 2021.07.TDS1. The octopus were reared by primary researcher.

Samples:

Twelve *O. bimaculoides* hatchlings (~6-7mm in mantle length and 7 days post-hatch) were brought in from the Marine Biological Laboratory in Woods Hole, Massachusetts. These hatchlings were delivered fully developed and able to survive on their own (Hanlon et al. 1985). They were raised to ~25mm (~4 months) before experiments began as they begin to forage at this point in their development (Forsythe & Hanlon, 1988). Of the twelve individuals, six octopus died from unknown causes before the start of experiment.

Diet:

The natural diet for *O. bimaculoides* in the wild is mollusks and crustaceans. To simulate this natural diet, the octopus were fed a combination of mysid shrimp and amphipods/copepods. Upon arrival to two-three months post-arrival, we decapitated the ghost shrimp and cut it into tiny pieces to be handed directly to the octopus. At that point, octopuses began hiding more, so we began feeding juveniles live food (King 2019). Food items were immobilized by placing into perforated 15ml centrifuge tubes that allowed octopus to reach inside. All food items were kept in separate aquaria with their own attachable filters as needed. Brine shrimp and ghost shrimp were fed every other day with TDO ChromaBoost and shrimp pellets respectively. Octopus individuals were fed twice daily until they were more than three months of age (King, 2003). Once they could eat on their own, they were fed once in the afternoon every other day since previous observations noticed eating occurring every other day and a decrease in food consumption to once a day.

Enrichment:

Hatchlings (< 2 months) were given enrichment starting four weeks after octopus were purchased. They were given a variety of textured and/or colored objects for one week at a time or novel food items for one day (Table 1). Enrichment is an important aspect of raising species with advanced cognition since it stimulates and encourages natural behaviors. Octopus are curious creatures that enjoy different textures and shapes (Cooke, et al., 2019). Introducing different food types encouraged problem solving and manipulation common in their natural habitats (Anderson et al., 2009). Even the change over from dead, hand-fed prey to hunting for live items served as enrichment since this required the use of problem solving and dexterity. During the first few months of development, octopus juveniles (> 2 months) preferred areas of concealment, so enrichment options used took this into account (Cooke et al., 201

Table 1: Enrichment Schedule

After four weeks of being in the lab, the individuals were introduced to various enrichment types, except during periods of experiments.

Week	Enrichment Type
Week 1	2 Artificial Goldfish (varying colors)
Week 2	PVC Pipe
Week 3	Artificial Jellyfish
Week 4 – Week 5	Tall/Bushy Artificial Plant
Week 6	2-inch PVC Pipe
Week 7- Week 8	No Enrichment
Week 9	All housing material moved to side under water flow
Week 10	Brine Shrimp introduced
Week 11	Food placed into 15 mL centrifuge tubes
Week 12 - Week 15	No Enrichment: In Experiment
Week 16- Week17	Tall/Bushy Artificial Plant
Week 18-28	No Enrichment: In Experiment

Octopus' Housing and Care:

Individuals were kept in the invertebrate laboratory at Nova Southeastern University, Halmos Oceanographic Campus in Dania Beach, FL. All animals were housed in separate aquaria, with dimensions: 60.96cm L x 30.48cm D x 60.96cm W for two, 60.96cm L x 31.75cm D x 60.96cm W for one, and 46.99cm L x 29.21cm D x 62.23cm W for six. All aquaria contained thirty pounds of crushed coral, 1-2 approximately 10cm in length artificial plants, and a 10 cm long terra clay pot for concealment (Walker et al., 1970). Plants and pots were spread throughout housing aquarium until two weeks before experiments began. Mesh lids were used to cover aquaria and the water level was kept 5-8 centimeters below the top to prevent escape. Each tank was maintained at 23-25 ℃ with heaters to encourage faster growth (Forsythe & Hanlon, 1988). Since octopus are sensitive to certain compounds such as ammonia (NH_3), nitrite (NO_2), and copper (Cu), water chemistry was checked regularly. Salinity was checked daily to ensure concentrations remained at a specific gravity of 1.026, pH of 8-8.4, NO_3, NO_2, and copper of 0, and $NH_3 < 30$. The use of a hydrometer and marine chemistry test kits ensured these water quality parameters, and the protein skimmer in the filtration sump assisted in minimizing high concentrations of organic waste, biological toxins, and phosphate. To maintain these water quality parameters, 20% water changes were completed as needed. The water in the system was treated routinely with activated carbon to prevent unwanted olfactory cues between individuals, and nitrifying bacteria (Turbo Start 900 or API Aqua Essential) to balance nitrite and ammonia levels (King, 2019). Two identical systems containing 6 tanks each were maintained. Of the six remaining octopus after the initial mortality event, five shared one system and one octopus was maintained in the second system. Figure 4 illustrates the holding system holding five octopus.

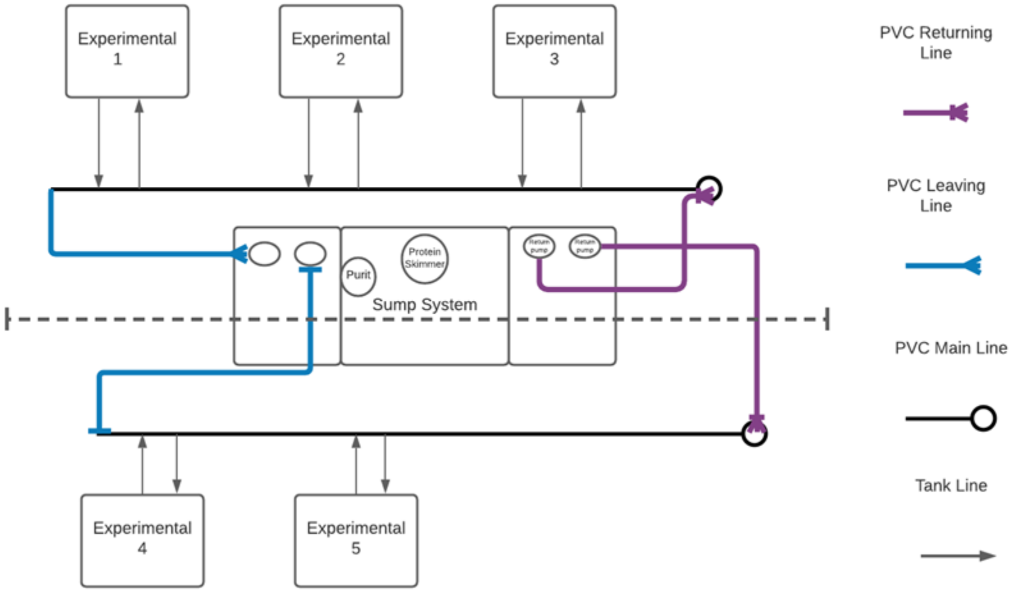


Figure 4: Connection Design for Holding Aquaria Filtration System

Five of the aquaria were connected by a series of PVC pipes to one sump system (114-227 liters), with a protein skimmer and overflow compartment.

