THE NEUROBEHAVIORAL MECHANISMS OF INK AS AN ANTIPREDATION STRATEGY

A Dissertation

by

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Submitted to the Graduate and Professional School of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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August 2021

Major Subject: Marine Biology

ABSTRACT

Inking is a chemical defense mechanism which has evolved independently in disparate taxa. The goal of this research was to investigate if the functional role of ink as an antipredator chemical deterrent has been conserved despite the divergent ancestry of three inking animal groups. I compared the ink from three unrelated taxa: California sea hares (*Aplysia californica*), common cuttlefish (*Sepia officinalis*) and pygmy sperm whales (*Kogia breviceps*). This research investigated the function of ink as a chemical deterrent by examining the chemical composition of ink, describing the chemosensory system of the predator affected by ink defenses, and quantifying the antipredator behavioral effects of ink. There was no single or cluster of amino acids which was consistently elevated across the three different inks. However, I recovered a large percentage of D-Amino acids which signal a connection to antimicrobial function and excretory pathways - the two evolutionary hypotheses for how ink arose. I also describe the microstructure for the olfactory system of sharks, common predators of inking organisms. I focus on bonnethead sharks and describe the distribution of sensory vs. nonsensory olfactory epithelium, mapped the pathway of the olfactory receptor axons through the olfactory lamellae, and investigated the lamellar morphological differences throughout the organ. I found that lamellae within the medial portion of the organ, which receives less water flow, had less sensory surface area and less secondary folds, leading to the hypothesis that these areas are less sensitive. Finally, I

demonstrate that all three inks elicit a negative response on shark swimming behavior. When sharks came in contact with an ink cloud, they deviated from their swimming path, exhibiting aversion responses. This research investigated the three components of a chemically mediated predator defense: the chemical makeup of that defense, the morphology of the predator's affected chemosensory system, and the behavioral effects of the interaction between the two.

DEDICATION

For mis abuelos and my grandparents, who came to the US with nothing so I could have everything, including a PhD.

Viva Cuba Libre. Patria y vida.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank the Chair of my committee, Dr. Christopher Marshall, who has been paramount in my training as a scientist. Through his mentorship, I was able to complete this dissertation while also gaining experience in a variety of areas of research. We've worked on everything from gator tongue histology to in water sea turtle work to dugong stable isotope analysis and many more side projects. Dr. Marshall taught me how to be a mentor to my undergraduate interns and an instructor to my students. I am grateful to learn from him on how to excel in all three areas of academia: research, teaching, and service. I have learned so much as one of his graduate students and I will forever be grateful for how much I have grown these last six years working with him.

I would also like to thank all my committee members: Dr. Karl Kaiser for his invaluable input and guidance on my chemical analysis chapter (and for continuously providing new columns after my ink clogged them up); Dr. Anja Schulze for her invertebrate knowledge and training on the SEM; Dr. David Wells for his knowledge on sharks and assistance with sample collection; and finally, Dr. Lene Petersen, who has been with me since the start of my degree. Dr. Petersen has been a great resource not only for my physiology questions but also for my "what kind of academic job do I want?" questions. I am so honored to work with this amazing team.

I am incredibly thankful for all the graduate students who have assisted me over the years. Thank you to Jillian Gilmartin for her help with stats, Tom Tinhan for teaching me to operate the university boats, Sarah Pruski for her help setting up my equipment at Moody Gardens, as well as Mariah Livernois and Jamie Thompson for their help in the field and the lab when COVID prevented us from having undergraduate interns. I would like to thank Laura Leonard in the Kaiser lab for being the first to attempt to separate out the *Kogia* ink from the "lunch". I would also like to thank MC Hanon for her training on the SEM.

This work would not be possible without the EcoMorph Lab members especially my undergraduate interns: Marco Chacon, Michael Bryan Gahn, Anahy Garza, Jeremesia Hamilton, Todd Jahns, Jordan Lynch, Sharleen Muñoz, Jill Otto, Rob Perkins, Rebekah Reaves, Justin Wilson, and Allison Zimmerhanzel. They not only watched sharks swim in circles for hours at Moody Gardens, but then watched hours of footage of those same sharks once again for kinematic analysis. I would like to further thank Michael Bryan Gahn for his immense effort in the free amino acid analysis portion of my dissertation. Additionally, I would like to thank Joshua Cullen and Carlos Peredo for their guidance as senior members of the lab. Finally, I would like to thank Aubree Jones who taught me histology from the ground up and still to this day is there if I need her guidance on a stain or imaging procedure.

I am indebted to all those who have supported this work. Thank you to

The National Resource for Aplysia, Superior Fish Company, D. Ann Pabst & William McLellan, and Christine Bedore for ink samples. Thank you to Katie St. Clair and the SLF staff who helped take care of my *Aplysia* and bonnethead sharks. Thank you to everyone at Moody Gardens including Roy Drinnen, Karen Holcroft, David Sanchez, Greg Whittaker, and the Quarantine Staff for their assistance with my behavioral studies. I would like to thank Dr. Tricia Meredith and Dr. Stephen Kajiura for teaching me so much about shark olfaction as well as Dr. Marianne Porter for her guidance and support in the end of my degree. Finally, thank you to RGSO and the MARB Department for all their academic assistance and advising.

I am so lucky to have a strong support system. I would like to thank the TAMUG graduate student community for making Galveston feel like home. I am thankful for our comradery and adventures over the years. I would like to thank the amazing organization, Minorities in Shark Science, for helping me find more people who look and act like me in the very straight white man world of shark science. I would like to thank Dr. Maria Cartolano for being my graduate school mentor and confidant. I would like to thank Jamie Thompson for being one of my strongest supporters and a top tier friend. Finally, I would like to thank my mom, Gisela, my dad, Tony, my brother, Mark, and my tia, Yayi, for believing in me when I didn't believe in myself and loving me unconditionally.

Galatians 6:9.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by a dissertation committee consisting of Dr. Christopher Marshall (Chair), Dr. Lene Petersen, Dr. Anja Schulze, and Dr. David Wells of the Department of Marine Biology and Dr. Karl Kaiser of the Department of Oceanography.

All work for Chapter 1 of the dissertation was completed independently. The data analyzed in Chapter 2 was collected in part by Michael Bryan Gahn. The data analyzed for Chapter 3 was collected in part by Marco Chacon, Joshua Cullen, Michael Bryan Gahn, Anahy Garza, Jeremesia Hamilton, Todd Jahns, Aubree Jones, Jordan Lynch, Sharleen Muñoz, Jill Otto, Rob Perkins, Rebekah Reaves, Justin Wilson, and Allison Zimmerhanzel.

Funding Sources

Graduate study was supported by TAMUG MARB IDP Graduate Assistantship Award, Bernice E. Powell Maritime Education Scholarship, SeaSpace MARS Graduate Award, and the Hispanic Scholarship Fund.

This work was also made possible in part by the TAMUG Marine Biology Department Mini Grant. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the TAMUG Marine Biology Department.

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CHAPTER I

INTRODUCTION

Chemically Mediated Defenses

Predator-prey interactions produce strong selection pressures that drive morphological, behavioral, and neurological innovations (Barbosa and Castellanos, 2005). A predation event consists of four steps: initial prey encounter or detection, followed by attack, capture, and prey ingestion. Predation defenses have evolved for each step of this process based on whether the threat is general or acute. General threats are when a predator is in the area searching for food. Defenses against general predation combat the first two steps of a predation event, initial prey encounter detection and attack, and often rely upon long distance senses such as chemoreception. An acute threat is when an attack is in place and is perceived visually and mechanosensorarily (e.g., being bitten, direct tactile hair stimulation, lateral line detection, etc.). These defenses focus on the latter two parts of the predation event (Brönmark and Hansson, 2012). Prey will evolve defenses against predation, which in turn result in predator innovations that counter these defenses creating an evolutionary arms race (Barbosa and Castellanos, 2005). Predator induced defenses in prey can result in morphological changes, such as an increase in body size or the development of spikes, thorns, neckteeth, and helmets. Predators can also induce changes in life history and behavior, such as changes

in maturation or changes in migration, as seen in diel vertical migration (Brönmark and Hansson, 2012).

One mechanism by which prey defend against predation is through chemically-mediated defense mechanisms. Some species sequester chemicals from their food, some produce their own chemical defenses *de novo*, while others can utilize defensive chemicals produced by their symbionts (Brönmark and Hansson, 2012). Just as the evolutionary arms race has influenced defensive chemical delivery and usage, it has also affected chemical composition. The spread of organic molecules is medium specific; molecular characteristics determine the solubility in the medium, volatility, and release/transport towards sensory epithelium. The evolution of appropriate marine signal molecules has been influenced by aquatic properties such as: the small diffusion constant of water, the higher predictability of ocean current direction and velocity as opposed to wind, and the polarity of the water molecule (Thewissen and Nummela, 2008).

There are three major categories of chemically mediated defenses. Prey may employ phagomimicry and set up a trap for their predators in which the same chemicals predators use to find injured prey are employed as a decoy. In this scenario, prey release secretions that mimic the chemical signal of a food item, acting as a stimulant for appetitive feeding. In response, predators attack the chemical cloud instead and the prey are able to escape (Aggio and Derby, 2008; Kicklighter et al., 2005; Shabani et al., 2007). Chemical defenses can also

manifest as alarm cues, which alert conspecifics to the presence of a predator and the severity of the threat. When exposed to these alarm cues conspecifics perform anti-predator behaviors. Some alarm cues are actively secreted. Examples include molluscs secreting alarm cues in their slime trails or during an inking event. Other alarm cues are released through the hemolymph or blood when a conspecific is injured. Alarm substances exist on a gradient and allow conspecifics to gauge the threat level based on the chemical concentration. Alarm cues also help prey species learn to identify threats. When prey species are exposed to alarm substances along with visual or mechanical cues, they can recognize the predator as a threat in the future. Chemically mediated defenses also exist in the form of defensive chemicals which may be used disrupt the sensory systems of predators by physically or physiologically when encountering such chemicals, or by being aversive (distasteful, irritant, toxic, etc.) to the predators (Brönmark and Hansson, 2012).

Ink as a Chemically Mediated Defense

The use of ink as a method of predation avoidance has been described for a variety of animal groups including sea hares (*Aplysia spp.),* cephalopods, crestfish, and an unusual family of toothed whales, kogiids (Aggio and Derby, 2008; Bush and Robison, 2007; Caldwell, 2005; Caldwell and Caldwell, 1989; Honma et al., 1999; Kamio et al., 2010; Kicklighter et al., 2005). When ink is used as a predatory defense, it is hypothesized to affect predator chemosensoy

systems as a chemical defense (Aggio and Derby, 2008; Kamio et al., 2010; Kicklighter et al., 2005) and vision, acting as a smoke screen (Bush and Robison, 2007; Caldwell, 2005). These three taxa produce ink of different colors and consistencies: *Aplysia* spp. produce a red ink which they can release in concert with opaline, a viscous clear substance; Cephalopods release a mix of black ink and mucus; and kogiids produce a brown, viscous ink with their liquid feces. Despite these compositional and visual differences, all three groups produce ink in the same context- in response to a predation threat. Although these three animal groups have different ecologies and are evolutionarily distant, they have evolved a similar mechanism of predator avoidance.

The effect of ink on predators has been tested for *Aplysia* and cephalopods (Aggio and Derby, 2008; Bush and Robison, 2007; Caldwell, 2005; Derby et al., 2013; Kamio et al., 2010; Kicklighter and Derby, 2006; Kicklighter et al., 2005; Love-Chezem et al., 2013; Nusnbaum and Derby, 2010a; Nusnbaum and Derby, 2010b; Nusnbaum et al., 2012; Shabani et al., 2007; Wood et al., 2010), but not kogiids. Additionally, the functional and chemical similarities between these three inks have not been tested. To fill this knowledge gap, this project focused on the effect of ink on a common predator: sharks.

Bonnethead Sharks as a Model System

Bonnethead sharks (*Sphyrna tiburo*) are a small member of the sphyrnid family. They are an appropriate model system since they are abundant in the

local waters, are well suited for captivity, and have broad cephalofoils that give easy access to their olfactory structures. Their broadly spaced nares give them a larger separation between olfactory rosettes, allowing for an enhanced ability to resolve odor gradients. Bonnetheads are also labeled as “Least Concern” by the IUCN allowing for the use of a non-threatened shark species in our experiments. However, because olfactory sensitivity is conserved throughout shark species (Kajiura et al., 2005), we can infer our results in bonnetheads to other shark species. Bonnethead sharks have also been successfully used in olfactory sensitivity studies (Meredith and Kajiura, 2010; Meredith et al., 2012). Furthermore bonnetheads also prey on squid, making them likely targets for ink defenses.

Fish Olfactory Systems

The use of olfaction in fish have been well described (Caprio, 1988; Døving et al., 1980; Hara, 1994; Laberge and Hara, 2001). In aquatic environments, chemicals dissolved in the water enter through the nares into the olfactory rosette and bind to G-protein coupled olfactory receptors on olfactory receptor neurons (ORNs) in the olfactory epithelium (Eisthen, 2004; Smeets, 1998). In teleost fish, the olfactory epithelium contains three main types of G-protein coupled ORNs: microvillus, ciliated, and crypt (Døving, 2007; Hansen and Finger, 2000; Hansen and Reutter, 2004; Hansen and Zielinski, 2005; Hansen et al., 2005; Thommesen, 1983; Yamamoto and Ueda, 1979a;

Yamamoto and Ueda, 1979b). Each individual ORN expresses one type of receptor gene that is correlated to its morphology, G-protein, and transduction channel. Ciliated sensory neurons have cilia-containing dendritic ends, express the “OR” type odorant receptor genes, are coupled with Golf/s type G-proteins, and utilize the cyclic nucleotide transduction channel (Cao et al., 1998; Hansen et al., 2004; Speca et al., 1999). The microvillus ORNs have tufted dendrites with microvilli, express the “V2R” type odorant gene, are coupled with Gao, Gaq, or Ga_{i3} proteins, and utilize the transient receptor potential transduction channel C2 (Hansen et al., 2004; Sato et al., 2005). Finally, the understudied crypt ORNs have an apical invagination (“the crypt”) which is thought to contain odor receptor proteins and transduction factors. Crypt ORNs express Galpha-o or Galpha-q proteins and are thought to express the “V1r” type receptor gene (Hansen et al., 2004; Pfister and Rodriguez, 2005). Each of these ORNs is additionally matched with a specific type of odor ligand. Ciliated ORNs respond to bile salts and conspecific alarm cues; microvillus ORNs respond to amino acids and nucleotides; and the crypt ORNs, though still understudied, are thought to respond to sex pheromones (Hamdani and Døving, 2007).

Cross adaptation experiments have proven that the receptor populations that bind to amino acids are independent of those that bind to bile salts and sex steroids in teleosts (Hara, 1994). Thommsen (1983) was able to correlate stimuli-specific electro-olfactogram (EOG) responses to areas of the olfactory epithelium with relative densities of specific ORNs. The EOG response

amplitudes were larger for amino acids in the central margin of the olfactory epithelium, where microvillus ORNs outnumber ciliated ORNs. Following this pattern, EOG responses were greater in response to bile salts in the lateral olfactory epithelium where ciliated ORNs had a greater density. Sato and Suzuki (2001) found no response to sex pheromones when recording intracellularly from microvillus and ciliated ORNs. However, they did not record from crypt ORNs. Schmachtenberg (2006) saw responses of crypt ORNs to mixtures of amino acids but not bile salts, polyamines, or a culture medium.

In addition, ORN type is correlated to different parts of the olfactory tract (OT) and nuclei in the telencephalon. The ORNs synapse onto the second order neurons, the mitral cells, within the olfactory bulb. These synapses, termed glomeruli, are specific to one type of ORN (**Figure I.1**). For example, 1000s of ciliated ORNs will synapse onto one mitral cell creating one ciliated ORN-associated glomeruli. The axons from these mitral cells project to the telencephalon through the olfactory tract. In teleosts, these axons are chemotopically arranged within the olfactory tract. Therefore, the olfactory tract can be divided into medial and lateral sections based on specific associations with ORN nerve fibers. The lateral olfactory tract (LOT) corresponds to the microvillus ORNs and responds to appetitive stimuli such as amino acids and nucleotides. The medial olfactory tract is further divided into medial (mMOT) and lateral (lMOT) tracts that correspond to ciliated and crypt ORNs, respectively. This division is seen within the olfactory nuclei located in the pallium of the

telencephalon as well (Hamdani and Døving, 2002; Hamdani and Døving, 2006; Hamdani and Døving, 2007). Electrophysiological studies on these tracts and the brain have demonstrated a correspondence to odorant ligand-specificity among ORNs as well. Stimulation of the mMOT, which transduces information from ciliated ORNs, elicits an alarm response; stimulation of the mMOT, which transduces information from crypt ORNs, elicits reproductive behavior; and stimulation of the LOT, which transduces information from microvillus ORNs, elicits feeding behaviors (Døving et al., 1980)

Amino acids and nucleotides, which are associated with microvillus ORNs, indicate the presence of food in teleosts. Lindsay and Vogt (2004) reported that amino acids elicited appetitive behavior in zebrafish. Amino acids detection capability by teleosts is in the nanomolar range (as low as 10^{-9} mol L^{-1}) , which is approximately the levels of free amino acids found in their natural environments. L-amino acids with unbranched and uncharged side chains are widely considered the most effective in producing behavioral responses (Hara, 1994). Specific amino acids receptor sites have been defined in teleosts including site TSA (L-theronine, L-serine, and L-alanine), site L (L-lysine) and site AD (D-alanine) (Cagan and Zeiger, 1978).

Interestingly, elasmobranchs only possess 2 of the 3 ORN types observed in teleosts and lampreys. Elasmobranchs lack ciliated ORNs, which are linked to the detection of bile salts, yet EOG recordings have shown that the elasmobranch olfactory system is able to detect these compounds. Additionally,

cross adaptation experiments have shown that different receptors respond to amino acids than respond to bile salts (Meredith et al., 2012). Alarm cues, the other olfactory stimuli connected with ciliated ORNs has yet to be studied in elasmobranchs.

Ink as Shark Deterrent

In this dissertation, I investigate the anitpredatory effects of ink from three different taxa: California sea hares (*Aplysia californica*), common cuttlefish (*Sepia officinalis*), and pygmy sperm whales (*Kogia breviceps*). I approach this question from three avenues: by quantifying the chemical composition of these three inks; describing the target of these ink defenses, the olfactory organ of the bonnethead shark; and investigating the interaction between these two- the behavioral response of a bonnethead shark to ink.

In Chapter 2, I compare the chemical composition of ink between *Aplysia*, *Sepia*, and *Kogia.* I look at the total free amino acid composition and investigate the hypothesis that the same chemical components have been selected for in the inks of these three disparate taxa. Additionally, I evaluate what percentage of the total dissolved organic carbon is made up by the measured free amino acids. Finally, I look at the relative composition of D-form free amino acids in each of the three inks and relate it to the current evolutionary hypotheses for how ink arose.

In Chapter 3, I describe the microstructure of the bonnethead olfactory organ. I describe the general morphology of the olfactory rosette and the sensory vs. nonsensory area on the lamellae. Furthermore, I track the projections of the olfactory receptor neurons through the lamellae, mapping the pathway from the epithelium to the olfactory bulb. Lastly, I look at how different morphological metrics differ along the medial to lateral gradient within the rosette to investigate my hypothesis that lamellar morphologies will differ based on their position within the olfactory organ.

In Chapter 4, I quantify the behavioral response of bonnethead sharks to ink to test my hypothesis that ink acts as a chemical deterrent. I measure the resulting swimming kinematics of sharks exposed to the three inks. I also compare ink to two controls: seawater (to control for mechanosensory stimulation) and food coloring (to control for color). Additionally, I expose sharks to food odor to elicit an attraction response. I compare swimming kinematic metrics (angle of deviation, body angle, angular velocity, speed, etc.) for all three inks, controls, and food odor. Finally, I looked at the long term effects of ink by calculating how much time sharks spent around the ink injection site following the introduction of stimuli.

In my concluding Chapter 5, I integrate the chemical components of ink, the morphology of the shark olfactory system, and the behavioral response of a shark to ink. These three components elucidate the form-function relationship between a prey’s chemical defense, a predator’s sensory system, and the

resulting predator-prey interaction. The novel nature of this research is discussed, including that it is the first reported data of any kind for *Kogia* ink. Future directions are discussed as well as broader impacts of this research in relation to changing ocean conditions.

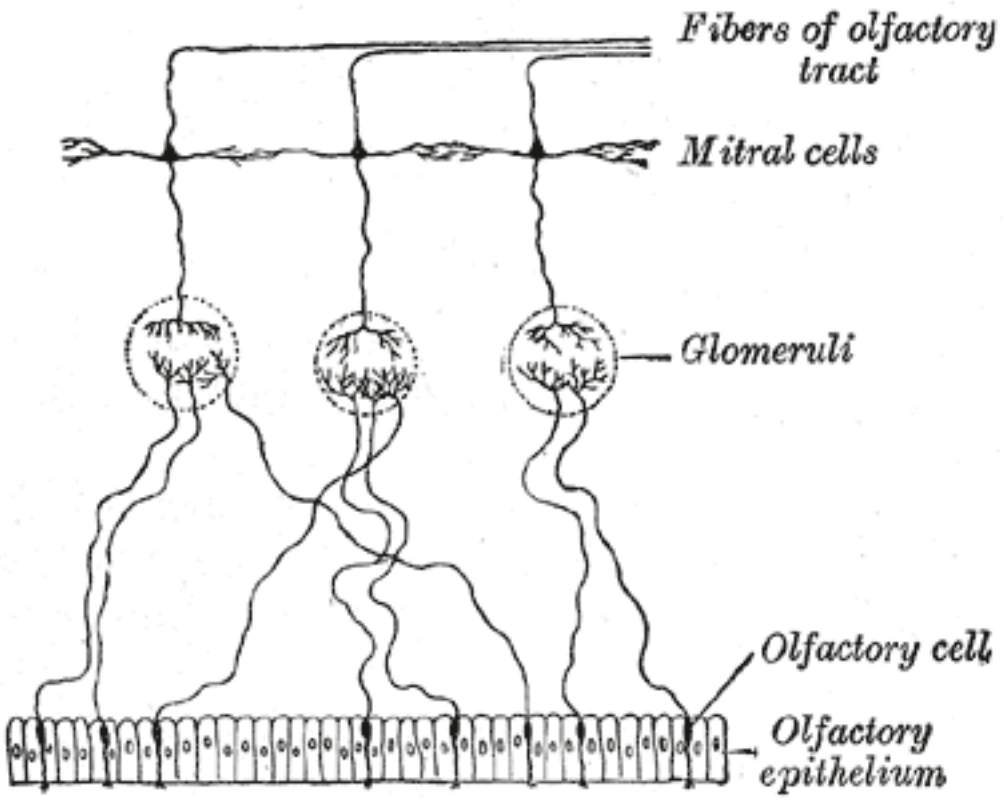

Figure I.1. Diagram of the olfactory system from Gray and Lewis (1918)

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CHAPTER II

COMPARISON OF FREE AMINO ACID COMPOSITION OF INK FROM CALIFORNIA SEA HARES (*APLYSIA CALIFORNICA)*, COMMON CUTTLEFISH (*SEPIA OFFICINALIS)*, AND PYGMY SPERM WHALES (*KOGIA BREVICEPS)*

Introduction

Predator-prey interactions elicit strong evolutionary selection pressures that drive organismal adaptations. Chemical defenses constitute one class of predation defense strategies. Inking is a visually striking predator defense used by a wide variety of taxa such as sea hares (*Aplysia* spp.), many cephalopods, as well as pygmy and dwarf sperm whales (kogiids) (Aggio and Derby, 2008; Bush and Robison, 2007; Caldwell, 2005; Caldwell and Caldwell, 1989; Derby, 2007; Kicklighter and Derby, 2006). Ink production sites among these organisms are not homologous. Instead, inking behavior is hypothesized to have arisen independently in each of these taxa. Despite their different origins, the function of inking has converged to produce a similar tactic that deters and avoids predation.

Ink is a multimodal deterrent that targets different sensory systems simultaneously. Ink can affect a predator's vision acting as a smoke screen, and chemoreception, as a chemical defense via phagomimicry or chemical deterrence. Phagomimicry is defined as the use of chemicals, by a potential prey species, that mimic the composition of injured prey and act as a stimulant for

appetitive feeding. In response, predators attack the chemical cloud instead, and the prey are able to escape (Aggio and Derby, 2008; Kicklighter et al., 2005; Shabani et al., 2007). Chemical deterrents disrupt the predator's sensory systems by either preventing chemosensation or by being aversive (distasteful, irritant, toxic, etc.) to the predators (Brönmark and Hansson, 2012)

Although the route of predator defense is similar, the composition, type of pigmentation, and density of the ink are dissimilar. The defensive secretion of California sea hares (*Aplysia californica)* is comprised of two substances, opaline and red algae pigmented ink. Cephalopods, such as the common cuttlefish (*Sepia officinalis)*, eject a mix of black melanin-based ink and mucus. Pygmy sperm whales (*Kogia breviceps*) produce purple viscous ink that includes melanin derived from melanocytes and mucus produced by goblet cells in their large intestine and release it along with fecal matter when threatened. Despite their differences, the inking behavior observed in these three disparate taxa is speculated to have arisen in response to two selection pressures: as an antimicrobial mechanism and as an excretion pathway. The investigation of ink, ink composition, and inking behavior provides us with a unique opportunity to investigate a relatively unusual chemical defense that likely arose multiple times in disparate taxa through convergent evolution.

One such evolutionary pathway hypothesized for the production of ink chemical defenses is through the sequestration of chemicals previously used as antimicrobials. L-amino acid oxidases (LAAOs) are antimicrobial compounds

found in the ink of *Aplysia* spp. as well as cephalopod ink. Antimicrobial compounds like LAAOs are found in *Aplysia* egg masses, their albumen gland, and their ink gland (Butzke et al., 2005; Iijima et al., 2003a; Iijima et al., 2003b; Jimbo et al., 2003; Kamio et al., 2009; Kamiya et al., 1989; Kamiya et al., 2006; Yamazaki et al., 1989; Yang et al., 2005). The ink of cuttlefish and squid also exhibits antimicrobial activity (Derby, 2014; Girija et al., 2011; Petkovic, 2013; Smiline et al., 2012; Vennila et al., 2010). Both cephalopods and *Aplysia* lay their eggs on benthic rocky substrate or in seagrass beds, making them susceptible to biofouling and predation. It is advantageous to have antimicrobial compounds in the egg masses and in the albumen gland that packages the eggs to both act as antipredatory and antifouling agents.

The antimicrobial compounds in ink have been shown to have direct, negative effects on predators. For example, *Aplysia* ink contains escapin, an LAAO ortholog. Escapin inhibits growth of Gram-positive and Gram-negative bacteria, including marine and pathogenic bacteria. Escapin also inhibits yeast and fungal growth. When released with opaline during an inking event, escapin oxidizes the L-lysine in opaline. This reaction produces millimolar concentrations of hydrogen peroxide, ammonia, keto acids, carboxylic acids, and other products which have antipredatory effects such as inhibiting ingestion and invoking escape responses in the predator ((Aggio and Derby, 2008; Kamio et al., 2009; Kicklighter et al., 2005; Nusnbaum and Derby, 2010; Yang et al., 2005). Gene sequence data has supported the hypothesis that LAAOs first appeared in the

albumen gland and egg masses of molluscs (Derby, 2007; Kamiya et al., 2006)). This evolutionary scenario also explains why the LAAOs present in the ink gland have antimicrobial function in addition to their antipredatory functions.

The selection of waste release as a predatory defense is a common hypothesis due to its potential use as a smoke screen and therefore blocks visual cues (Derby, 2007). The addition of chemical deterrent components in fecal excretion was adaptive since it would also confer a multimodal sensory defense; visually as well as chemically. This evolutionary phylogeny for ink is also supported by the inking strategy of members of Kogiidae. *Kogia* ink is a mix of fecal matter and red, viscous ink. Histological analysis has shown that the ink is produced in the jejunum, ileum, and upper colon of the digestive track. Additionally, molluscan ink glands (and the opaline gland in *Aplysia*) are thought to have arisen originally as excretory structures. Although Aplysia ink glands no longer show a morphological connection to the excretory system, cephalopod ink sacs do maintain that digestive anatomical connection. Specifically, cephalopod ink sacs form as a diverticulum from the hindgut and ink is released near the anus (Derby, 2014). The presence of diet derived products in *Aplysia* ink also provides support for this past excretory connection. *Aplysia* mainly consume red algae and their ink contains the modified red algal pigments phycoerythrobilin and aplysioviolin from their diet (Kamio et al., 2010). In fact, sixty-five percent of the dry-mass of *Aplysia californica* ink is comprised of phycoerythrobilin (Prince et al., 1998). The digestive, opaline, and ink glands of

Aplysia accumulate additional secondary metabolites from their red algal diet. Defensive compounds (such as LAAOs) are also synthesized and packaged within these glands (Johnson et al., 2006). In cephalopods, the color of the ink is due to the addition of the dark pigment, melanin. While melanin likely originally arose as a photo-protectant, it is hypothesized to be an exaptation as a camouflage agent and a detoxifier of heavy metals. Eumelanin, a type of melanin, in cuttlefish ink chelates metal ions and free readicals. Therefore, ejecting melanin-based ink provides a route for excretion and detoxification of heavy metals. Therefore, the ink sac of cephalopods may have had an early role in the excretion of heavy metals through the production of melanin. Melanin, though not thought to be an anitpredatory defense has been hypothesized to be a carrier for these bioactive molecules (Derby, 2014; Hong and Simon, 2006; Ichihashi et al., 2001; Raimundo and Vale, 2008).

While *Aplysia* and cuttlefish ink have been chemically investigated (Derby et al., 2007), *Kogia* ink has not. Despite the divergent ancestry of these three animal groups, I set out to investigate whether the chemical composition of these inks has been similarly selected for. Specifically, I compiled a profile of L- and D-form free amino acids (FAAs) for each of the three inks to investigate potential convergence of ink composition. Although FAA analysis has been performed for both *Aplysia* and cephalopod ink, they only looked at L-FAAs. Unlike L-amino acids, D-amino acids have an amino group on the right side instead of the left and rotate plane-polarized light clockwise instead of

counterclockwise. Additionally, D-amino acids indicate the presence of bacterial activity and bacterially derived organic matter. Additionally, any observed changes in D-AA content in the ink samples throughout their time in storage could indicate several things, like bacterial growth behavior (exponential versus stationary). These changes could also reflect the intrinsic stability of bacterially derived organic matter compared to the other organic matter present in the ink. I hypothesized that there would be an overlap across the three inks in the most abundant FAAs. This is the first comparative study of the chemical composition of ink from these three groups. Additionally, to my knowledge, this is the first published chemical analysis of ink from any vertebrate animal.

Methods

Ink Collection

Ink was collected and pooled from 13 live specimens of *Aplysia californica* purchased from the National Resource for *Aplysia* at the University of Miami (Key Biscayne, Florida). *Aplysia* were placed in a 1 L beaker and agitated via mechanical stimulation until there was a visual secretion of ink. They were removed from the beaker upon cessation of inking. *Sepia* ink was acquired from Superior Fish Company (Royal Oak, MI). This ink was compared to *Sepia officinalis* and *Sepia bandensis* ink sacs (provided by Christine Bedore, Georgia Southern University, Statesboro, Georgia) via the HPLC methods described below. The free amino acid profile confirmed that the *Sepia* ink came from *Sepia*

officinalis. Sepia ink (56.6 mg) was diluted in 1 ml of mili-Q water to prepare it for analysis. *Kogia breviceps* ink was collected from the large intestine of 2 euthanized, stranded whales (University of North Carolina Wilmington Marine Mammal Stranding Program) and frozen immediately after necropsy. "*Kogia A"* was a male that stranded on June 19, 2015 and "*Kogia B"* was a female that stranded on December 22, 2018. Each ink was stored frozen (-20°C) until used for analysis. *Kogia A*'s sample was too heterogeneous to be analyzed as is, so it was filtered through a 2 μm filter to separate out the ink from the fecal matter.

HPLC Apparatus and Fluorescence Detection

Analysis of L- and D-form free amino acids (FAAs), dopamine, and l-3,4-dihydroxyphenylalanine (L-DOPA) was performed with an Agilent Technologies 1260 High Performance Liquid Chromatography system, equipped with a degasser, binary pump, autosampler, column compartment, fluorescence detector (FLD) and OpenLab ChemStation CDS (Kaiser and Benner, 2005). Compounds were separated using an Agilent Poroshell 120 EC-C18 reverse phase column (4.6x100mm, 2.7 um) with an Agilent Poroshell 120 EC-C18 guard column (4.6x5mm, 2.7 um) at 35°C. Ultrapure water was produced using a Millipore A10 purification system (Merck KGaA, Darmstadt, Germany). Compounds were separated using a dual solvent, gradient elution profile with solvent A (A), 48 mM potassium phosphate monobasic adjusted to pH 6.25 with 50% (v/v) sodium hydroxide, and solvent B (B), 93:7 methanol:acetonitrile

solution. The linear gradient started with 100% A to 39% B at 13.3 min, 54% B at 19.2 min, 60% B at 21.3 min, 80% B at 22 min, maintained 80% B until 23 min, then returned to 100% A at 24 min with an additional 1 minute for equilibration. The flow rate was 1.5mL min^{-1} with a total run time of 25 min. Before separation, free amino acids were derivatized with isobutyryl-L-cysteine (IBLC) and ortho-phthaldialdehyde (OPA). The fluorescent derivatives were detected at an excitation wavelength of 330nm and emission wavelength of 450 nm at a PMT gain of 10. Standards and peaks were calibrated, integrated and analyzed using Agilent OpenLAB Control Panel. Calibration curves were obtained from standards ranging in concentration from 100 nM to 2 μM.

Preparation of ink for fluorescence detection

Pure Filtration

Stock ink samples were diluted with 10mM ascorbic acid Milli-Q solution then filtered using 0.2 μm nylon filter (VWR). Immediately after filtration, ink was further diluted with 10mM of ascorbic acid in Milli-Q water to minimize possible oxidation of dopamine and L-DOPA. The resulting filtered dilutions were then run alongside standards.

Centrifuge

To avoid the possibility that the filtration process stripped the ink of potential compounds of interest, a second stock ink sample was centrifuged to produce a precipitate pellet rather than filtration. Stock ink samples were

transferred into Agilent autosampler vials and diluted with 10mM ascorbic acid in Milli-Q. Samples were then placed inside Fisher Scientific model 228 centrifuge for one hour at 3300rpm. The supernatant was then collected and transferred to autosampler vials, ensuring not to collect the precipitated pellet. The precipitate pellet was then placed under a custom-made drying stand and treated with N_2. Once dry, 1mL 10mM Ascorbic Acid in Milli-Q water was added to each sample and vortexed using Fisher Vortex Genie 2^{TM} every 15 minutes for 4 hours. The vials were placed back into the centrifuge and spun for an additional hour at 3300 rpm. The supernatant was collected, added to autosampler vials and set aside for chromatography.

Identification of L-DOPA and Dopamine

To ensure that the ink preparation method did not remove dopamine and L-DOPA, prior to centrifugation, a subset of ink samples were artificially spiked with the following amounts of both compounds: Aplysia with 10μM, Sepia with 40μM, and Kogia 125 μM. The artificially spiked ink was overlayed with the un-spiked, stock ink sample, and the standard. This confirmed consistent retention times across samples and ensured L-DOPA and dopamine were not removed in the process of ink preparation. This also ensured proper identification of both L-DOPA and dopamine.