Experiment I: Spatial Learning

Methods:

During the experiment octopus were given one brine shrimp inside a 15ml centrifuge tube for each trial, and one free swimming ghost shrimp on Saturdays. During weeks without experiments, they were given one free swimming ghost shrimp every other day until Sunday.

Experimental Design:

During the experiment, the housing equipment (pot and plants) were moved to the side of each aquarium closest to the flowing water. Each aquarium had a video camera (ZOSI 1080P 8CH Security Camera System) overhead recording all activity in two-hour increments to a hard drive in the laboratory. The pump from the sump to the aquaria was turned off during experiment to decrease reflection on water surface during recording.

Three-dimensional, identical prints of Texas Holey Rocks were printed with white PLA (polylactic acid) filament in a 3-D printer. They were then glued to a platform and/or each other to allow easy removal and addition to aquaria. Each platform has an increasing number of rocks present, beginning with two, three, and then four, to create increasing structural complexity (Figure 5). Rock 1 has the dimensions: 16.51cm L x 63.5cm W x 15.24cm H. Rock 2 has the dimensions: 15.24cm L x 20.32cm W x 12.7cm H. 3-D printed rocks were anchored with bricks. The habitat platform was inserted starting ten minutes before experimentation session began to allow for all rock formations to be placed before experiments began.

During experiments, black cardstock paper was attached to the front of the aquaria with Velcro to allow for easy removal during set up. Remaining sides were covered with taped black cardstock paper to prevent distraction from other octopus, outside light sources, and movement. Lamps with 40 watts of red-blue light were placed over aquaria for twelve hours a day, which included the four hours of experimentation. Since these octopus are already known for their exploratory movements, this encouraged their natural habits (Boal et al., 2000; Wells et al., 1964).

Experimental Aquaria Rotation:

Octopus were divided into groups with three individuals per group. Due to pre-experiment mortality, only six individuals of the original twelve individuals were left to perform the experiment. The groups were as follows.

- Group 1: Individual 1, 4, 5
- Group 2: Individual 8, 10, 12

Each group were introduced to the experimental habitats in randomized order (Table 2). The subjects were not fed 24 hours before each experiment day to encourage foraging. (Wells et al, 1968; Boal et al., 2000). Two trials (9-11am and 4:30-6:30pm) were done on experiment days (Monday, Wednesday, and Friday).

If no mortality was experienced, the individuals went through an eight-week training period, for a total of 16 training trials per individual, where they were rotated through all three levels of experimental habitats for a total of six trials a week with two trials for each of the three habitats (Figure 5) (Alves et al., 2008; Boal et al., 2000; Bowers et al., 2020; Fiorito & Scotto, 1992).

For Example: Individual 1 (Group 1) for the trials had Habitat 1 (low complexity) on Day 1, Habitat 2 (medium complexity) on Day 3, and Habitat 3 (high complexity) on Day 5.

They then spent one week off before being introduced to the aquaria again. They will take an additional four weeks off and then be tested again for one trial (Table 2 & 3). The training period is used to examine learning, while the extended period looks at memory.

Statistical models for Experiment I

Linear Mixed Effects Model: This experiment was attempting to determine the effect of two explanatory variables: 1) "time" (i.e., time away from habitat; categorical with 2 levels: 1wk and 1mth) and 2) "complexity" (i.e., habitat complexity; categorical with three levels; low, medium, and high) on "pathway length" (i.e., distance traveled prior to finding food, which is indicative of activity) and "time to find food" (i.e., total time taken for octopus to find the food item once added to the experimental arena). Note that learning would lead to a shorter pathway length to food source and time to find food as trials progress. There were also three individuals participating at once in each "round" of trials. To account for the repeated measure within individuals and the potential for greater variation within rounds than among round, each trial included a random effect in which individual was nested within trial. Models are outlined below.

1. PathwayLength (activity) ~ Time*Complexity + (1|Trial/Individual)
2. TimeToFindFood (learning) ~ Time*Complexity + (1|Trial/Individual)