Results

Filtering vs Centrifugation

The centrifugation method yielded either an equal or higher recovery of almost all amino acids in comparison to the filtering preparation method. This was especially prominent in the two melanin-based inks from *Sepia* and *Kogia*. The centrifugation method recovered higher concentrations for all FAAs except D-Ala, L-Ala, D-Asp, and L-Met for *Aplysia* ink; D-Asp, L-His, L-Met, for *Sepia* ink; and D-Ser for *Kogia* ink (**Figure II.1**). It is important to note that some FAAs were only detected using the centrifugation method; these included L-Lys in *Aplysia* ink, D-Ser and L-Phe in *Sepia* ink, and L-His in *Kogia* ink. In total, the centrifugation method recovered 26% higher concentrations of FAAs in *Aplysia* ink, 91% higher concentrations in *Sepia* ink, and 136% higher in *Kogia* ink.

The centrifugation methodology worked significantly better for both melanin-based inks, *Sepia* and *Kogia*. Melanin is a large granule which aids in the formation of a substantial pellet after centrifugation, making it easier to wash and collect from these pellets. When these melanin-based inks are filtered, the large melanin granules are trapped within the filter, clogging it and potentially keeping FAAs from being collected in the filtrate. **Figure II.2** shows an example of this issue with *Sepia* ink- the black, opaque ink become clear after passing through the filter. *Aplysia* ink, a red algal pigment-based ink, does not clog the filter, and did not undergo a color change when filtered, or form a substantial pellet when centrifuged.

Concentration and distributions of FAAs

Concentrations of FAAs in ink ranged from the micro to the millimolar range (**Table II.2**). In total, I recovered 2,951 μM of FAAs in *Aplysia* ink, 138 μM in *Sepia* ink, and 257 μM in *Kogia* ink. The most abundant FAAs in *Aplysia* were Taurine (78% of total FAAS), L-Ser (5%), and Glycine (3%). *Sepia* ink FAAs were Taurine (37%), L-Ala (14%), and L-Val (12%); *Kogia* A ink FAAs were D-Ala (15%), L-Ala (14%), and Taurine (10%). Kogia B ink FAAs were L-Glu (19%), L-Ala (13%), and Glycine (10%). *Aplysia* ink contained no D-Ser or L-Arginine (L-Arg). The least abundant FAA for both *Sepia* and *Kogia* B ink was D-Ser (0.03 and 0.02% respectively). The least abundant FAA for *Aplysia* ink was D-Ala (0.06%) and L-His for *Kogia* A ink.

Each organism ink had a unique FAA composition; no single FAA, or cluster of FAAs were consistently elevated across all three inks. However, there was overlap in the most abundant FAAs between similar inks. For both invertebrate inks, *Aplysia* and *Sepia*, Taurine was by far the most abundant amino acid. Taurine also ranked in the top 3 most abundant FAAs for *Kogia* A. Both melanin-based inks, *Sepia* and *Kogia*, did show overlap in some of their most abundant FAAs with L-Ala comprising 9-14% of their total FAAs.

The measured FAAs explained varying amounts of dissolved organic carbon (DOC) among the three inks (**Table II.3**). Measured FAAs explained 3-4% of total DOC for *Kogia* B and *Aplysia* ink respectively but 16% of DOC in

Sepia ink and 38% of *Kogia* A. The individual FAAs also varied in how much they contributed to the total DOC. For *Aplysia* ink, Taurine had the highest contribution with 2% of total DOC, followed by L-Ser and L-Met both with 0.2%. Taurine, along with L-Val, is also the highest contributor to DOC for *Sepia* ink, both making up 3% of the total DOC. The third most abundant FAA in *Sepia* ink was L-Leu (2%). The largest contributors to total DOC in *Kogia* ink are L-Leu (4%), D-Glu (4%), and L-Ile (4%) for *Kogia* A and L-Glu (0.5%), L-Val (0.2%), and L-Ala (0.2%) *Kogia* B (**Table II.2**).

A surprising large amount of D-form FAAs were recovered in all three of the inks (**Figure II.3 and II.4**). *Kogia* ink especially had high concentrations of D-form FAAs that comprised 5% of its chiral FAAs (enantiomeric FAAs which have both an L- and D-form). *Sepia* ink contained 3% D-FAAs and *Aplysia* contained 2% D-FAAs. The highest D-FAAs were D-Ala in *Kogia* (8%) and *Sepia* ink (4%) and D-Glu in *Aplysia* ink (2%). Since D-FAAs are associated with bacterial presence, I compared D-FAA composition over time to account for possible bacterial growth. There was no significant difference in D-FAA composition for any of the inks over time (**Figure II.4**). The greatest change was *Aplysia* ink which actually decreased in total D-FAAs concentration by 2%.

Discussion

Comparison to previously published data

My *Aplysia* FAA results were comparable to previously published data (**Table II.4**). When compared to a similar analysis on maricultured *Aplysia californica* from the same source in Derby et al. (2007), I found similar concentrations in both total and individual FAA concentration. Most of my concentrations were higher with the exception of L-Arg and L-Tyr. It should also be noted that there is a reported difference in concentration between maricultured and wild caught *Aplysia*. Wild *Aplysia* exhibited much greater FAA concentrations but at a similar percentage composition of maricultured *Aplysia* (Derby et al., 2007). Although my *Aplysia* ink samples had lower levels of FAAs than ink from wild *Aplysia*, they still had higher levels than wild *Aplysia* hemolymph and mucus (563-715μM), bolstering the evidence that ink is a more concentrated substance than other *Aplysia* secretions and has evolved for chemical defense.

In contrast, my *Sepia* results do not agree with reported values for *Sepia officinalis* (**Table II.5**; Derby et al., 2007). I quantified the presence of previously unreported FAAs such as L-Arg, L-His, L-Met, L-Phe, and L-Tyr. However, most of my values were markedly lower. I only recovered 125 μM of total FAAs compared to Derby et al. (2007)'s 2491 μM. The majority of this difference was due to Taurine. Derby et al. (2007) reported a Taurine concentration of 2088 μM while I only recovered 52 μM. This difference could be attributed to a difference

in sample preparation. I had to dilute my *Sepia* ink in mili-Q water to prepare it for HPLC analysis, which could explain the differences observed in this study. Previous studies identified dopamine and l-3,4-dihydroxyphenylalanine (L-DOPA) as important constituents of ink (Fiore et al., 2004; Gilly and Lucero 1992; Lucero et al., 1984; Russo et al., 2003). Analysis performed in this study did not identify quantifiable concentrations of these two compounds in any of the ink samples (**Figure II.5**). **Figure II.6** show chromatographs of standards overlayed with unspiked and spiked ink. Peaks for both dopamine and L-DOPA were only observed in spiked samples, proving that ink samples did not have quantifiable amounts of either compound and that the preparation methods were not removing either compound. It is a possibility that large neighboring unknown fluorescent peaks may have previously been misidentified as either of these compounds. L-DOPA has a retention time of ~13.33 minutes and is closely bordered by Taurine, which has a retention time of ~13.12 minutes and L-Ala with a retention time of ~13.54 minutes (**Figure II.5**). Samples artificially spiked with L-DOPA showed fluorescent peaks overlapping with the L-DOPA standard at 13.33 minutes, but the unspiked stock ink samples did not. Similarly, dopamine has a fluorescent peak at a retention time of ~21.11 minutes but also has neighboring peaks. There is a close unidentified peak with a retention time of ~20.75 minutes which I suggest could have been misidentified for dopamine in previous studies (**Figure II.6**). The chromatography applied here is optimized to achieve the separation of enantiomeric amino acids and superior to normal

amino acid analysis, allowing for a more efficient separation of amino acids and other unidentified compounds.

Comparison of Kogia samples

I analyzed two *Kogia* ink samples. One sample, *Kogia* A, had been stored frozen since collection in July 2015 (four years prior to analysis in June-October 2019). The second sample, *Kogia* B, was collected in December 2018, frozen, and analyzed between June-October 2019. I observed large differences in FAA concentrations between the two samples. *Kogia* A had FAA concentrations at the milimolar level while *Kogia* B had FAA concentrations in the micromolar level (**Table II.2**). I was able to recover 9332 μM of FAAs from *Kogia* A and 257 μM from *Kogia* B. There was also a disparity in how much of the total DOC was explained by the measured FAAs. *Kogia* A's FAAs explained 38% of the DOC while *Kogia* B's only explained 3% (**Table II.3**). The relative concentration of each FAA to the total concentration in both samples had similar values with some notable exceptions. In *Kogia* A, D-Ala was 15% of the total FAA concentration while it only made up 4% of total FAA concentration in *Kogia* B. Conversely, L-Glu made up only 9% of *Kogia* A's total FAA concentration but 19% of *Kogia* B's concentration (**Table II.2**). It is unclear if these disparities are due to variation between individual *Kogia*, differences in sample collection, or time elapsed between collection and analysis. However, there was no change in overall D-FAA presence, leading us to believe there was no active bacterial

growth over time between analyses and that time prior to analysis was not a factor (**Figure II.4**). Furthermore, despite the difference in concentration, the relative concentration and composition of FAAs was mostly consistent across the two samples. Due to the dissimilarities between the two *Kogia* samples, I suggest more samples and analyses are needed to investigate the level of individual variation in ink chemistries.

FAA composition

For all three inks, all total FAA recovery was well above reported background seawater total amino acid ranges for the Atlantic (70-200 nM) and the Pacific Ocean (90-490 nM; Druffel et al., 1992; Lee and Bada, 1977). Individual FAA concentrations were also above sensitivity thresholds for elasmobranchs and teleosts which range from 10^{-9} to $10^{-6.9}$ (Caprio, 1988; Hara, 1994; Meredith and Kajiura, 2010). Individual FAAs such as Taurine, L-Glu and D-Glu are also above the published threshold for the spiny lobster chemosensory systems. The measured FAA concentration for *Aplysia, Sepia*, and *Kogia* inks are distinguishable from background seawater. Additionally, all three inks have concentrations within the sensitivity ranges of the chemosensory systems of spiny lobsters, bony fish and sharks, notable predators of all three inking taxa included in this study.

The most abundant FAAs in each species' ink was relevant to their predator-prey relationships. Taurine comprises almost 80% of the FAAs in

Aplysia ink and is also the most abundant FAA in *Sepia* ink. Both crustaceans and fish have specialized receptors for Taurine that are sensitive at the submicromolar level (Caprio, 1988). Alanine and Serine rank among the most stimulatory amino acids to both the elasmobranch and teleost olfactory systems (Caprio, 1988; Meredith and Kajiura, 2010; Tricas et al., 2009; Zeiske et al., 1987). Alanine is one of the most abundant amino acids in both *Kogia* and *Sepia* ink; Serine is the second most abundant FAA in *Aplysia* ink. Glycine is the third most abundant FAA in both *Kogia* and *Aplysia* ink and is a potent gustatory stimulus among some fish (Caprio, 1998). Glutamic acid was also one of the most abundant FAAs in *Kogia* ink samples. Glutamic acid, along with Glycine, stimulate the elasmobranch olfactory system (Meredith and Kajiura, 2010; Tricas et al., 2009). Interestingly, recent work has shown that changes in seawater pH or increased P_{CO_2} decrease fish olfactory sensitivity to amino acids such as L-Ser, L-Arg, and L-Leu but not L-Glu, suggesting a different transduction pathway for this amino acid (Velez et al., 2021).

D-Amino Acids and Evolutionary Hypotheses

Historically, it was assumed that organisms except bacteria were composed solely of L-amino acids. At one time is was presumed that mammals especially not possess D-AAs. However, recent work has demonstrated the presence of D-AAs in a variety of taxa including marine invertebrates and mammals. For example, D-Ala has been found in octopus muscle and in the

gastrointestinal biome of mice (Corrigan, 1969; Matsumoto et al., 2018; Preston, 1993). D-AAs have also been found in the brain of both vertebrates and invertebrates. D-Asp in particular is thought to act as a neurotransmitter and has been found in the nervous system of *Aplysia* (D'Aniello et al., 1993; Spinelli et al., 2006). Structurally, D-Asp is similar to L-Glu, a prominent neurotransmitter, and it has been hypothesized to be a substitute for L-Glu at excitatory Glu receptors. D-Asp has been shown to induce currents in *Aplysia* PVC cells could also play a modulatory role (Fieber et al., 2010).

Considering the current ink evolutionary hypotheses, I was particularly interested in the correlation of D-AAs with antimicrobial activity and excretory pathways. Defensive chemicals in ink are hypothesized to first have arisen as antimicrobial agents. D-AAs have been shown to improve antimicrobial activity and stability, inhibiting bacterial growth. When substituted for L-AAs in antimicrobial peptides, D-AAs have been used to enhance antimicrobial performance in the medical field. D-AA antimicrobial peptides have defended against pathogenic bacteria and maintained antimicrobial activity in conditions where their L-AA counterparts lost efficacy over time. Additionally, D-AA substitution can increase antimicrobial peptide resistance to proteolysis (Carmona et al., 2013; Hamamoto et al., 2002). Furthermore, the presence of D-AAs significantly reduces the formation of biofilms, multicellular communities formed by bacteria for protection. A mixture of D-AAs will cause the release of fibers that link the cells together in the biofilm, triggering disassembly. D-AAs

have been shown to prevent the formation of biofilms produced by pathogenic bacteria (Hochbaum et al., 2011; Kolodkin-Gal et al., 2010; Sanchez Jr et al., 2014). The ability of D-AAs to enhance antimicrobial efficacy may play a factor in the role of ink as an antimicrobial substance. *Aplysia* and cephalopod ink contain antimicrobial compounds like LAAOs. These chemicals are similar to those found in the egg masses and albumen glands of these invertebrates. *Sepia officinalis* even injects ink into their eggs acting as a photoprotectant, antimicrobial, and antipredatory deterrent (Boletzky, 2003; Derby, 2014). Mechanisms, such as ink production, that promote survival as well as prevents predation of eggs should be highly selected for since this would increase fitness.

Furthermore, inking has been hypothesized to have arisen via the co-option of a gut excretory pathway. This connection is further supported by the presence of these D-forms of amino acids in the gut, as they have a potentially important function in the gut flora of metazoans. In mice, the concentrations of amino acids, including D-Asp and D-Ser, in the brain are correlated to their presence in gut microbiota, leading to the hypothesis that amino acid metabolism in the brain can be altered by manipulating gut microbiota (Kawase et al., 2017; Sasabe et al., 2016). D-AAs including D-Ala, D-Asp, and D-Glu are commonly found in the mammalian intestine and in even higher numbers in the colon (Sasabe et al, 2016). Furthermore, D-AAs found in the ink samples (D-Ala, D-Asp, D-Glu, and D-Ser), have been linked to bacteria in the mammalian colonic lumen, especially those belonging to the phylum Firmicutes. Firmicutes

is a prominent bacterium in the gut microbiome of *Kogia* (Bai et al., 2021; Matsumoto et al., 2018). *Kogia* produce ink in their digestive tract, an obvious connection between inking and an excretory pathway. Although less morphologically linked, *Aplysia* and *Sepia* are thought to have co-opted ink from a pigment excretion pathway (for red algal pigment and melanin, respectively).

The presence of these D-AAs in the ink samples provide further support for the leading hypotheses for ink evolution, strengthening the connection between ink, antimicrobial activity, and excretion. Although these three taxa have evolved ink for similar purposes, their chemical compositions are not conserved. There are three independent compositions of FAAs among the three inks, albeit with some overlap. Ink production and inking provides an unusual phenomenon to investigate a predator defense mechanism and the evolution of those systems. Each of these taxa use ink in a different habitat and against different predators, yet they have the same functional benefits: opaque coloration for visual disruption and high levels of FAAs for chemosensory stimulation. Since ink has such a strong chemical component, its functionality may be affected by future changes in ocean chemistry, such as ocean acidification. It is important to understand these chemically mediated predator prey interactions currently, as we are faced with changing ocean conditions.

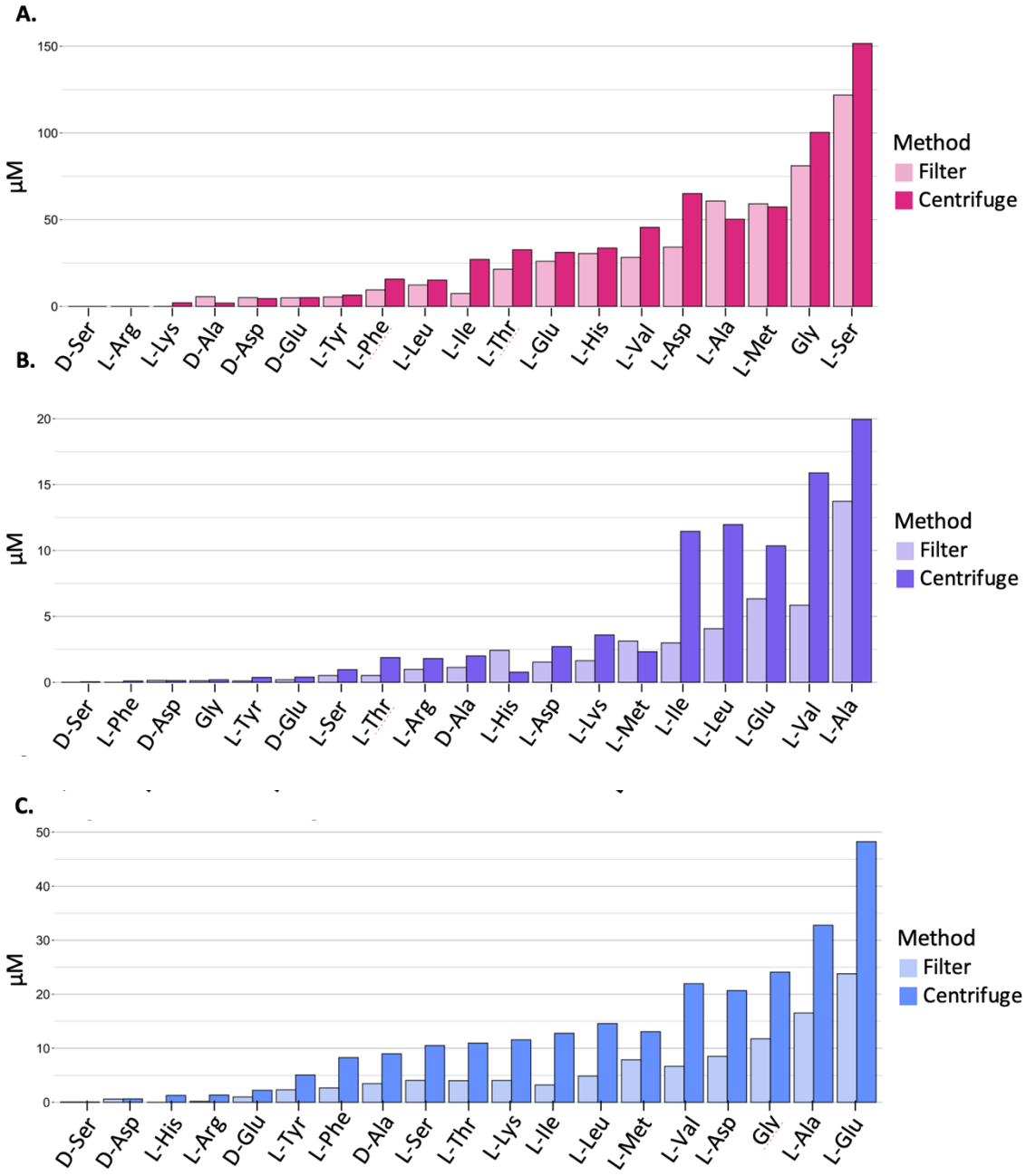


Figure II.1. Amount of FAAs recovered from *Aplysia* ink (**A**), *Sepia* ink (**B**), and *Kogia* B ink (**C**) through the filtration method (lighter color) vs the centrifugation method (darker color).