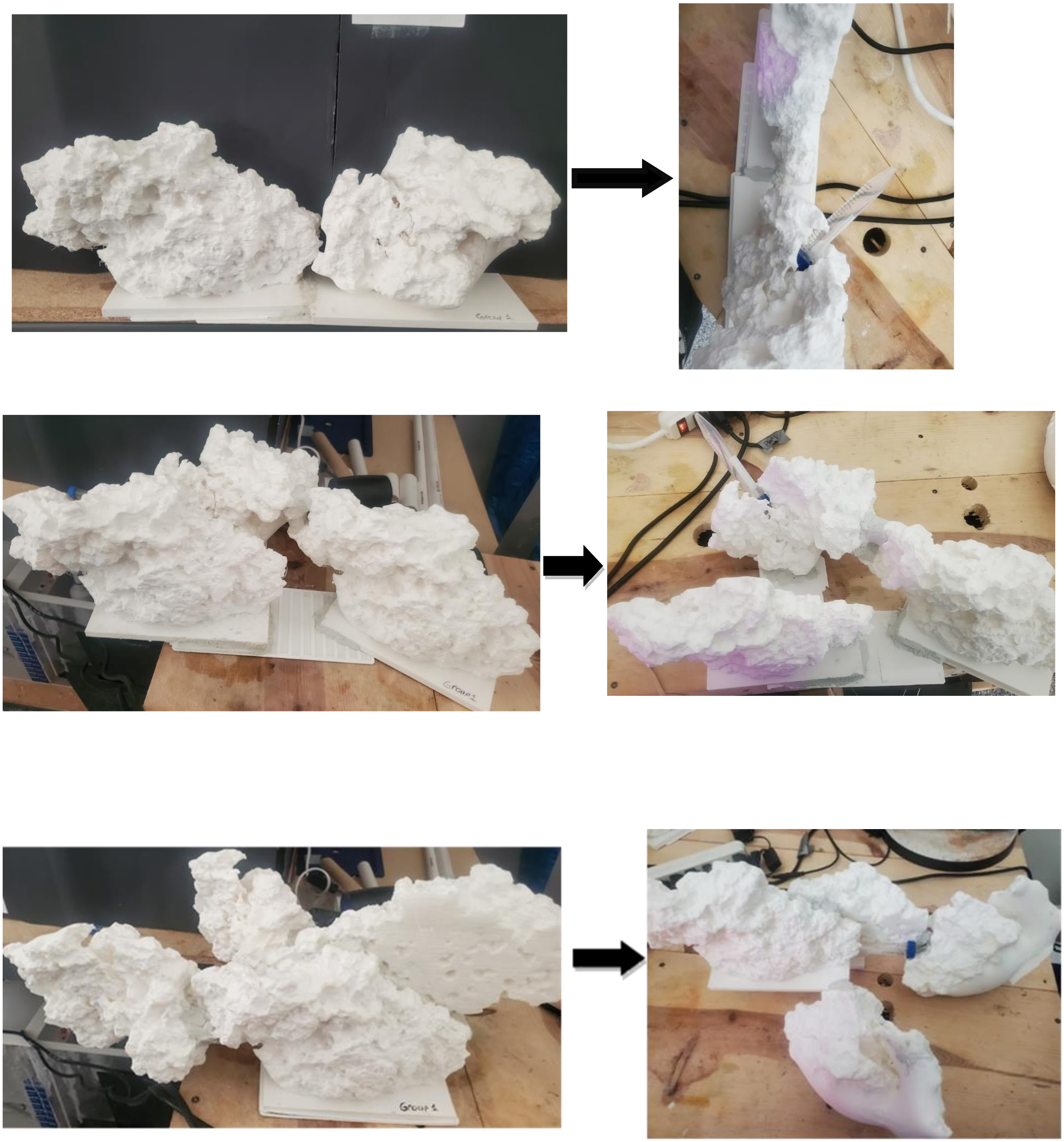


Figure 5: **3D Printed Rock Habitat Designs**

Three different 3D printed rocks were replicated and organized into three different habitats. Each habitat was of increasing complexity: low complexity (top), medium (middle), and high (bottom).

Table 2: Weekly Schedule for Introduction to Different Complexity Tanks

Each group member was tested twice a day for two hours, once in the morning and once in the afternoon. Each individual started at a different level of complexity and rotated through each level over three days as indicated below. Both groups were tested each day.

Group Number	Individual	Complexity Schedule (AM & PM)	Schedule for Experiment
1	Individual 1	low complexity (experimental tank 1) to high complexity (experimental tank 3)	Day 1, 3,5
1	Individual 4	high complexity (experimental tank 3) to medium complexity (experimental tank 2)	Day 1, 3, 5
1	Individual 5	Medium complexity (experimental tank 2) to low complexity (experimental tank 1)	Day 1, 3, 5
2	Individual 8	low complexity (experimental tank 1) to high complexity (experimental tank 3)	Day 1, 3, 5
2	Individual 10	high complexity (experimental tank 3) to medium complexity (experimental tank 2)	Day 1, 3, 5
2	Individual 12	high complexity (experimental tank 3) to medium complexity (experimental tank 2)	Day 1, 3, 5

Anticipated Results:

Despite early conclusion of experiment, it can be expected that there would have been a steady decrease in the time spent exploring the rocks by all octopus with a more direct path to the food item being seen (Figure 6). This expected trend would have led to a decrease in time from ~ 3000 seconds (50 min) to around ~1200 seconds (20 min) to complete the habitat as trials progressed. This could be seen in as early as trial six for about 75% (9 of the 12) individuals with a plateau around trial ten at ~300 seconds (5 min) (Figure 7A; Boal et al., 2000; Scata et al., 2016). When the short-term memory test was completed at trial seventeen, it was expected that 75% of the octopus juveniles would take slightly longer than trial sixteen (~420 seconds or 10 min) but would still complete the habitats in less time than trial one (Figure 7A; Borrelli &

Fiorito, 2008).. The same would be true for trial eighteen testing long-term memory (Figure 7A; Borrelli & Fiorito, 2008).

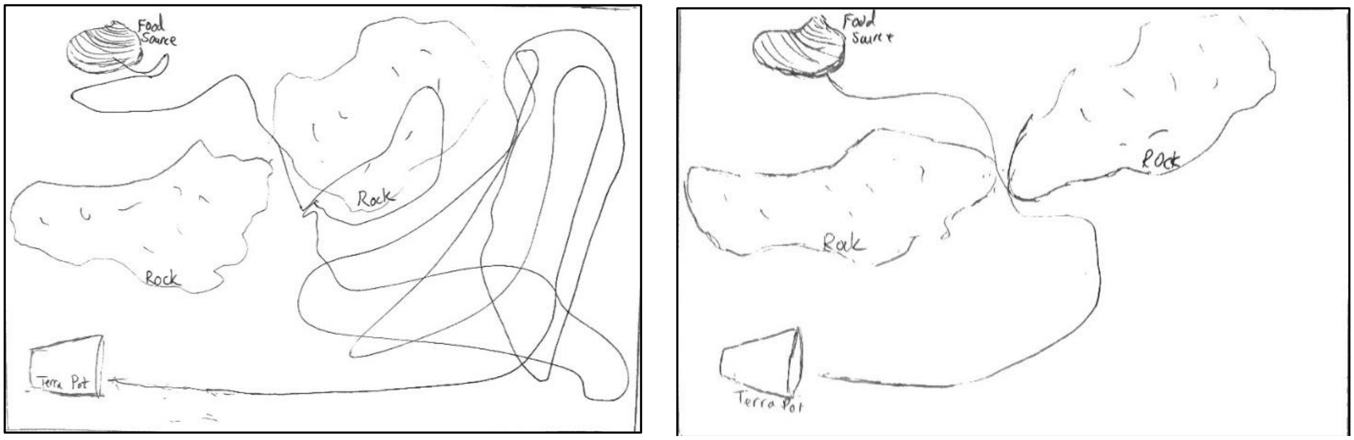


Figure 6: Example of Theoretical Pathway Changes

It was expected that pathways would start as longer and more indirect as the octopus explored the novel habitat (left), but as training continued, the pathway would become shorter, more direct towards the food source (right). However, in the experiment, individuals rarely explored this novel habitat, hiding while the rocks were in their aquaria. Thus, this experiment was discontinued.

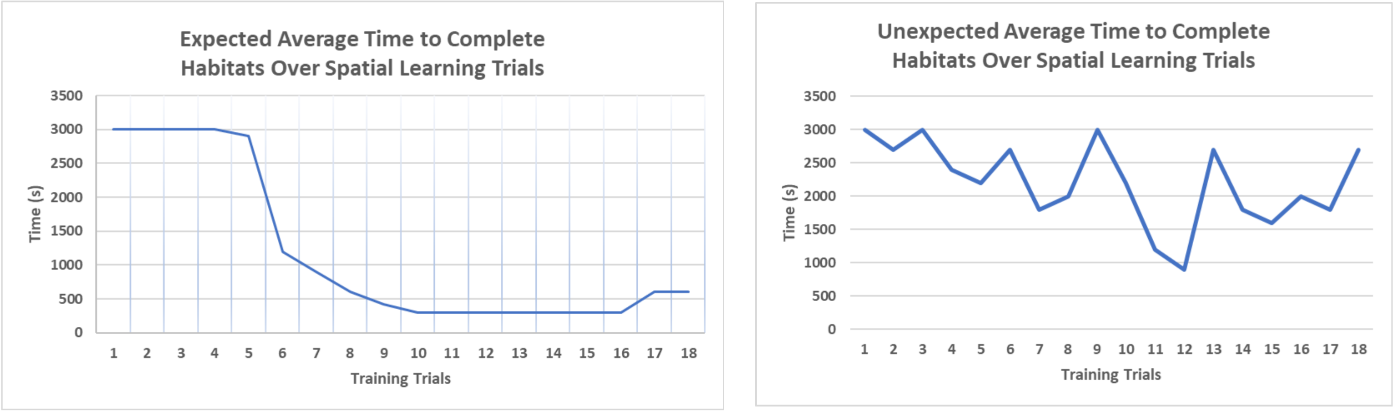


Figure 7: Theoretical Comparison of Expected vs. Unexpected Results of Spatial Learning and Memory

A. (left) shows the potential decrease in time to complete each trial as the octopus continued to be introduced to each habitat complexity over 8 weeks. B. (right) shows potential result for octopus that do not showcase spatial learning. Amount of time to complete habitat navigation is random. Trial 17 represents the trial for short term memory and Trial 18 represents the test for long-term memory.

Results:

During the four months before the training trials began, seven of the twelve octopus were found deceased – upside down with the arms curled around their bodies – for unknown reasons. All individuals had been seen eating the day before they died. No observations of skin abnormalities such as lesions/ulcers were documented. Water quality was deemed within the optimal range.

Trials were recorded for 2 octopuses (octopus 4 and 5) over the first three weeks with a total of ~60 minutes of video. Octopus 4 explored the aquarium for ~25 minutes following the addition of the rock formations. However, this individual was only observed crawling over the rocks once before continuing exploration elsewhere. Octopus 5 was seen for ~2 minutes crawling along the rock formations and conical tube containing the brine shrimp but later swam away. Both of these octopus were only observed during the evening trials, and none were observed exploring the rocks during the morning trials. No other octopus was observed during any scheduled recording times. Due to this issue, the experiment was discontinued after the first three weeks of training, and a new experiment was designed.

Experiment 2: Food Preference

Methods:

The first part of this experiment determined the food preference of *O. bimaculoides* for future trials. For one week a gram of fresh shrimp, ghost shrimp (the food item used for the juveniles up to this point), crab, and scallops were offered, each in a separate glass jar marked one through four (Ambrose,1984; Anderson et al., 2009; García-Fernández et al., 2019; Hanlon & Forsythe, 1985; Solorzano et al., 2009). The jars were placed on their sides, openings facing in the same direction and were ~13cm from each other (5x the mantle length of the octopus). They formed a diamond in the middle of the aquaria (Figure 8). Each jar sat in the middle of a ~7.62 cm diameter ring of black aquarium rocks. Each ring represented a zone (1-4) and was numbered to match the jar with the respective food item. Anything outside the rings was considered the neutral zone. The order of the jars in the diamond positioned zones was randomized for each octopus (Maselli et al., 2020).

For example: Octopus 1 had the following order from the top of the diamond in the clockwise direction:4, 3, 2, and 1. Octopus 2 had the following order from the top of the diamond in the clockwise direction: 1, 2, 3, and 4.

Figure 8: Configuration of Zones and Rocks for Food Preference Experiment

Ring of black aquarium rocks were considered zones with space outside them being the neutral zone. One glass jar labelled between 1 and 4 was placed in each.

Figure 9: Camera Design for Food Preference and Associative Learning Experiments

Cameras were attached with rubber bands to interlocked PVC tubes and placed directly over zones.

The ZOSI cameras were placed over each aquarium with the use of a PVC stand to have all four jars in the field of view when recording the experiment (Figure 9). The food samples remained in the aquarium for 24-hour sessions every day. The recordings were switched from every other day after two sessions to every day to ensure all activity was captured.

During the sessions, behavioral responses were observed: hiding (i.e. not visible within the aquaria), eating (i.e. actively interacting with food item), exploring (i.e. actively investigating the jars within zones), resting (i.e. inactive but visible), and swimming (i.e. motion without touching the floor).

The time spent doing each activity for each zone was recorded, as well as how many times a switch (i.e. change in activity and/or zone) took place during the session. The amount of time spent with each food item (i.e. time in the food item jar) out of the total time spent with all food items (i.e. total time spent in all jars) was also measured to assist in determining preferred food.

For the second part, the comparison of frozen versus fresh options would have been done for the preferred food option, with three jars containing the least favored food and one random jar containing the favored food choice.