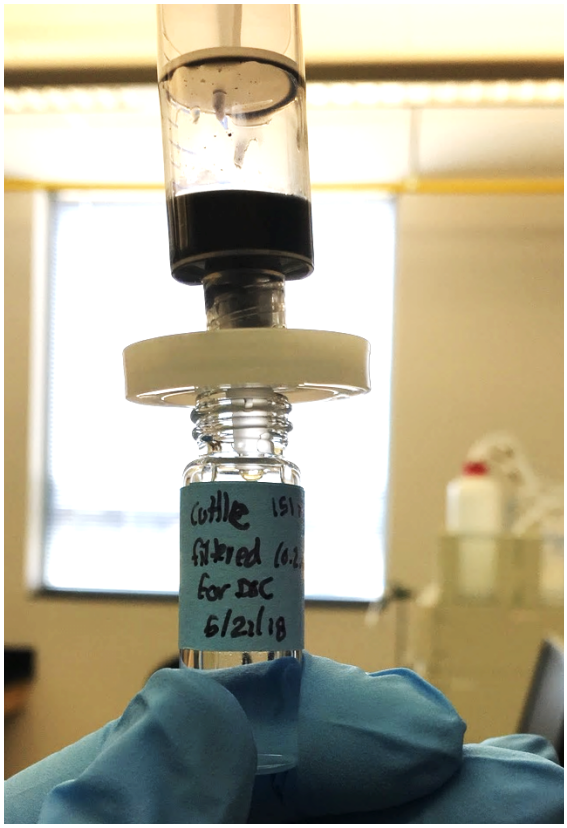


Figure II.2. *Sepia* ink passed through 0.2 □m nylon filter. The top, dark black ink is the unfiltered ink. The vial below contains filtered, clear ink. Melanin granules are caught in the filter, removing the dark color of the ink in the process of filtration.

Table II.1. Abbereviations for FAAs mentioned in the study.

Free Amino Acid (FAA)	*Abbreviation*
D-Alanine	D-Ala
D-Aspartic Acid	D-Asp
D-Glutamic Acid	D-Glu
D-Serine	D-Ser
Glycine	Gly
L-Alanine	L-Ala
L-Arginine	L-Arg
L-Aspartic acid	L-Asp
L-Glutamic acid	L-Glu
L-Histidine	L-His
L-Isoleucine	L-Ile
L-Leucine	L-Leu
L-Lysine	L-Lys
L-Methionine	L-Met
L-Phenylalanine	L-Phe
L-Serine	L-Ser
L-Threonine	L-Thr
L-Tyrosine	L-Tyr
L-Valine	L-Val
Taurine	--

Table II.2. FAA composition for each of the three inks expressed in μM, as a percentage of the total FAA concentration, and as a percentage of the total dissolved organic carbon (DOC) in the inks.

FAA	*Kogia* A			*Kogia* B		
	Concentration (μM)	% of total FAAs	% of total DOC	Concentration (μM)	% of total FAAs	% of total DOC
D-Alanine	1367	145%	1%	9	4%	0.06%
D-Aspartic Acid	95	1%	0.3%	1	0.2%	0.01%
D-Glutamic Acid	41	0.4%	4%	2	0.9%	0.02%
D-Serine	19	0.2%	0.2%	0.06	0.02%	>0.01%
Glycine	915	10%	2%	24	9%	0.11%
L-Alanine	1293	14%	0.05%	33	13%	0.22%
L-Arginine	75	0.8%	0.06%	1	0.5%	0.02%
L-Aspartic Acid	297	3%	2%	21	8%	0.18%
L-Glutamic Acid	792	8%	2%	48	19%	13%
L-Histidine	11	0.1%	0.4%	1	0.5%	0.02%
L-Isoleucine	327	4%	4%	13	5%	0.17%
L-Leucine	517	6%	4%	15	6%	0.19%
L-Lysine	379	4%	1%	12	5%	0.15%
L-Methionine	191	2%	3%	13	5%	0.14%
L-Phenylalanine	309	3%	0.9%	8	3%	0.17%
L-Serine	543	6%	3%	11	4%	0.07%
L-Threonine	499	5%	2%	11	4%	0.10%
L-Tyrosine	171	2%	3%	5	2%	0.10%
L-Valine	578	6%	2%	22	9%	0.24%
Taurine	914	10%	2%	7	3%	0.03%
TOTAL	**9332**			**256.46**		

Table II.2 (cont). FAA composition for each of the three inks expressed in μM, as a percentage of the total FAA concentration, and as a percentage of the total dissolved organic carbon (DOC) in the inks.

	Aplysia			*Sepia*		
FAA	**Concentration (μM)**	**% of total FAAs**	**% of total DOC**	**Concentration (μM)**	**% of total FAAs**	**% of total DOC**
D-Alanine	2	0.06%	<0.01%	2	2%	0.2%
D-Aspartic Acid	4	0.2%	0.01%	0.1	0.09%	0.02%
D-Glutamic Acid	5	0.2%	0.01%	0.4	0.3%	0.06%
D-Serine	0	0%	0%	0.04	0.03%	>0.01%
Glycine	100	3%	0.1%	0.2	0.1%	0.01%
L-Alanine	50	2%	0.08%	20	14%	2%
L-Arginine	0	0%	0%	2	1%	0.3%
L-Aspartic Acid	65	2%	0.1%	3	2%	0.3%
L-Glutamic Acid	31	1%	0.08%	10	8%	2%
L-Histidine	34	1%	0.1%	0.8	1%	0.1%
L-Isoleucine	27	0.9%	0.09%	11	8%	2%
L-Leucine	15	0.5%	0.05%	12	9%	2%
L-Lysine	2	0.07%	0.01%	4	3%	0.7%
L-Methionine	57	2%	0.2%	2	2%	0.4%
L-Phenylalanine	16	0.5%	0.07%	0.1	0.07%	0.03%
L-Serine	152	5%	0.2%	1	0.7%	0.09%
L-Threonine	33	1%	0.07%	2	1%	0.2%
L-Tyrosine	7	0.2%	0.03%	0.4	0.3%	0.1%
L-Valine	46	2%	0.1%	16	12%	3%
Taurine	2305	78%	2%	51	37%	3%
TOTAL	**2951**			**138**		

Table II.3. Percentage of DOC explained by quantified FAAs for each of the three inks.

Ink	DOC (μmol L^{-1})	FAA % of DOC
Aplysia	21,12	4%
Sepia	3,167	16%
***Kogia* A**	110,163	34%
***Kogia* B**	45,100	3%

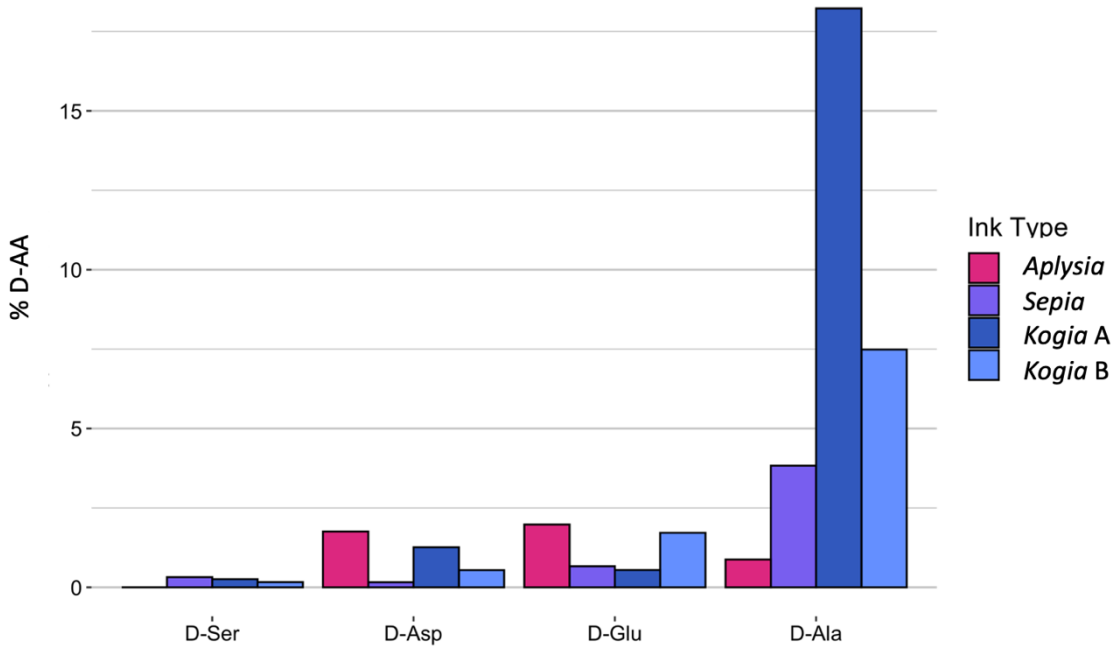


Figure II.3. Percentage made up by each D-form FAA of the chiral FAAs in all three inks.

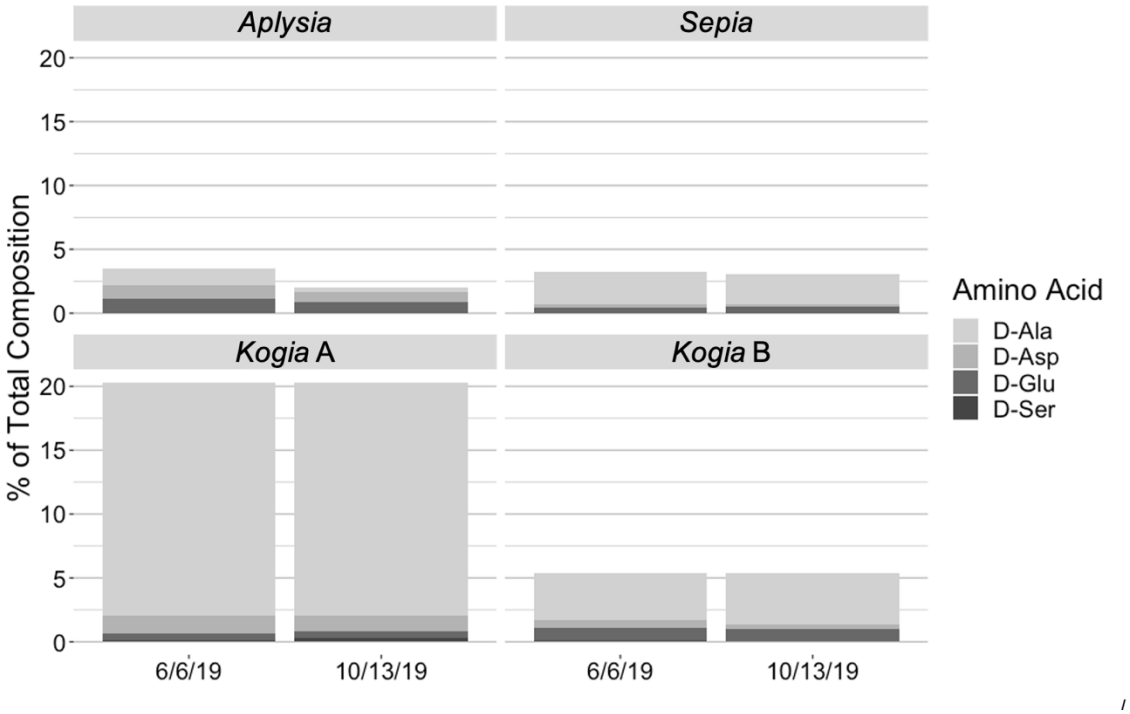


Figure II.4. D-Amino Acid Composition for each of the three inks expressed as a percentage of the total FAA concentration. Both samples of *Kogia* are included to show lack of change in D-AA, suggesting a lack of bacterial growth despite the difference in age of samples.

Table II.4. Comparison of FAAs quantified for *Aplysia californica* ink in my study and Derby et al., 2007

FAA	Mariculture Aplysia Ink (Derby et al., 2007)		*Aplysia* Ink (My study)	
	Concentration (μM)	Recalculated* % of total FAAs	Concentration (μM)	Recalculated* % of total FAAs
Glycine	62	3%	100	3%
L-Alanine	21	1%	50	2%
L-Arginine	23	1%	0	0%
L-Aspartic acid	62	3%	65	2%
L-Glutamic acid	27	1%	31	1%
L-Histidine	20	1%	34	1%
L-Isoleucine	1	0.05%	27	1%
L-Leucine	4	0.2%	15	1%
L-Lysine	1	0.05%	2	0.07%
L-Methionine	6	0.3%	57	2%
L-Phenylalanine	1	0.05%	16	1%
L-Serine	7	0.4%	152	5%
L-Threonine	3	0.2%	33	1%
L-Tyrosine	13	0.7%	7	0.2%
L-Valine	15	0.8%	46	2%
Taurine	1743	87%	2305	78%
Recalculated* Total	**2009**		**2940**	

*Recalculated based on the FAAs that both studies quantified. All D-FAAs were excluded from my totals and L-Asparagine, L-Cystine, L-Glutamine, L-Proline, and L-Tryptophan were omitted from Derby et al., 2007).

Table II.5. Comparison of FAAs quantified for *Sepia officinalis* ink in my study and Derby et al., 2007

	Sepia officinalis ink (Derby et al., 2007)		*Sepia officinalis* ink (My study)	
FAA	**Concentration (µM)**	**Recalculated* % of total FAAs**	**Concentration (µM)**	**Recalculated* % of total FAAs**
Glycine	6	0.2%	1	0.8%
L-Alanine	134	5%	<1	0.03%
L-Arginine	0	0%	1	0.6%
L-Aspartic acid	32	1%	2	2%
L-Glutamic acid	135	5%	<1	0.2%
L-Histidine	0	0%	2	1%
L-Isoleucine	10	0.4%	20	16%
L-Leucine	17	0.7%	2	2%
L-Lysine	2	0.08%	<1	0.3%
L-Methionine	0	0%	16	13%
L-Phenylalanine	0	0%	2	2%
L-Serine	20	1%	<1	0.1%
L-Threonine	17	1%	12	9%
L-Tyrosine	0	0%	12	10%
L-Valine	30	1%	4	3%
Taurine	2088	83%	52	41%
TOTAL	**2491**		**125**	

*Recalculated based on the FAAs that both studies quantified. All D-FAAs were excluded from my totals and L-Asparagine, L-Cystine, L-Glutamine, L-Proline, and L-Tryptophan were omitted from Derby et al., 2007).

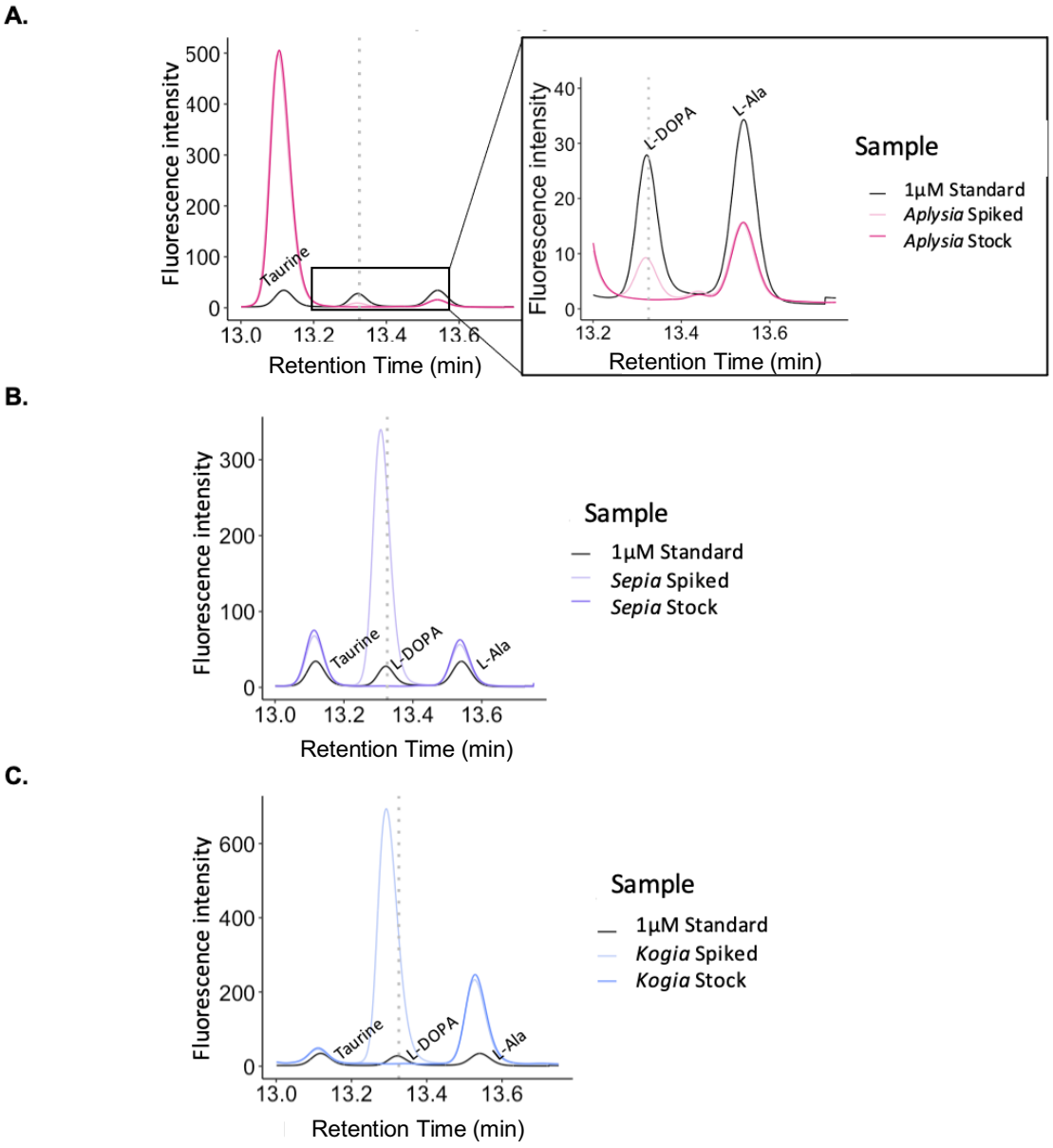
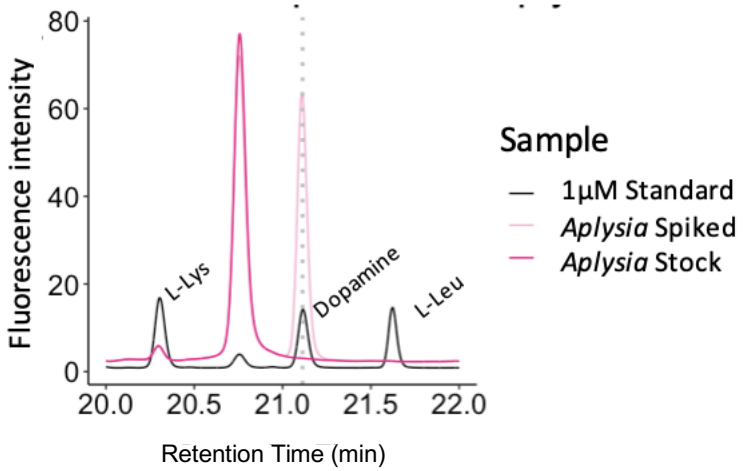
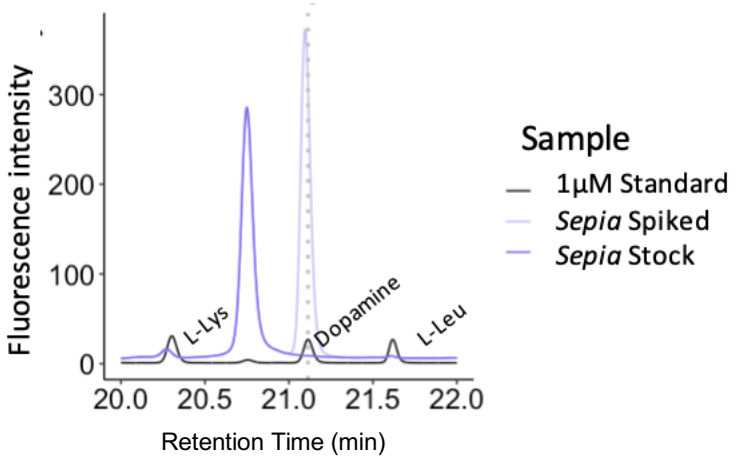


Figure II.5. Chromatographs for *Aplysia* ink (**A**), *Sepia* ink (**B**), and *Kogia* B ink (**C**) centered around L-DOPA's retention time (~13.33). A 1µM standard (black) is overlayed with a sample artificially spiked with L-DOPA (lighter color) and the stock, unspiked sample (darker color). In all three inks, only the spiked samples have fluorescence peaks matching the L-DOPA standard.

A.


Fluorescence intensity
80
60
40
20
0
L-Lys
Dopamine
L-Leu
20.0
20.5
21.0
21.5
22.0
Retention Time (min)
Sample
1µM Standard
Aplysia Spiked
Aplysia Stock

B.


Fluorescence intensity
300
200
100
0
L-Lys
Dopamine
L-Leu
20.0
20.5
21.0
21.5
22.0
Retention Time (min)
Sample
1µM Standard
Sepia Spiked
Sepia Stock

C.

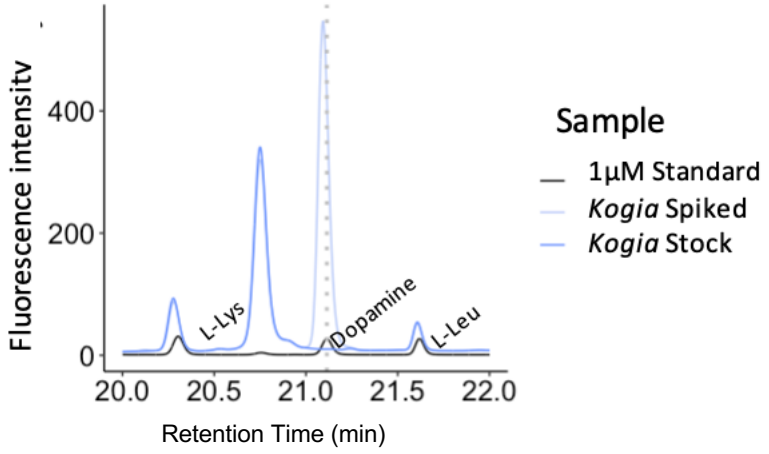

Fluorescence intensity
400
200
0
L-Lys
Dopamine
L-Leu
20.0
20.5
21.0
21.5
22.0
Retention Time (min)
Sample
1µM Standard
Kogia Spiked
Kogia Stock

Figure II.6. Chromatographs for *Aplysia* ink (**A**), *Sepia* ink (**B**), and *Kogia* B ink (**C**) centered around L-Dopa's retention time (~21.11). Either a 500 or 1μM standard (black) is overlayed with a sample artificially spiked with Dopamine (lighter color) and the stock, unspiked sample (darker color). In all of three inks, only the spiked samples have fluorescence peaks matching the Dopamine standard.

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CHAPTER III

MICROSTRUCTURE OF THE BONNETHEAD SHARK (*SPHYRNA TIBURO*) OLFACTORY ROSETTE

Introduction

Sharks have often been regarded as “swimming noses” with superior smelling ability by the poplar media. However, sharks have not lived up to the hype, exhibiting similar olfactory capabilities as teleosts (Meredith and Kajiura, 2010). While their olfactory sensitivities are similar, the morphology of their olfactory structures differ in olfactory bulb shape and location and olfactory sensory receptor type (Camilieri-Asch et al., 2020a; Caprio, 1988; Lisney and Collin, 2006; Reese and Brightman, 1970; Schluessel et al., 2008; Theisen et al., 1986; Zeiske et al., 1986; Zeiske et al., 1987; Zielinski and Hara, 2006). Olfactory morphologies also differ among sharks, including variations in olfactory bulb insertion and olfactory peduncle length, as well as surface area and structure of the nares and olfactory rosettes (Kajiura et al., 2005; Meredith and Kajiura, 2010; Schluessel et al., 2008; Smeets, 1998; Yopak et al., 2015). These differences have been related to neuroecology rather than differences in sensitivity or phylogeny (Meredith and Kajiura, 2010; Schluessel et al., 2008; Yopak et al., 2015)

Some sharks, such as bonnethead sharks (*Sphyrna tiburo*), have an incurrent and excurrent naris to allow for unidirectional water flow. In these sharks, water travels through the incurrent naris and into the incurrent canal of

the olfactory rosette. This canal extends across the rosette and its many lamellae (Figures 3.1-3.2). Within the incurrent canal, water passes over the lamellae and flows in between their secondary folds, which are covered by olfactory epithelium. Chemicals dissolved in the water bind to G-protein coupled olfactory receptors neurons (ORNs) on the surface of the olfactory epithelium (Eisthen, 2004; Smeets, 1998). Water then flows out through the excurrent canal, passing by the posterior ends of the lamellae, and out the excurrent nares (Abel et al., 2010; Rygg et al., 2013; Zeiske et al., 1987). Hammerhead sharks in particular possess prenarial grooves that direct the flow of the water into the incurrent naris (Kajiura et al., 2005; Rygg et al., 2013).

The olfactory epithelium is divided into nonsensory and sensory parts. While some shark species have a patchy distribution of epithelium, most have nonsensory epithelium at the margins of lamellae, while the sensory epithelium is centrally located. The nonsensory epithelium is made up of goblet cells and is covered by small microvilli. The sensory epithelium contains the receptor cells, ciliated supporting cells, basal cells and goblet cells (Meredith et al., 2013; Schluessel et al., 2008; Zeiske et al., 1987). The ciliated supporting cells are thought to move mucus and water across the olfactory epithelium. The former is more probable since Zeiske et al. (1987) reported no net water movement in the olfactory organ in stationary lemon sharks (*Negaprion brevirostris*).

Deep to the olfactory epithelium is an olfactory nerve layer, of the ORN axons originating in the epithelium (Figure 3.2a). The axons of ORNs diverge in

the olfactory bulb and then synapse at the glomeruli. Glomeruli are spherical structures, similar to ganglia of the peripheral nervous system in other vertebrates, and are distributed throughout the bulb in described species (Butler and Hodos, 2005; Meredith et al., 2013). Within the glomeruli, ORN axons synapse onto mitral cells (second- order olfactory neurons). The axons of these mitral cells form part of the olfactory peduncles that communicates sensory information from the olfactory bulb to the telencephalon (**Figure III.1**; Laberge and Hara, 2001; Yopak et al., 2015).

The unique shape of sphyrnids (hammerhead sharks) head has led to many competing and synergistic hypotheses about its function and potential sensory advantages (Kajiura and Holland, 2002; Kajiura et al., 2005; Meredith and Kajiura, 2010; Tricas et al., 2009). Its distinctive olfactory morphology in particular has led to hypotheses of enhanced olfactory performance. Recent investigations into the flow of water within the structure (Abel et al., 2010; Rygg et al., 2013) demonstrate that this complex structure exhibits a differential pressure system between the two nares that induces flow through the olfactory chamber. Additionally, hammerhead olfactory structures regulate flow internally via the gaps between the lamellae. This leads to differential fluid velocities in different parts of the olfactory chamber. Particularly, lamellae at the medial end of the olfactory chamber experience a near-stagnant recirculation of water (**Figure III.2**; Rygg et al., 2013).

The objective of this study was to describe the microstructure of the olfactory rosette of another sphyrnid, bonnethead sharks (*Sphyrna tiburo*). Additionally, given the variability of water flow within the sphyrnid olfactory rosette, I investigated differences of individual lamellae based on their positioning within the rosette. Specifically, I investigated both the amount of sensory area and number of secondary folds. Both of these metrics serve as a proxy for sensitivity, providing connections between sensitivity and water flow within the olfactory organ.

Methods

Sample Collection

Two recently deceased bonnethead sharks (*Sphyrna tiburo*) were donated by local fisherman caught from the waters surrounding Galveston, TX. Olfactory systems including brains, olfactory tracts and rosettes were immediately removed and placed in 10% buffered formaldehyde for preservation. These structures were kept intact and connected to preserve orientation.

Scanning Electron Micrography (SEM)

Rosettes were cut longitudinally, separating the right and left lamellae. Then, every third lamella was excised placed. Lamellae were dehydrated using an ethanol dehydration series (30%, 50%, 70%, 70%, 80%, 80%, 95%, 95%,

100%) for 10 minutes per bath. Lamellae were then further dehydrated with Hexamethyldisilazane (HDMS), mounted on carbon stubs, and sputter coated with gold/palladium before they were imaged in a Hitachi TM3000 scanning electron microscope.

Light Micrography (LM)

Rosettes were dissected into 10 transverse sections, each containing both sides of the lamellae and the olfactory bulb. Rosette sections were processed for paraffin histology using a Leica tissue processor (Leica Biosystems Inc., Buffalo Grove, IL, USA) under vacuum. Samples were processed through a dehydration series of alcohol, followed by infiltration of xylene and paraffin. Samples were then embedded as cross-sections in paraffin block and sectioned at 7 μm on a rotary microtome. Sections were mounted onto 1% gel subbed slides and stained with Toluidine Blue. Digital micrographs were collected using a Nikon E-400 (Nikon Instruments Inc., Melville, NY, USA) Eclipse light microscope fitted with a Spot Insight (Diagnostic Images, Sterling Heights, MI, USA) digital microscopy camera.

Image Analysis

Three lamellar morphological metrics were collected along the medial-to-lateral gradient. Each lamella was assigned a position, with 1 being the most lateral and 24 being the most medial. These metrics were measured for both

SEM and LM samples via ImageJ 1.52q (Schneider et al., 2012). The number of secondary folds and the percentage of the total surface area covered by sensory epithelium was calculated for each lamella. During SEM processing, the lamellae were dehydrated and shrank considerably. To account for this and to be able to compare across methodologies, I measured the surface area of each lamella as a percentage of the lamella with the largest surface area. Surface area measurements did not account for secondary folding. The nonsensory area was defined by the presence of either nonsenory epithelium or the olfactory nerve layer. For LMs, the most superficial sections were used, which showed both the nonsensory epithelium and the starting position of the olfactory nerve layer (**Figure III.3**). The sensory area was defined by the presence of sensory epithelium.

Results

Microstructure of the olfactory rosette

The bonnethead olfactory rosette is made up of paired lamellae joined by a central raphe which attaches to the olfactory bulb (**Figure III.2 A**). Water flows through the incurrent channel, passing between the two lamellae then back out the excurrent channel, passing the bottom of the lamellae (**Figure III.2**). The nonsensory epithelium is concentrated around the inner margins of the lamellae. Deep to the nonsensory epithelium is the olfactory nerve layer (**Figure III.4 and**

III.5 A,D). The nonsensory epithelium is covered in microvilli and contains both goblet and mucus cells (**Figure III.5 B-C**). The sensory epithelium is covered in ciliated support cells and has secondary folds that increase the surface area. I did not observe olfactory knobs, the dendritic swellings of olfactory receptor cells, reported in other shark species (Camilieri-Asch et al., 2020b; Schluessel et al., 2008; Theiss et al., 2009). However, I did observe putative olfactory knobs *sensu* Schluessel et al. (2008) in areas of sensory epithelium with lower cilia density (**Figure III.5 E and F**).

Pathway of the olfactory nerve layer

I mapped the path of the olfactory (**Figure III.6**). Superficially, the olfactory nerve runs from the bulb, along the inner margins of each lamella and branches out into the middle of the secondary folds (**Figure III.4 and III.5 D**). Deeper into the lamellae, the olfactory nerve layer courses centrally and expands anteriorly. It continues to expand toward the outer lamellar margins until it forms two loops at the anterior and posterior ends of the lamellae. This pattern was observed in all sectioned lamellae, regardless of their position within the rosette.

Morphological difference along the medial-to-lateral gradient

Morphologically, I did observe variation in lamellae based on their positioning within the rosette (**Figure III.7**). The percentage of the sensory area

remained relatively consistent throughout the majority of the rosette, and ranged between 96-86% of the total area. However, the medial lamellae show a decrease in percentage, reaching a low of 60% at the most medial lamella. The trends in number of secondary folds varied by methodology. For LM samples, fewer folds were counted on the lamellae on the medial and lateral ends of the rosette and more folds were counted within the central lamellae. SEM samples showed less variation between the lateral and central lamellae, but a decrease in the medial lamellae was observed. Finally, lamellae within the center of the rosette had higher surface areas than lamellae at the lateral or medial ends.

Discussion

Bonnethead sharks exhibit similar olfactory morphology to other sharks. Lamellae are covered with nonsensory epithelium on the inner margins and sensory epithelium centrally and to the outer margins. I found sensory epithelium in both the “troughs” and “peaks” of the secondary folds, similar to spiny dogfish (*Squalus acanthias*) and small-spotted catsharks (*Scyliorhinus canicular*; Theisen et al., 1986). The only other published SEM of sphrynid shark (scalloped hammerheads, *Sphyrna lewini*) reported projections of the nonsensory epithelium into the sensory epithelium, which was not observed in this study (Schluessel et al., 2008). Secondary folds within the lamellae are covered in dense ciliated supporting cells. Unlike teleosts, elasmobranchs do not possess ciliated receptor cells. Instead, they have microvillus and crypt ORNs

(Camilieri-Asch et al., 2020b; Ferrando et al., 2006; Hansen and Zielinski, 2005; Northcutt, 1978; Schluessel et al., 2008; Takami et al., 1994; Theisen et al., 1986; Theiss et al., 2009; Zeiske et al., 1987). Some sharks have olfactory knobs, which are the dendrites of microvillus ORNs that reach the epithelial surface. I did not observe these in bonnethead lamellae. The supporting ciliated cells were dense, possibly obscuring these structures from view. Previous SEMs of scalloped hammerheads also did not report olfactory knobs (Schluessel et al., 2008). However, I did find similar structures within areas of sparser cilia coverage. It is possible that this could be a variant morphology of olfactory knobs, but the function of these structures remains unknown.