Results:

It was anticipated that the octopus would prefer the blue crab over the other options due to that preference naturally (Anderson et al., 2009; King 2019).

This experiment was stopped after one week as the video footage indicated that only octopus 8 move among the jars and around the aquarium. Note that in this one individual, there was no obvious pattern. On average, during a 24-hour filming, octopus 8 spent ~1 minute exploring (inside a zone), ~40 minutes swimming, ~23 hours and 8 minutes hiding, 0 time swimming, ad 0 time resting. The individual was never seen entering the jars themselves. The remaining aquaria were checked for presence of juvenile octopuses. The remaining octopuses were not found, and the experimental trials were discontinued. Shortly after discontinuation, octopus 8 was not found and assumed deceased.

Experiment 3: Associative Learning (Suggested for Future Study)

Methods:

The following methods were not completed as planned due to the early mortality of the remaining octopus individuals. They are included for future reference. Below are the methods described as if mortality of octopus did not occur.

The ZOSI cameras remained recording for 24 hours every other day. One jar was marked with a black x pattern, while the other three jars were marked with the horizontal black lines. The one with the x had the preferred food option (i.e. the food item with the highest percentage of visits in last experiment) and the one with the horizontal lines had the least preferred option (i.e. the food item with the lowest percentage of visits in last experiment). The jars were randomly positioned in the aquaria in each session to ensure learning of pattern and not location. The zones were still used as before, and the same behaviors and variables were observed. The experiment started with four trials (i.e., session every other day as before) and increased as needed for each individual to reach a higher percentage (i.e., time with food item) for the preferred food versus the least preferred food.

The jars were then switched so that the preferred food source was now in the jar with horizontal lines. Time was then recorded for how long it took for the individual to begin associating the new jar with the preferred food choice. Experiments once again began with four trials and increased as needed. Once a higher percentage (i.e., time with food item) was observed, experiment was stopped.

For the last part of the experiment, a 50ml centrifuge tube with ~1 inch holes along the sides was partially buried into the substrate and used to hold the preferred food. The tube was marked with the horizontal lines seen on the jar. Three jars were marked with the x pattern but contained the least preferred food source. The position of each jar and the centrifuge tube was randomized each session and still remained in their assigned zones. The same variables and behaviors were observed as previously stated. This experiment would train the octopus to enter a controlled setting where there is live food, allowing for use in future spatial learning test.

Results

This part of the experiment was not able to be completed, so no results are available for discussion.

Experiment 4: Brain Sectioning:

Methods

The whole octopus brain (n=4) was imaged using histology techniques with the following ages: 3 weeks, 8 weeks, 11 weeks, and 13 weeks. First, the tissues were decalcified in Formical-4 for 24 hours to remove the beak and radula. The octopus were then rinsed with distilled water and stored in 70% ethanol (ETOH).

For paraffin embedding, samples were placed in microcentrifuge tubes with 2mL of the following solution(each solution was removed with a pipette between each step) for these specific periods of time: 80% ETOH for 10 minutes, 90% ETOH for 15 minutes twice, 100% ETOH for 20 minutes thrice, and finally xylene for 25 minutes four times. Then, 2 mL of paraffin was added, and the tissues were incubated overnight at 56°C. The following day the xylene/paraffin mixture was changed, and incubation was continued at 56°C. The changing of the xylene/paraffin mixture was repeated twice, once per hour. The entire octopus was then moved to a mold of fresh, melted paraffin. Each octopus was allowed to cool overnight. Paraffin embedded samples were cut into longitudinal sections in 10µm layers.

These sections were then stained in a 12-compartment staining rack. The compartment was filled to 150 mL to the top of the unfrosted portion of the slides. In compartment 1 and 2 the slides were deparaffinized in xylene for 10 minutes. In compartment 3, the slide was rehydrated in 100% ETOH for 10 minutes. In compartment 5 the slide was rehydrated in 95% ETOH for 2 minutes (150 mL = 143 mL 95% ETOH + 7mL DI H_2O). In compartment 6, the slides were rehydrated in 70% ETOH for 2 minutes (150 mL = 105 mL 95% ETOH + 45mL DI H_2O). The slides were washed dipped 10 times in distilled water. In compartment 7, the slides were stained in Harris' hematoxylin for 2 minutes. The slides were washed in running tap water for 5 minutes. In compartment 8, differentiation in 1% of acid alcohol was done for 30 seconds. Slides were washed again in running tap water for 3 minutes. In compartment 9, the samples were blued in saturated lithium carbonate for 30 seconds and the washed in running water for 5 minutes. Compartment 5 was used to rinse the slides 10x in 95% ETOH. Compartment 10 was used to counterstain the slides in Eosin Y for 60 seconds. Compartment 5 was used to dehydrate the slides in 95% ETOH for 5 minutes. Compartments 3 was then used to dehydrate the slides in 100% ETOH for 10 minutes. Compartments 11 and 12 were used to clear slides with xylene for

5 minutes. The coverslips were mounted with Permount before the xylene dried and the slides were allowed to dry for 24 hours. The slides were then visualized with a compound microscope under 50x and 100x and the brain sizes of octopus at each age (3-13 weeks) was measured with the use of the LAS X program. Area of the sample visible on the slide was measured as well as the area of the brain. The length and width of the brain was measured as well.

Results

In four octopuses from age 3 weeks to 13 weeks (n=4, 1 octopus per age category), the percentage of brain size relative to body size of the octopus hatchlings grew by eight percent with an average brain area being 1.98 mm^2 and average sample area (i.e., octopus visible on microscope) 3.67 mm^2 (Table 4). The brain took up ~52% of the sample area in the 3-week-old sample, 55% in the 8-week-old sample, 48% in the 11-week-old sample, and 60% in the 13-week-old sample (Table 4). On average the brain occupied ~4% of the sample. The average length and width of the brains were 2.13 mm and 1.23 mm respectively but varied from age to age.