The relationship between the ORN axons and the olfactory bulb has been described for a handful of species of elasmobranchs. In most vertebrates, ORN axons project to differential locations within the olfactory bulb according to their odorant class (Friedrich and Korsching, 1998; Hamdani and Døving, 2007; Nikonov and Caprio, 2001; Xu et al., 2000). Some elasmobranchs exhibit a chemotopic arrangement within their olfactory bulb, similar to teleosts (Døving et al., 2011; Hansen and Zielinski, 2005; Hansen et al., 2003; Hansen et al., 2004; Nikonov and Caprio, 2001; Sato and Suzuki, 2001). In spotted catsharks (*Scyliorhinus canicula)*, for example, crypt ORNs correspond to ventral glomeruli while microvillus ORNs project in numerous axon bundles within the remainder of the glomeruli (Ferrando et al., 2009). However, other elasmobranchs, such as bonnetheads, have somatotopically arranged olfactory bulbs, with each ORN

projecting to the olfactory bulb glomeruli immediately anterior to it (Dryer and Graziadei, 1993; Meredith et al., 2013). Whether this somatotopic arrangement continues through the peduncle and into the telencephalon remains to be investigated. To my knowledge, this is the first time the olfactory nerve layer, which contains the axons projecting from the ORNs to the bulb, has been tracked through a shark lamella.

I report differences in sensory area, degree of folding, and size of lamellae along the medial-to-lateral gradient within the rosette of bonnethead sharks. Medial lamellae had less sensory surface area and fewer secondary folds than centrally or laterally located lamellae. Rygg et al. (2013) described the medial portion of the hammerhead olfactory organ as a near-stagnant, recirculating area. The results suggest that lamellae in this region may have less olfactory sensitivity, possibly correlated with lack of flow at this part of the organ. While Rygg et al (2013)'s model was based on the smalleye hammerhead (*Sphyrna tudes*) olfactory system, bonnethead sharks have a similar olfactory morphology. A computational fluid dynamics model should be produced to see if these patterns hold up within the bonnethead olfactory organ. While these two metrics can be seen as proxies for olfactory sensitivity, physiological testing of olfactory sensitivity along this medial to lateral gradient need to be conducted to confirm my hypotheses.

Additionally, I observed differences in lamellar total surface area with the larger lamellae occurring in the center of the olfactory organ. These lamellae

may be more robust to withstand the higher flow velocities experienced in most of the rosette. Lamellae in the medial region are smaller but likely experience lower velocities. Lamellae are also smaller in the lateral portion of the organ than in the center, however, the flow around these lamellae has not been modeled in any species. The histologically processed lamellae also had fewer secondary folds in these lateral lamellae. The flow within the organ at this location should also be modeled to inform the relationship between lamellae morphometrics and flow.

Despite differences in the number of secondary folds based on positioning, I observed secondary folds through the entirety of the lamellae regardless of positioning in the rosette. This is a pattern shared by most elasmobranchs. However, lemon sharks (*Negapiron brevirostris*) lack secondary folding on the dorsal base of centrally located lamellae (Meredith and Kajiura, 2010). In contrast, clearnose skates (*Raja eglanteria*) and brown-banded bamboo sharks (*Chiloscyllium punctatum*) lose secondary folding in their ventral free margin (Schluessel et al., 2008; Takami et al., 1994).

Differences along the medial-to-lateral gradient within the olfactory rosette should be taken into account for future studies of olfactory morphology, especially for sharks with elongated olfactory organs like sphrynids. Sharks with differently shaped olfactory organs may produce different flow patterns and exhibit different configurations of lamellar morphologies. A combination of

morphology, physiology, and fluid dynamics will lead to a better understanding of how the shark olfactory organ samples chemical stimuli in its environment.

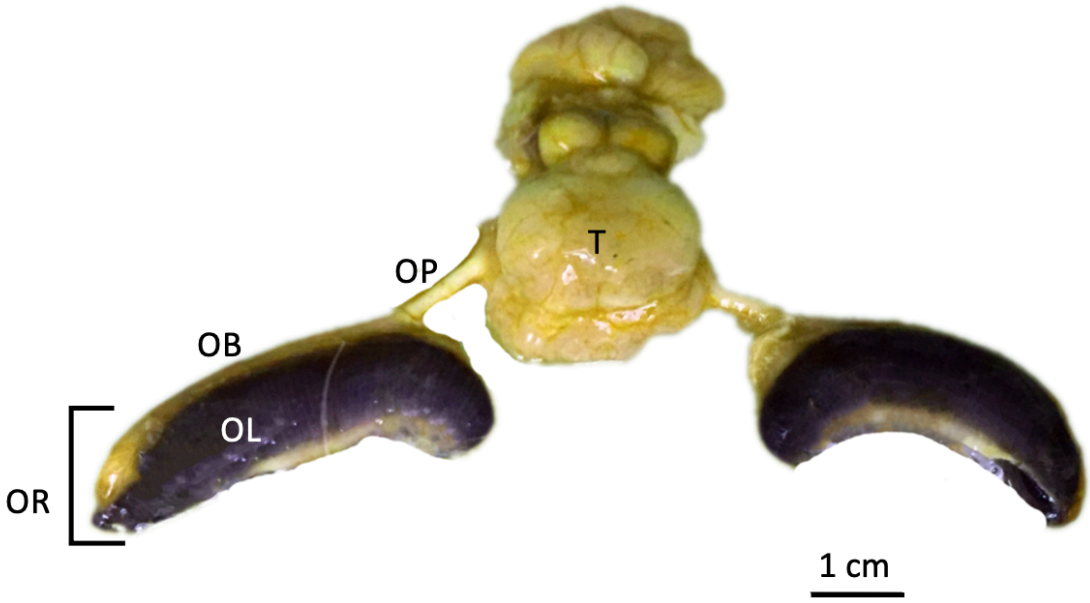

Figure III.1. Gross anatomy of the bonnethead shark (*Sphyrna tiburo*) olfactory system. OB- olfactory bulb, OL- olfactory lamellae, OP- olfactory peduncle, OR- olfactory rosette, T- telencephalon

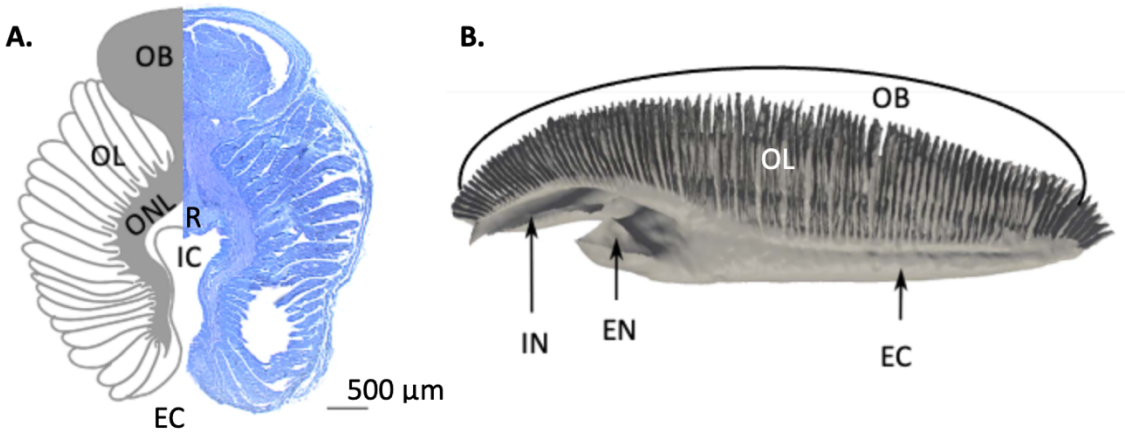

Figure III.2. Morphology of two sphyrnid olfactory organs. A cross section through the olfactory rosette of a bonnethead shark (**A**) and the 3D anatomy of the olfactory rosette from a smalleye hammerhead shark (**B**; Rygg et al., 2013). EC- excurrent canal, EN- excurrent naris, IC- incurrent canal, IN- incurrent naris, OB- olfactory bulb, OL- olfactory lamellae, ONL- olfactory nerve layer, R- raphe. **A** stained with toluidine blue.

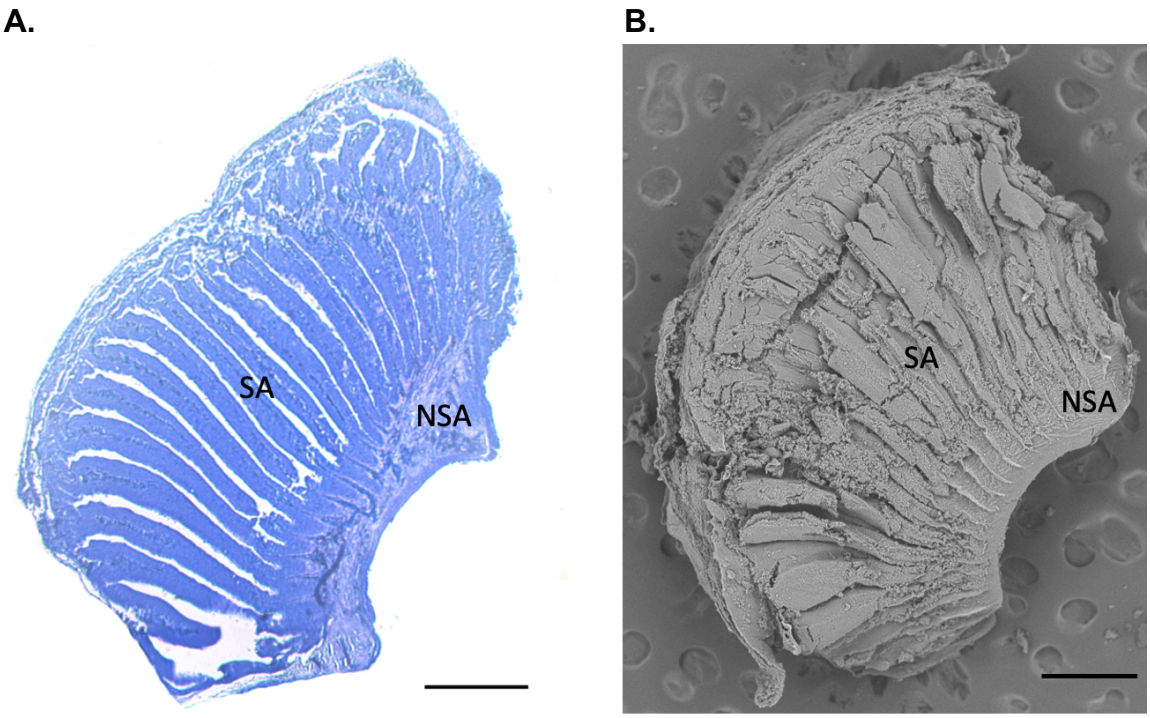

Figure III.3. Definition of sensory area (SA) vs nonsensory area (NSA). Light micrographs of lamellae stained with toluidine blue (**A**) and scanning electron micrographs of lamellae (**B**). Scale bars are 500 μm.

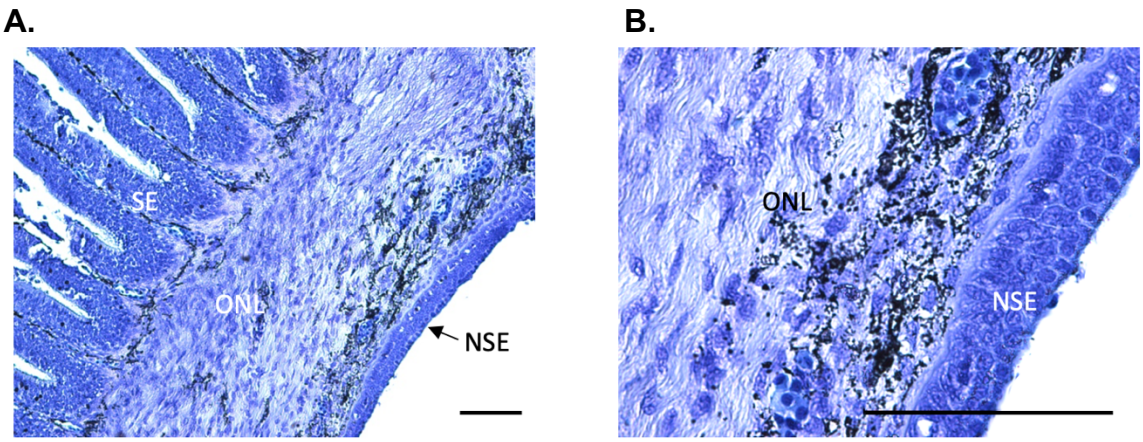

Figure III.4. Light micrographs of the sensory epithelium, olfactory nerve layer, and nonsensory epithelium at 10x (**A**) and 40x (**B**). SE- sensory epithelium, NSE- nonsensory epithelium, ONL- olfactory nerve layer. Scale bars 100 μm.

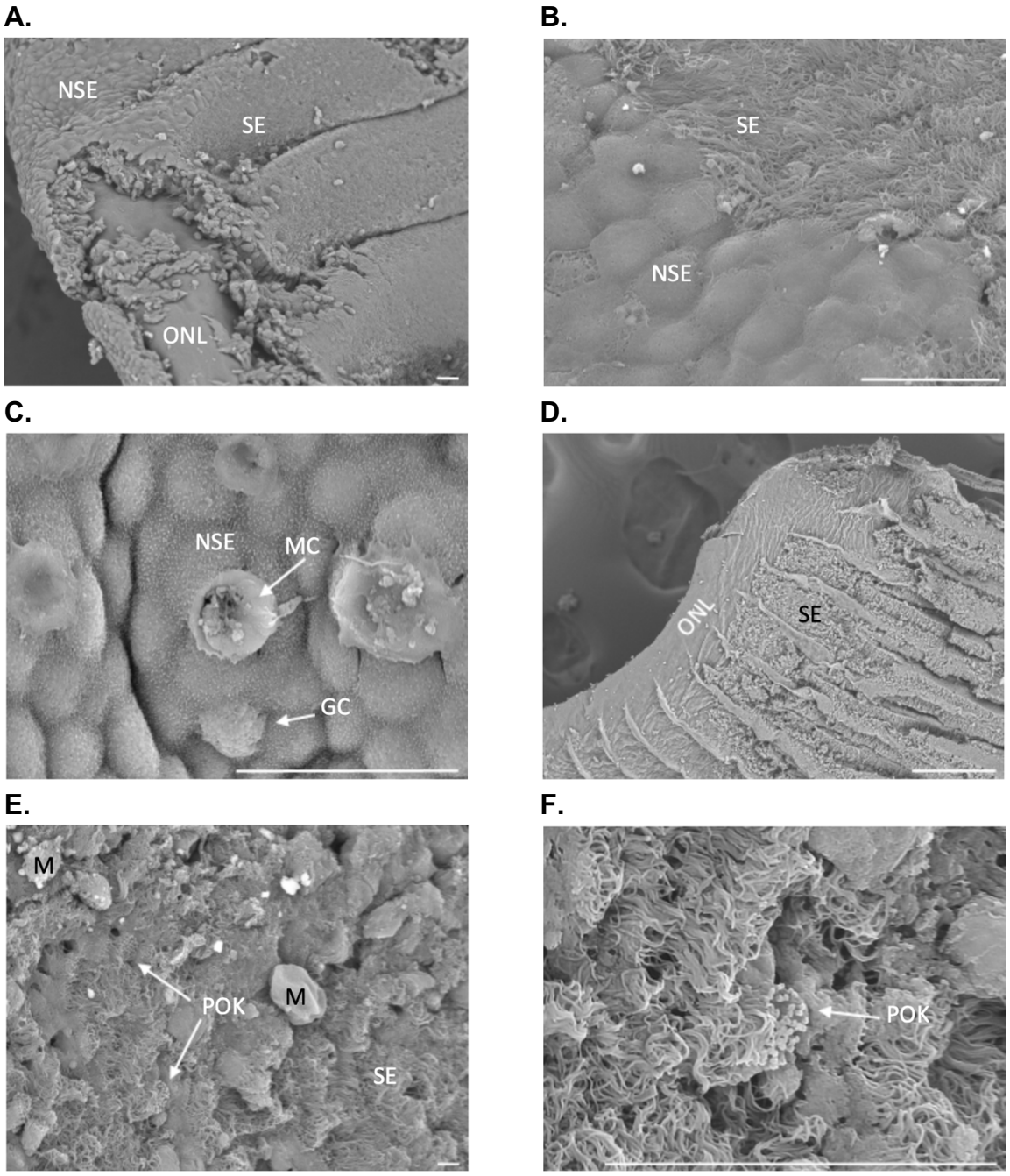

Figure III.5. Scanning electron micrographs of the olfactory lamellae. **A**. The nonsensory vs sensory epithelium with secondary folding. Part of the nonsensory epithelium is removed, showing the olfactory nerve layer

underneath. **B**. A closer look at the nonsensory epithelium with microvilli and the sensory epithelium with cilia. **C.** Nonsensory epithelium covered in microvilli with mucus cells and goblet cells. **D.** The nonsensory epithelium has been removed, leaving the olfactory nerve layer. **E.** Sensory epithelium with putative olfactory knots. **F.** Close up of a putative olfactory knot. GC- goblet cell, NSE- nonsensory epithelium, M- mucus, MC- mucus cell, ONL- olfactory nerve layer, POK- putative olfactory knot, SE- sensory epithelium Scale bars = 20 μm.