The 11-week old octopus didn’t follow a trend, but the area of the sample was also smaller due to a more compressed orientation of the octopus during Paraffin embedding. There was also noticeably more development in the different lobes of the brain as the octopus aged, with 13-week-old individual showing distinct optic, pedal, and vertical lobes while both 8-week and 11-week-old individuals’ brain lobes are still forming with only the optic lobe somewhat distinguishable (Figure 10). The size of the optic lobes did not vary much with age, possibly due the tissue forming the lobes were still present, just not fully developed into distinct oval-shaped areas (Table 4; Figure 10)

Table 4: Brain Histology Measurements of *Octopus bimaculoides* from Ages 3 Weeks to 13 Weeks

The brains of four octopus were stained and measured under 50x. With one potential outlier at age 11 weeks, area of the brain relative to area of sample increased with age. Three weeks brain took up 52% of sample area, 8 weeks took up 55%, 11 weeks took up 48%, and 13 weeks took up 60%. Length and width of brain, however, varied with age. The area of the two optic lobes didn't vary much from age to age.

Octopus Age	Area of sample (mm^2)	Area of brain (mm^2)	Length of brain (mm)	Width of brain (mm)	Area of optic lobe (left) (mm^2)	Area of optic lobe (right) (mm^2)
3 weeks	3.88	2.01	2.26	1.16	0.300	0.778
8 weeks	3.78	2.09	1.96	1.55	0.280	0.096
11 weeks	3.24	1.57	2.01	0.924	0.356	0.412
13 weeks	3.79	2.26	2.28	1.43	0.885	0.787

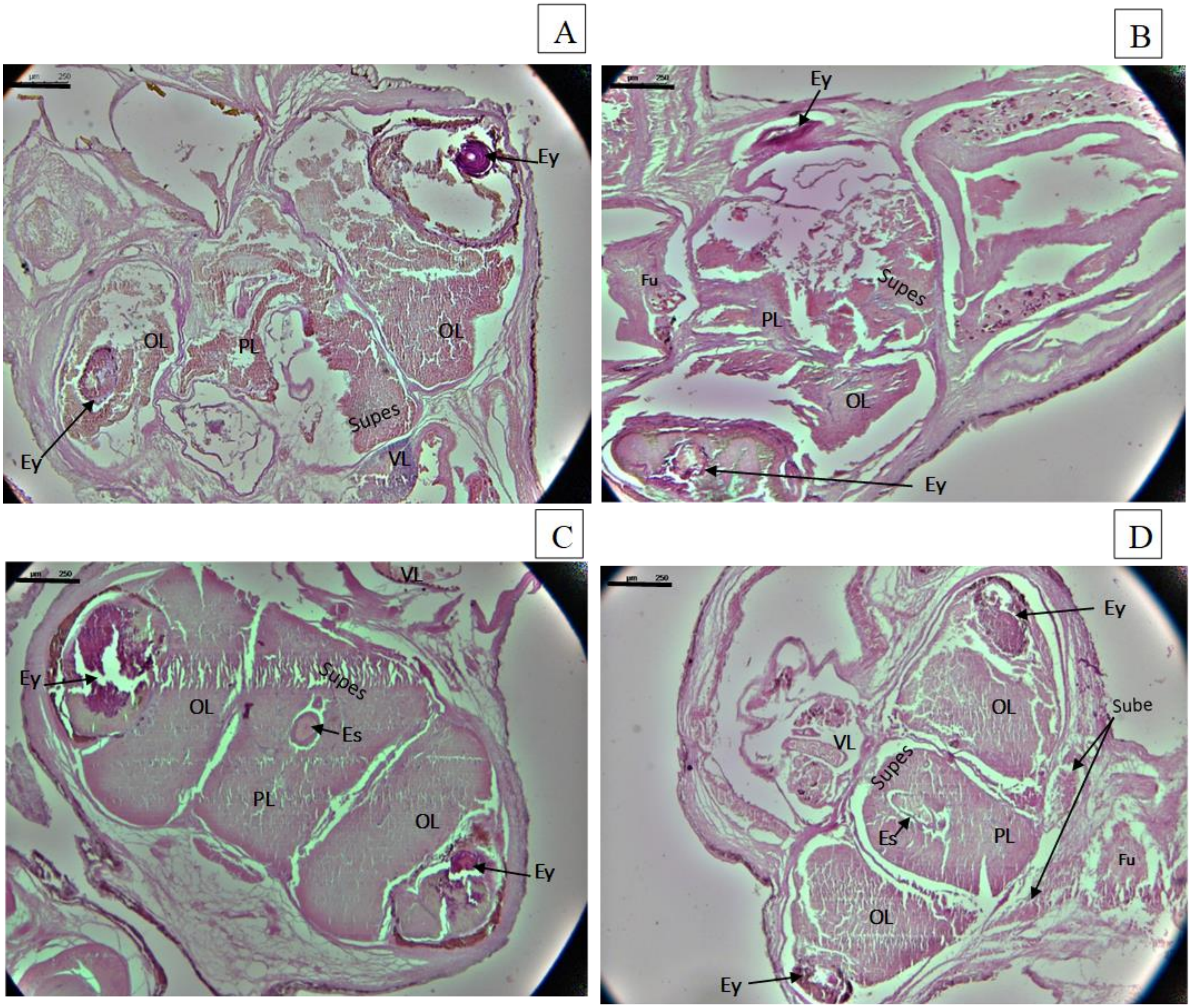


Figure 10: Brain Histology of *Octopus bimaculoides* from Ages 3 Weeks to 13 Weeks

The brains of four octopus were stained and measured at 50x. There ages from left to right, A. 3 weeks, B. 8 weeks, C. 13 weeks, and D. 11 weeks. Octopus specimens at all ages were too large to fit entirely in view and so overall size was estimated for mantle length. Images are labelled as follows: Es (esophagus), Ey (eye), Fu (funnel), PL (pedal lobe), OL (optic lobe), VL (vertical lobe), Sube (subesophageal mass), Supes (supesophageal mass) (Deryckere et al., 2021; Shigeno et al., 2015).

Discussion

Animal Care and Behavioral Observations:

Although two octopus had their designated area in the aquaria (separated by acrylic board) they managed to “squeeze by” the border and were found resting alongside their neighbor. This was observed ~8 times for seven hatchlings/juveniles. Since they were not always visible at the same time, it was not always clear which octopus was being seen – especially with the slipping from one side of the aquaria to the other. This is one of the few species where this occurs without cannibalism and aggression, especially with small size and adequate food (Gutnick & Kuba, 2018; Hanlon & Forsythe, 1985).