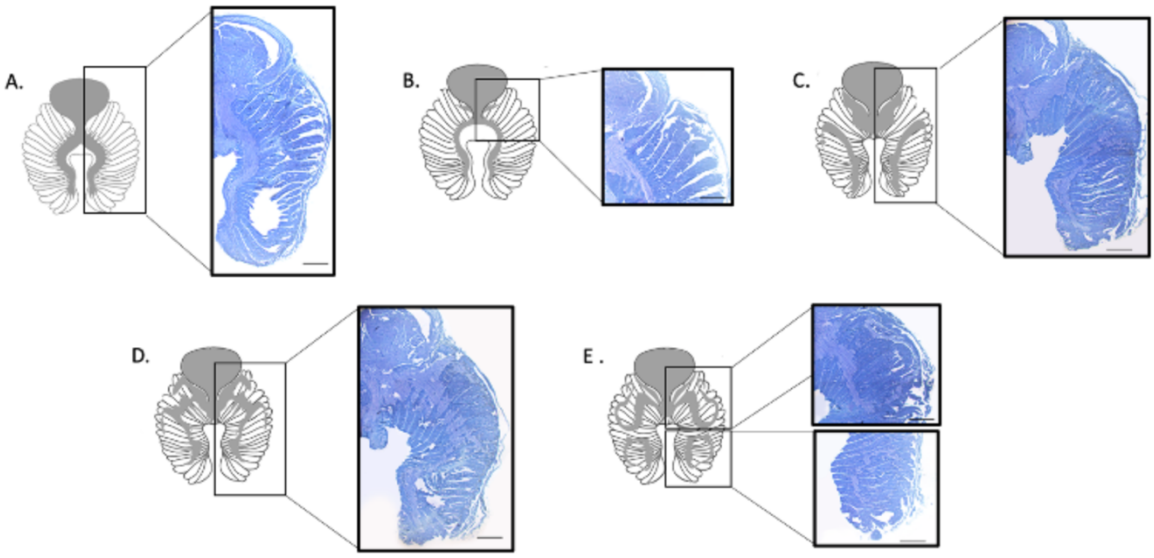

Figure III.6. Sections from superficial (**A**) to deep (**E**) showing the olfactory nerve layer innervating the lamellae. Scale bars = 500 μm.

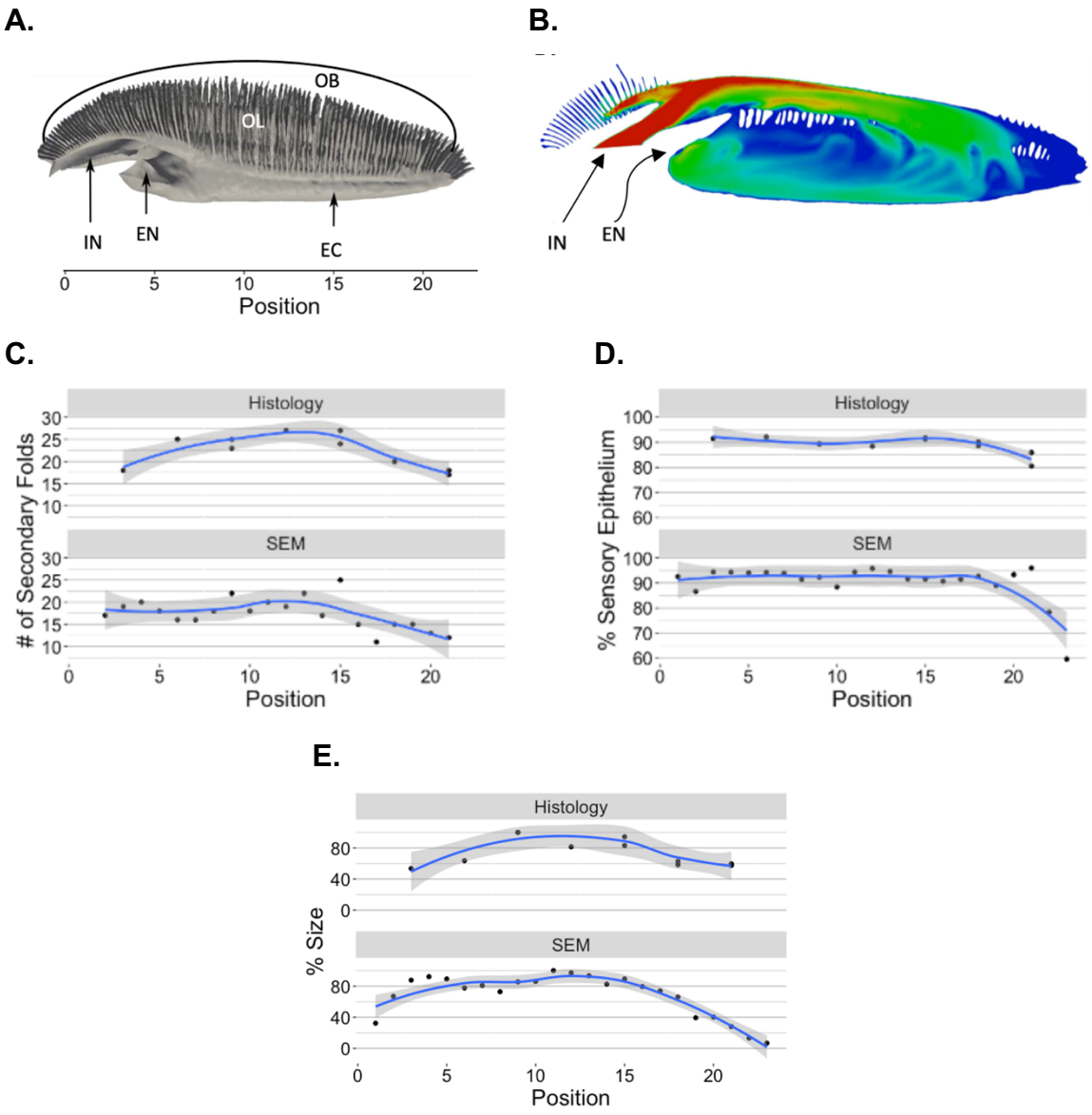

Figure III.7. Lamellar morphological patterns throughout the rosette. 3D anatomical model (**A**) and calculated velocity fields (fine mesh; **B**) of smalleye hammerhead olfactory rosettes from Rygg et al., 2013. Lamellar position is defined along the medial to lateral gradient from 0 (most lateral) to 23 (most medial). Trends in calculated morphological metrics, such as amount of secondary folds (**C**), percentage of total surface area covered by sensory

epithelium (**D**), and total surface area standardized as a percentage of the largest lamella (**E**), are visualized with a less smooth curve. All surface areas did not account for secondary folding. IN- incurrent naris, EN- excurrent naris

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CHAPTER IV

A NATURAL OCCURING SHARK REPELLENT: INK HAS A NEGATIVE EFFECT ON SHARK SWIMMING BEHAVIOR

Introduction

Inking is an impressive predator defense used by a limited number of metazoan groups such as sea hares, cephalopods, crestfish and an unusual family of toothed whales, kogiids. The functional uses of ink (phagomimicry vs. deterrent vs. alarm cues) and the method of defense (chemical vs. visual vs. social) are areas of research that are actively being investigated and researched. When ink is used as a predatory defense, it is hypothesized to affect predators chemically, acting as a deterrent (Aggio and Derby, 2008; Kamio et al., 2010; Kicklighter et al., 2005; Love-Chezem et al., 2013) and visually, acting as a smoke screen (Bush and Robison, 2007; Caldwell, 2005). However, the physiological mechanisms of the use of ink as a deterrent are still poorly understood. There is strong evidence for multiple functional pathways of ink: ink affects predators' visual and chemical sensory systems as well as alerting conspecifics of predation threat.

Previous behavioral studies that address the function of ink have only explored the effect of one type of ink and have largely focused on invertebrate predators. For example, the majority of sea hare (*Aplysia sp.*) ink behavioral work has been tested on spiny lobsters (Aggio and Derby, 2008; Kicklighter et

al., 2005; Love-Chezem et al., 2013; Shabani et al., 2007), sea anemones (Kicklighter and Derby, 2006; Wolfe et al., 2016) and blue crabs (Kamio et al., 2010; Wolfe et al., 2016). As far as vertebrate predators, there is one anecdotal paper on the effect of octopus ink on green sea turtle hatchlings (Caldwell, 2005). There have only been a handful of fish- focused studies that investigate effect of sea hare ink on four wrasse species and catfish (Nusnbaum and Derby, 2010a; Nusnbaum and Derby, 2010b; Nusnbaum et al., 2012; Sheybani et al., 2009) and squid ink on grunts, flounder and catfish (Derby et al., 2013; Wood et al., 2010). Nusnbaum and Derby (2010b) also included bonnethead sharks, however these behavioral assays used food pellets soaked in ink, which only investigated the gustatory effect of ink.

Current ink behavioral studies suffer from two important data gaps: their focus on invertebrate based ink and their single species approach. There are no published behavioral assays using ink from vertebrates, such as kogiids. Likewise, there is an absence of comparative studies using ink from more than one animal source. This study uses three types of ink to investigate in effect as a deterent on a vertebrate, bonnethead sharks (*Sphyrna tiburo*). Ink was used from two commercially available species, California sea hares (*Aplysia californica*), common cuttlefish (*Sepia officinalis*). In addition, my lab had access to an usual ink, that of pygmy sperm whales (*Kogia breviceps*). This is the first behavioral assay using ink from kogiids or any inking vertebrate.

Bonnethead sharks are an ideal animal model system to test the effects of ink on a predator. They are a small member of the hammerhead family, are abundant in local waters, are well suited for captivity, and have broad cephalofoils, which provide easy access to their olfactory systems. Their broadly spaced nares give them a larger separation between olfactory rosettes, allowing for an enhanced ability to resolve odor gradients. Although they have a greater head width and more olfactory lamellae than carcharhinids, there is no significant difference in olfactory epithelium surface between them, suggesting that sphyrnids do not possess any advantage in olfactory acuity to carcharhinids (Kajiura et al., 2005; Meredith and Kajiura, 2010). Bonnetheads are also labeled as “Least Concern” by the IUCN allowing us to use a non-threatened shark species in my experiments. Bonnetheads also prey on squid, making them likely targets for ink defenses. However, because the olfactory sensitivity is conserved throughout shark species, I can infer behavioral results from bonnethead sharks to the other sharks, especially those that prey upon sea hares and possibly pygmy sperm whales.

The goal of this this study was to test the effect of ink from three disparate taxa on freely swimming bonnethead sharks. I hypothesized that ink would be a deterrent effect, negatively impacting the normal swimming behavior of bonnethead sharks.

Methods

Ink and Animal Collection

Ink was collected and pooled from 13 *Aplysia californica* purchased from the National Resource for Aplysia at the University of Miami (Key Biscayne, Florida, USA). *Aplysia* were placed in a 1L beaker and agitated via mechanical stimulation until there was a visual secretion of ink. They were removed from the beaker upon cessation of inking. Cuttlefish ink was acquired from Superior Fish Company (Royal Oak, MI, USA). This ink was compared to *Sepia officinalis* and *Sepia bandensis* ink sacs (provided by Christine Bedore, Georgia Southern University, Statesboro, Georgia, USA) via chemical analyses and confirmed to be *Sepia officinalis. Kogia breviceps* ink was extracted from gastrointestinal (GI) tracts from a euthanized, stranded male whale (Georgia Department of Natural Resources and the Georgia Marine Mammal Stranding Network, Georgia, USA) and frozen immediately after necropsy. Kogia GI tract samples were filtered through a 2 μm filter to separate out the ink from the fecal matter.

Each ink was stored frozen (-20°C) until used for analysis. To standardize the ink, I controlled for concentration of dissolved organic carbon (DOC). Because *Aplysia*, *Sepia*, and *Kogia* all release ink of different color, volume, and densities, standardizing by DOC allowed us to use a standard volume for each of these inks and compare across these diverse species. Ink was standardized to have a DOC concentration of 2 mmol L^{-1}, a concentration closest to the ink

with the lowest DOC. *Aplysia* was diluted down from 3.8 mmol L^{-1}, *Sepia* from 2.1 mmol L^{-1}, and *Kogia* from 110.2 mmol L^{-1}.

Bonnethead sharks were captured for this study using hook and line in the waters surrounding Galveston, TX, USA. Sharks were transported and held in the Aquarium at Moody Gardens (Galveston, TX, USA) quarantine facility and remained in their care throughout and after the study. At the cessation of these experiments, these sharks were put on display at the Aquarium at Moody Gardens.

Experimental Design

I recorded the responses of six free swimming bonnethead sharks to the three inks. Sharks were marked with AgCl for identification purposes following the Aquarium at Moody Gardens' protocol. 5 mL of three types of ink from *Aplysia, Sepia*, and *Kogia.* were introduced into the path of free swimming bonnethead sharks. In addition to the inks, sharks were exposed to 5 mL of two controls: food coloring (to control for color) and seawater (to control for mechanosensory stimulation). Sharks were also exposed to food odor to both ensure that they were still responding to chemical cues and to demonstrate an attraction response. During preliminary dye tests, a 5 mL injection created a cloud equal to or larger than the length, width, and height of the shark's cephalofoil. Sharks were individually taken from their holding tank, marked with waterproof cattle-marker, put into the experimental tank (30-meter diameter

circular mesocosm), and left to acclimate for a minimum of 3 hours. Each trial consisted of the injection of the stimuli into the water, the shark coming in contact with the stimuli, and a minimum of 15-minute period of recovery. Sharks were kept in the experimental tank for a maximum of 2 weeks.

The introduction of ink into the experimental tank was tightly controlled. There were two injection sites in the tank (**Figure IV.1**). As logistically possible, the order of injections was as follows: Control, Food Odor, Control, Ink. The controls and inks were randomized each trial. The injections were introduced when the shark was one body length away from the ink injection site. If sharks started exhibiting unnatural behavior, the trial was stopped and all trials since the last appropriate response were discarded. Unnatural behavior was defined as avoidance to food odor or controls. Furthermore, trials were only included if the shark was correctly positioned at the start of the injection (one body length away, facing the injection site). I collected 300 acceptable trials total (5 trials per 6 treatments per 6 sharks; 5 x 6 x 6 = 180 trials total).

Kinematic Variables

Kinematic responses were recorded and analyzed (ProAnalyst, XCitex, Inc., Cambridge, MA, USA) to test the hypothesis that ink changes normal swimming behavior. A 30x30 cm calibration square was lowered into the water before each set of trials. I collected two kinematic variables: Maxium Angle of Deviation and Body Angle. Due to the statistically non-normal nature of the data,

differences among stimuli was calculated via a Kruskal-Wallis test with Dunn posthoc testing and p-values adjusted using the Benjamini-Hochberg method.

The Maximum Angle of Deviation (MAD) was measured by plotting a midsagittal line from the last gill slit through the cephalofoil in the frame before the stimulus was injected into the water. The maximum angle the shark deviated from that line was defined as the MAD. A deviation towards the injection site was recorded as a positive response while a deviation away was recorded as a negative response. I also calculated secondary kinematic variables that informed the intensity of the behavioral response. The distance between the cephalofoil and the point of injection was defined as the "Distance from End of Deviation" (**Figure IV.2**). The time it took to get to the max deviation was defined as "Time to Max Deviation".

When measuring the Body Angle (BA), kinematic analysis began in the frame prior to the introduction of the stimuli when the body angle was closest to 180°. The measured Body Angle was the angle formed between the cephalofoil, the anterior dorsal fin cranial insertion and the tail base measured as though the shark would turn away from the injection site. If the angle was less than 180º, the shark turned away from the injection site. Alternatively, if the the shark turned toward the injection site, the angle was > 180º. Kinematic analysis ended when the body returned closest to 180° (**Figure IV.3**). Maximum Angular Velocity, Average Speed, and Maximum Speed was also calculated for this window of frames.

To look at the long term effects of ink, I observed the shark's swimming behavior following the stimuli injection. Using the kinematic software, I defined the Injection Radius as a circle around the injection site with a radius equal to half the distance between the two injection sites (Figur 4.4). For a subset of sharks (N=3), the time spent within the Injection Radius following stimuli introduction was quantified and compared among the inks and controls. Sharks were said to be within the "Injection Radius" when they entered that circle. The amount of time the shark spent within the Injection Radius every minute for ten minutes was quantified and analyzed.

Results

Maximum Angle of Deviation (MAD)

The MAD was significantly different between the inks and controls (Fig. 4.5; Dunn Test; P= <0.05). The inks elicited MADs with means ranging from -95.55º to -117.21º while food coloring and seawater only elicited MADs with means of 0.86º (± 20.19º SD) and 7.60º (± 24.23º SD) respectively. Food odor caused sharks to deviate towards the injection site, with an average MAD of 161.65º (± 159.81 SD). Sharks took significantly longer to reach their maximum deviation and were significantly farther away from the injection site at their MAD when exposed to ink compared to the controls or food odor. These data speak to the degree of the aversion response elicited by the inks.

Body Angles

The BA, was significantly more acute for all three inks than the controls and food odor (Fig 4.6; Dunn Test; P = <0.05). When exposed to ink, sharks turned away from the injection site forming mean BA that ranged between 112.36-123.67º (± 25.02- 25.13º SD). However, exposure to the controls elicited an average BA of 181.83º (± 31.92º SD for food coloring and 188.40º (± 32.78º SD) for seawater. Sharks exposed to food odor had an average BA of 227.38º (± 21.81º SD a significantly larger angle than the responses to seawater.

The Maximum Angular Velocities showed significant differences among the controls and two of the three inks. *Sepia* and *Kogia* eliciting significantly higher angular velocities than both controls (Fig 4.6; Dunn Test; P = <0.05). *Aplysia*, however, provoked a significantly lower average velocity than *Sepia* and was not significantly different than food coloring. Additionally, both Average and Maximum Speed were significantly higher than the controls for all of the inks and food odor (Fig 4.7; Dunn Test; P = <0.05).

Injection Radius

There was no significant difference in time spent within the Injection Radius in the ten minutes following introduction of the stimuli. Additionally, there was not significant trends over time either on the whole or at the stimuli level (Fig 4.8; Kruskal-Wallis; P > 0.05). This suggests that ink, at the concentration and volume that I used, does not have a long term effect. However, the size of

the tank could also be a limiting factor, not allowing the shark the space to avoid the injection site area.

Discussion

Like other behavioral assays, the experiments demonstrated the deterrent effects of ink (Aggio and Derby, 2008; Caldwell, 2005; Derby et al., 2013; Kicklighter and Derby, 2006; Kicklighter et al., 2005; Love-Chezem et al., 2013; Nusnbaum and Derby, 2010a; Nusnbaum and Derby, 2010b; Sheybani et al., 2009; Wolfe et al., 2016; Wood et al., 2010)**.** In this study, both MAD and BA measures demonstrated that bonnethead sharks having an aversive reaction to ink from *Aplysia*, *Sepia*, and *Kogia*. Food odor elicited the opposite response, causing sharks to circle back towards the injection site and even occasionally "mouth" and bite at it. Kinematic variables following exposure to seawater or food coloring did not evoke a change from normal swimming behavior. The lack of response to food coloring suggests that color alone is not a strong enough deterrent to negatively impact swimming behavior.