Six octopuses were moved to their own separate aquaria on a separate system. When this occurred, the system was stable, but then ammonia levels spiked slightly as well as the nitrite levels. Although a water change was done and the chemical levels treated, the ammonia levels decreased back to zero while the nitrite increased until it was at 5ppm. Nitrite remained high for about two months with values reaching 5ppm regularly. The levels finally decreased to zero right before the start of the spatial learning experiments with continued use of Turbo Start 900 and API Aqua Essential. It is hypothesized that the movement of the octopuses could have stressed the system (personal communication with Dr. Lisa Abbo, veterinarian at the Marine Biological Laboratory). Whether chemical fluctuations caused the death of five of the octopuses is unclear, but it has been seen that levels up to 500 mg/L of nitrite could be withstood without changes to feeding habits (Hanlon & Forsythe, 1985). Temperature changes of about one degree occasionally occurred but was still within desired range (Forsythe & Hanlon, 1988; personal communication with staff at the Marine Biological Laboratory). All octopus were observed behaving and eating normally the day before their death. The date of death of the other octopus that shared the same system is unknown since it survived the chemical changes then went into hiding.

Experimental Design

A layer of 7-10 cm of crushed coral was used in the aquaria to mimic the texture of the habitats that *O. bimaculoides* would naturally experience. Although an important aspect of recreating habitats with the 3-D printed rock formation, the use of coral allowed the octopus to hide and inhibited routine health checks and observations of feeding habits. Based on this experience, I recommend future experiments either remove the substrate, or use a separate experimental arena with crushed coral. The clay terra cotta pot would provide the octopus a place to take shelter but would allow the researcher better access to monitor the octopus' health.

Another possible issue was that octopus juveniles remained diurnal the first couple months, then switched to nocturnal during the last couple of months before the start of experiments. It is not uncommon for their activity habits to have an ontogenetic shift (Hofmeister & Voss, 2017; Meisel et al., 2006). This change in behavior caused a decrease in visual accounts of octopus behavior during the day and when experiments began. This was addressed by attempting to change day-night schedule with a light source (discussed below) and then by leaving experiment running for 24 hours. The food item used, ghost shrimp, also was not their preferred food item - despite its use for four months - and so it was not a strong enough incentive for the octopus to reappear during the day. While it can be used to raise *O. bimaculoides*, giving a more variable food source as they grew may have been beneficial both for health, as well as motivation and visualization (García-Fernández et al., 2019; Hanlon & Forsythe, 1985; Solorzano et al., 2009).

The light source could have also encouraged the octopus to stay hidden during the day. The red-blue light is more indicative of daytime than night causing confusion (Figueroa et al., 1995). Since all available lamps couldn't be set to the ideal red light only, this combination was used for uniformity. A switching of light sources so that red light during the day and daylight during the night would have assisted in switching them to normal hours, but this would require removing any outside light sources in the lab. The lights were switched to fully red spectrum for the food preference trials, but did not reveal a difference in visualization times, most likely due to the previous comment about light sources in the lab and the likely mortality of the octopus before the start of the experiment. It should also be mentioned that light sources have not been found to effect feeding frequency (Garrido, et al., 2017). Since cameras did have night vision, observations were attempted without lights before new experiments, but it was found that distinction of octopus from aquaria items and substrates were impossible from a distance.

Although, not a regular occurrence, there were three times during the care of the octopuses where the power in the lab went out temporarily over the weekend. The length of time of these occurrences are unknown since the outages were reported afterwards but could have extended anywhere form 1-12 hours. This issue could have caused fluctuations in the temperature and water quality since the lab space itself tended to run cold and loss of power would have shut off the outflow pump. Another species that tends to live in the Pacific Ocean, *Octopus ocellatus* (the webfoot octopus), was found to decrease feeding habits when the temperature varies (Segawa & Nomoto, 2002). The filtration was not expected to be a concern since there were a large volume of water in comparison to size of octopus allowing for system to still be stable for oxygen levels and chemical balances during the power outage (DeRusha et al., 1989; Toonen, 2003). It is unclear if pathogens could have served a role in the octopus death. No white spots were observed on the individuals ruling out bodonid parasites on the skin or gills, and since there was no crowding issues and no skin condition were observed, ulcers were ruled out (Forsythe et al., 1991; Hanlon et al., 1984). However, the lack of obvious external symptoms does not rule out pathogenic bacteria such as bacterial septicaemia that can affect octopus hatchlings or rickettsial-like organisms (RLO) that can prevent gaseous exchange by blocking the gills (Farto et al., 2019). In the future, routine tests for microorganisms in the water could ensure that only beneficial microorganisms are allowed to thrive.

The octopus used in the experiment were about 25mm in mantle length. Their small size paired with the large aquarium size and large rock formations may have limited results. At this size, this species does not travel as much through complex environments as seen here. It is also possible that size of rocks prevented full view of octopus as they did explore habitat. Either waiting until they are of a larger size or potentially scaling down aquarium and rocks could account for this in future research. The overhead camera used also could not pick up the size of the juveniles clearly and could have impeded accurate counts of sightings on recordings. A stronger camera or one positioned directly overhead in a much smaller arena may work better. The camera here was positioned at approximately an angle of 45˚.

Brain Histology

From age 3 weeks to 13 weeks the percentage of brain size relative to body size grew by eight percent (Table 4). This is expected since octopus are known to grow throughout their lives with exponential growth occurring from hatchling to 156 days old. The brain also became separated into more distinct lobes as the age increased (Figure 10). Although the full size of the specimen could not be determined due to the orientation of the body when embedded, research has shown that the brain size can be twice the size of the octopus itself as seen in *Octopus vulgaris*. Its brain size, however, is found to be almost twice its body size as it grows, weighing roughly 2 grams when its body is only about 10 kilograms. (Forsythe & Hanlon, 1988; Packard & Albergoni, 1970; Yamazaki, et al. 2002). The visibility of the vertical lobe as early as 3 weeks shows that octopus of this age have the capability for learning and memory (Shomrat et al., 2008).

Connecting cognition to anatomy is a valuable resource for research comparing cognition across cephalopods. Histology allows to visually compare brain size relative to body size and the presence/absence of brain lobes at various age groups and could indicate whether some cephalopods develop larger brains quicker than others. It also serves as a valuable comparison to brain structure seen in vertebrates, such as the presence of the vertical lobe – area of learning and memory – further indicating the presence of advance function (Chung et al., 2022; Hochner et al., 2003; Sanders, 1975; Shomrat et al., 2008; Wells, 1978). Although this may not necessarily indicate a higher capability of learning and memory in different species, it may indicate at what age it develops. One study showed that *S. officinalis* (common cuttlefish) in the late embryonic age introduced to crab while in the egg, preferred this food once they hatched, suggesting that cephalopods may develop cognition earlier than expected (Darmaillacq et al., 2008). The use of histology combined with behavioral and genetic studies would serve as an invaluable resource to determine whether brain size is correlated with greater capacity for learning and memory, and/or whether genetics plays a role as well.

Conclusions

Overall, there is limited knowledge on octopus health and what can cause an individual to shift from lacking any external symptoms of ill health to mortality in a little as one day (Locatello, et al., 2013; O'Brien et al., 2018). Future research focused on diseases and other health issues present in cephalopods and specifically the species used here, would be beneficial for research and husbandry overall. This project served as a reminder to ensure that the experiment design model corresponds to the size and age of the animal being tested. While these experiments were not fully completed, the methodology with results and observations are discussed with suggestions for future direction to further our understanding of spatial learning and associative learning in cephalopods of all age ranges. Since this is a limited area of study, it also encourages cognition research of less examined species.

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<u>Appendix I:</u>

Figures and Tables

Table 3: AM & PM Experimental Aquaria Introduction Schedule

This chart shows what level each individual in each group is introduced to for every day until end of experiment. Each color indicates a specific individual.

		Group	1	1	1	2	2	2
Day	Complexity	Individuals	1	4	5	8	10	12
1	Low		🟦			🟪		
	Medium				🟨			⬛
	High			🟩			🟥	
2	Low							
	Medium							
	High							
3	Low			🟩			🟥	
	Medium		🟦			🟪		
	High				🟨			⬛
4	Low							
	Medium							
	High							
5	Low				🟨			⬛
	Medium			🟩			🟥	
	High		🟦			🟪		
6	Low							
	Medium							
	High							
7	Low							
	Medium							
	High							
8	Low		🟦			🟪		
	Medium				🟨			⬛
	High			🟩			🟥	
9	Low							
	Medium							
	High							
10	Low			🟩			🟥	
	Medium		🟦			🟪		
	High				🟨			⬛
11	Low							
	Medium							
	High							
12	Low				🟨			⬛
	Medium			🟩			🟥	
	High		🟦			🟪		
13	Low							
	Medium							
	High							

14	Low							
	Medium							
	High							
15	Low		🟦			🟪		
	Medium				🟨			⬛
	High			🟩			🟥	
16	Low							
	Medium							
	High							
17	Low			🟩			🟥	
	Medium		🟦			🟪		
	High				🟨			⬛
18	Low							
	Medium							
	High							
19	Low				🟨			⬛
	Medium			🟩			🟥	
	High		🟦			🟪		
20	Low							
	Medium							
	High							
21	Low							
	Medium							
	High							
22	Low		🟦			🟪		
	Medium				🟨			⬛
	High			🟩			🟥	
23	Low							
	Medium							
	High							
24	Low			🟩			🟥	
	Medium		🟦			🟪		
	High				🟨			⬛
25	Low							
	Medium							
	High							
26	Low				🟨			⬛
	Medium			🟩			🟥	
	High		🟦			🟪		
27	Low							
	Medium							
	High							
28	Low							
	Medium							
	High							
29	Low		🟦			🟪		
	Medium				🟨			⬛
	High			🟩			🟥	

30	Low							
	Medium							
	High							
31	Low			🟩			🟥	
	Medium		🟦			🟪		
	High				🟨			⬛
32	Low							
	Medium							
	High							
33	Low				🟨			⬛
	Medium			🟩			🟥	
	High		🟦			🟪		
34	Low							
	Medium							
	High							
35	Low							
	Medium							
	High							
36	Low		🟦			🟪		
	Medium				🟨			⬛
	High			🟩			🟥	
37	Low							
	Medium							
	High							
38	Low			🟩			🟥	
	Medium		🟦			🟪		
	High				🟨			⬛
39	Low							
	Medium							
	High							
40	Low				🟨			⬛
	Medium			🟩			🟥	
	High		🟦			🟪		
41	Low							
	Medium							
	High							
42	Low							
	Medium							
	High							
43	Low		🟦			🟪		
	Medium				🟨			⬛
	High			🟩			🟥	
44	Low							
	Medium							
	High							
45	Low			🟩			🟥	
	Medium		🟦			🟪		
	High				🟨			⬛

46	Low							
	Medium							
	High							
47	Low				🟨			⬛
	Medium			🟩			🟥	
	High		🟦			🟪		
48	Low							
	Medium							
	High							
49	Low							
	Medium							
	High							
50	Low		🟦			🟪		
	Medium				🟨			⬛
	High			🟩			🟥	
51	Low							
	Medium							
	High							
52	Low			🟩			🟥	
	Medium		🟦			🟪		
	High				🟨			⬛
53	Low							
	Medium							
	High							
54	Low				🟨			⬛
	Medium			🟩			🟥	
	High		🟦			🟪		
55	Low							
	Medium							
	High							
56								
57								
58								
59								
60								
61								
62								
63	Low		🟦			🟪		
	Medium				🟨			⬛
	High			🟩			🟥	

64	Low							
	Medium							
	High							
65	Low			🟩			🟥	
	Medium		🟦			🟪		
	High				🟨			⬛
66	Low							
	Medium							
	High							
67	Low				🟨			⬛
	Medium			🟩			🟥	
	High		🟦			🟪		
68	Low							
	Medium							
	High							
69								
4 weeks later								
97	Low		🟦			🟪		
	Medium				🟨			⬛
	High			🟩			🟥	
98	Low							
	Medium							
	High							
99	Low			🟩			🟥	
	Medium		🟦			🟪		
	High				🟨			⬛
100	Low							
	Medium							
	High							
101	Low				🟨			⬛
	Medium			🟩			🟥	
	High		🟦			🟪		
102	Low							
	Medium							
	High							