While there were usually no significant differences among inks, *Sepia* consistently had the highest average for each kinematic variable (**Figure IV.5 – IV.7**). After coming in contact with a *Sepia* ink cloud, sharks responded with larger deviations from their original swimming paths (MAD = -117.21º ± 64.21º SD) and swam farther away from the injection site (End of Deviation = 68 cm ± 29.11 SD). Sharks also responded with more acute body angles (BA = 112.36º ±

25.13º SD), with the greatest angular velocity (Maximum Angular Velocity = 272.53 s^{-1} ± 82.80 SD), and had fastest average and maximum speeds (Average Speed= 45.25 cm/s ± 13.60 SD.; Maximum Speed = 116.45 cm/s ± 37.05 SD.) after exposure to a *Sepia* ink cloud.

Ink has been suggested to work via phagomimicry, which involves secretions that mimic the chemical composition of a food source. When predators come in contact with a phagomimetic substance, they engage in feeding behaviors, attacking the substance rather than the prey (Aggio and Derby, 2008; Kicklighter et al., 2005; Shabani et al., 2007). The data does not support this route of chemical defense against sharks. The responses to all three inks were overwhelmingly negative, not mimicking the attractive behavior I saw after exposure to food odor. However, the lack of decoy-like effects of ink observed in my study could be due to the method of ink introduction. In squid, ink is mixed with varying amounts of mucus to create different types of secretions. Squid will sometimes change to a darker color before discharging a pseudomoroph, a gelatinous, mucus-heavy mass of similar size and color to the squid. Then, the squid will change to a lighter color and jet away, leaving the predator to bite the pseudomorph, getting a mouthful of of aversive chemicals. This decoy behavior is thought to rely on visual signals rather than chemical, as seen in pahgomimetic defenses. Wood et al (2010) found that pseudomorphs made of ink elicited significantly higher biting responses from predatory fish than their control of carboxymethylcellulose pseudomorphs. However, they found that

fish bit ink and colored carboxymethylcellulose at similar rates, suggesting pseudomorphs entice misdirected predatory behavior through visual cues rather than phagomimetic chemical cues.

Like squid, *Aplysia* ink is also released with a viscous secretion - opaline. When opaline comes in contact with water, it polymerized and becomes highly viscous. This sticky substance contains milimolar concentrations of free amino acids and can cover and adhere to the appendages and chemosensory structures of predators. When spiny lobsters, a common model for testing the effects of *Aplysia* defenses, were exposed to opaline they spend significant amounts of time cleaning their mouthparts and appendages. Opaline also reduces chemosensory abilities of spiny lobsters by physically blocking the receptors and impacting chemically-driven motor behaviors (Love-Chezem et al., 2013). Because I only investigated the effects of a non-viscous ink cloud, the deterrent effect elicited in sharks by *Aplysia* ink may be amplified when combined with opaline.

In addition to deterrence, exposure of ink to predators may elicit other unusual behaviors. For example, excessive grooming has been noted when exposing predators, such as spiny lobsters, to ink (Kicklighter et al., 2005). Anecdotally, I did see some parallels in bonnethead shark behavior in this study. While sharks have a lessened capability to clean their chemosensory structures than other predators, I did record four occurrences of sharks rapidly increasing speed and darting around the tank after exposure to *Sepia* ink. Bonnethead

sharks do not manually move water across their chemosensory structures. Water flows passively into their incurrent nares, across sensory olfactory epithelium, and out the excurrent nares. Sharks also swim with their mouths open, allowing water to flow passively through their oral cavity, coming in contact with their tastebuds. This quick movement around the tank may have been an attempt to flush their chemosensory systems of the *Sepia* ink.

I propose that ink is affecting the shark olfactory system as well as the gustatory system. Previous work has shown that bonnethead sharks do reject shrimp soaked in *Aplysia* ink (Nusnbaum and Derby, 2010b). Due to the unidirection flow of the shark oral cavity, gustation may come into play when coming in contact with an ink cloud. However, there was no food item that came into direct contact with the oral taste buds in my study. Future studies should test the olfactory system explicitly, either by directly injecting ink into the shark's nares via a headmount or by testing the shark olfactory system's response to ink via electrophysiological experiments. My study investigated effect of ink on an unmotivated, free swimming shark. Further studies should focus on a food motivated shark, investigating how a shark responds to ink in the presence of a food item. This would speak to the intensity of the ink aversion response and its ability to interrupt a predation event.

I did not record any significant differences in time spent within the Injection Radius, which suggests that ink has a short term, acute effect (**Figure IV.8**). It should be taken into account that this may be due to the restrictions of

the tank, not allowing sharks the option to avoid the injecton site. This study is limited by the challenges of standardizing ink from three very different animal groups. *Aplysia* release as little as 0.5mL of ink while adult *Kogia* can contain up to 12L of ink (Caldwell and Caldwell, 1989). Additionally, I standardized the ink by dissolved organic carbon, achieved by diluting each ink mmol L^{-1}. *Aplysia* was diluted down from 3.8 mmol L^{-1}, *Sepia* from 2.1 mmol L^{-1}, and *Kogia* from 110.2 mmol L^{-1}. These standardizations were necessary to make the inks comparable, however the inks may be differently effective at their full concentration and volume.

In conclusion, the data proves that ink acts as a deterrent, negatively impacting the swimming behavior of sharks. Upon contact with an ink cloud, sharks deviated from their normal swimming path, bending away from the ink, and swimming in another direction. Each of the three inks I looked at, from *Aplysia californica*, *Sepia officinalis*, and *Kogia breviceps,* had the same deterrent-like effects. Sharks had the complete opposite response to food odor which acted as an attractant. Sharks had no reaction to either food coloring or seawater, ruling out disturbance via mechanical stimulation or color. In each of these inking animals, I conclude that ink is used as a chemical defense. Although these three taxa live in different oceanic environments with variable ecologies, they have developed a similar chemical deterrent which is effective in repelling sharks, a common predator.

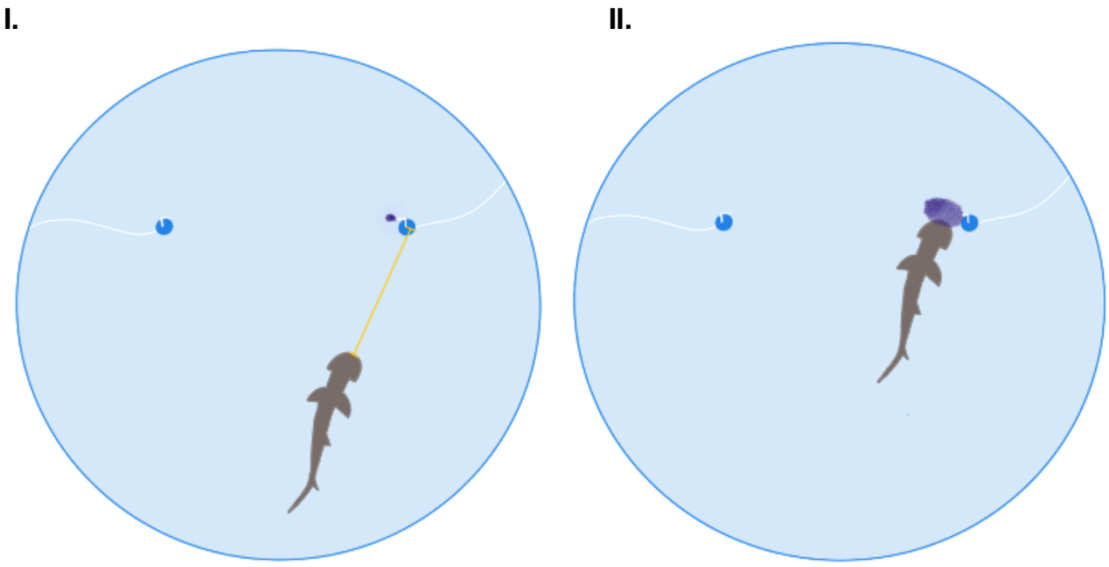
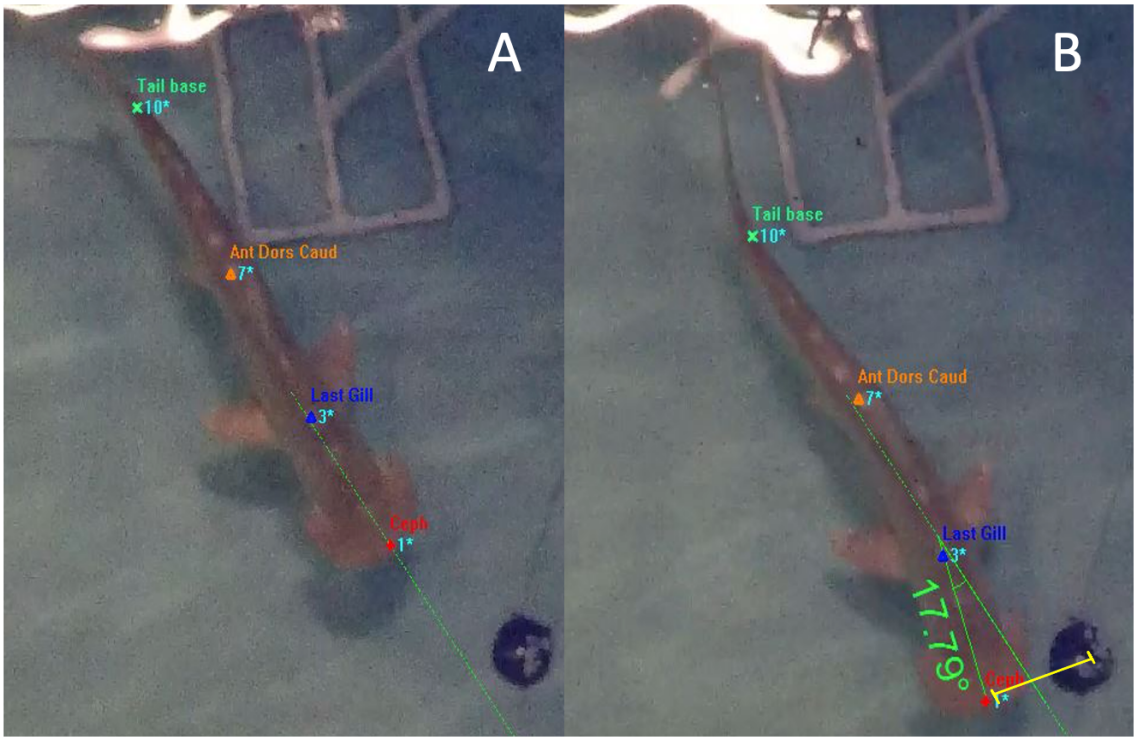


Figure IV.1. The experimental tank was set up with two ink injection sites. When a shark was one body length away from the injection site, 5 mL of ink was expelled (**I**) so that the shark swam through an ink cloud about the same size of its cephalofoil (**II**).

I.


A
Tail base
×10*
Ant Dors Caud
7*
Last Gill
3*
Ceph
1*
B
Tail base
×10*
Ant Dors Caud
7*
Last Gill
3*
177.79°
Ceph
1*

II.

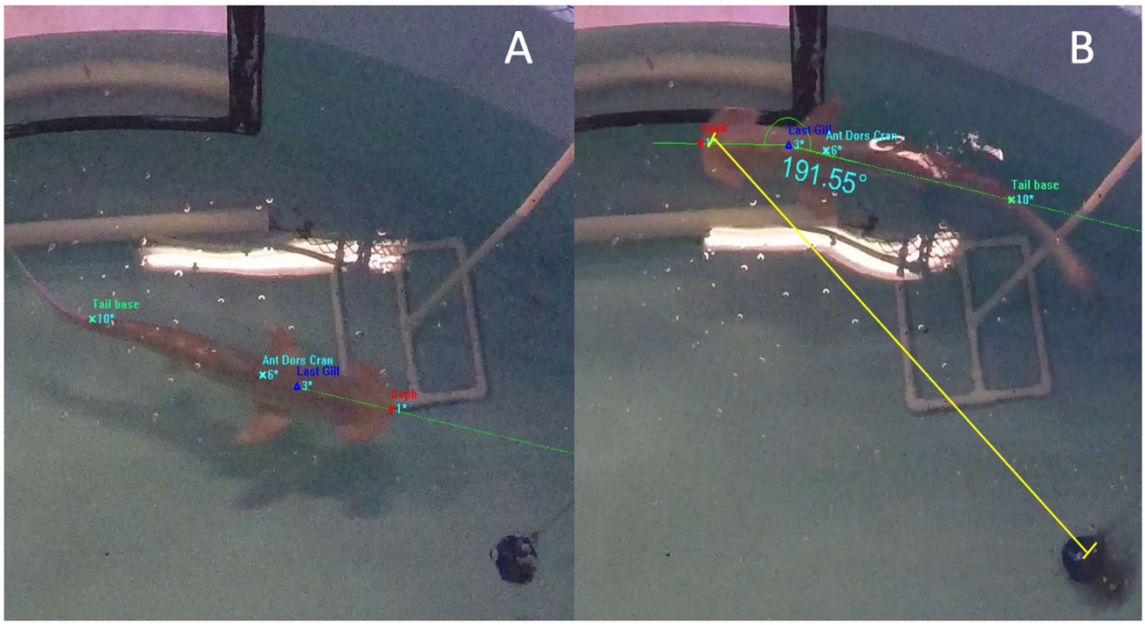

A
Tail base
×10*
Ant Dors Cran
×6*
Last Gill
3*
Ceph
1*
B
Ceph
Last Gill
3*
Ant Dors Cran
×6*
191.55°
Tail base
×10*

Figure IV.2. Maximum Angle of Deviation (MAD) for responses to seawater (**I**) and *Sepia* ink (**II**). The angle of deviation is the angle from the original swimming path (*A*) to the maximum deviation (*B*). Additionally, the distance (cm) from the cephalofoil to the injection site, at the maximum deviation, was measured.

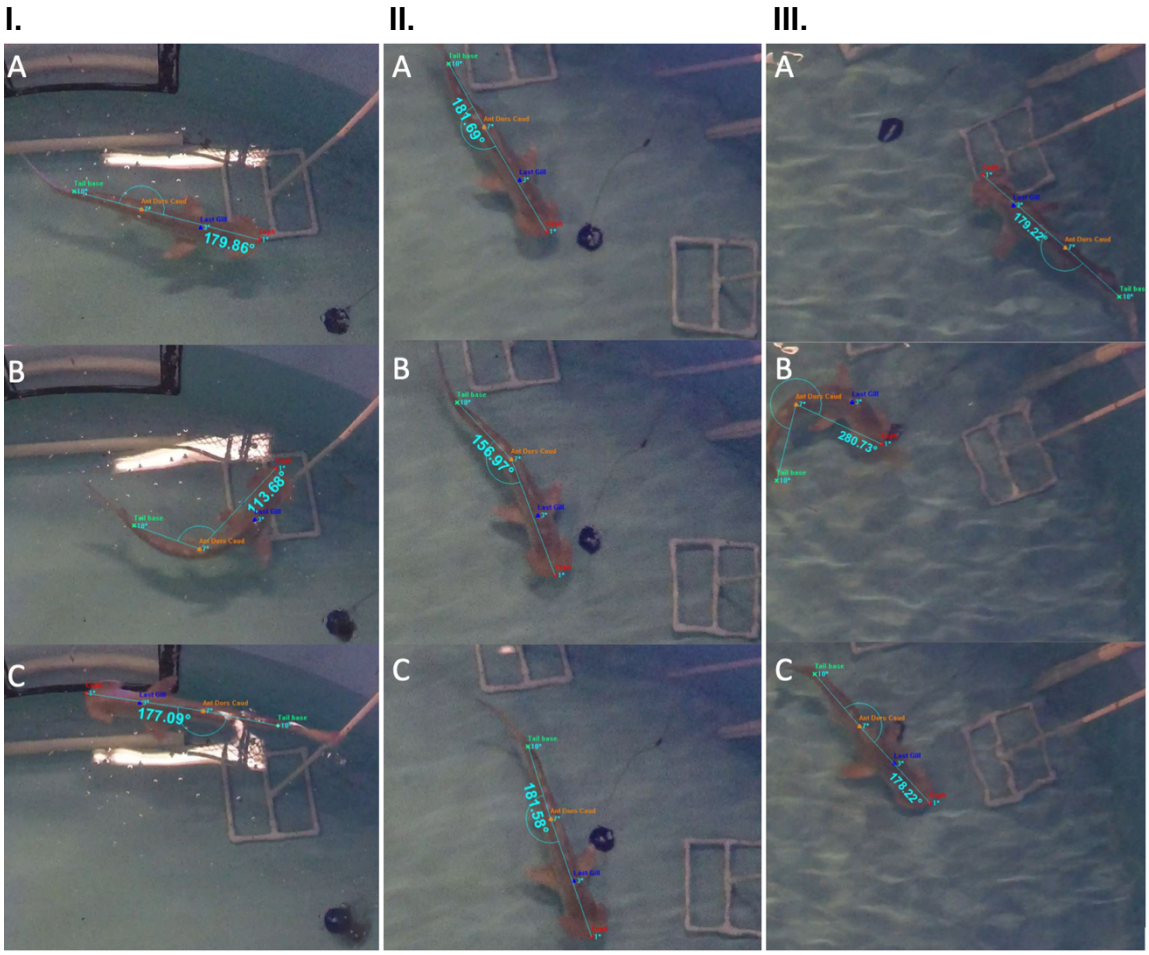


Figure IV.3. Measuring Body Angle (BA) in response to *Sepia* Ink (**I**), seawater (**II**), and to food odor (**III**). *A* shows the starting point, when the body angle is the closest to 180°, *B* shows the Body Angle (measured angle away from the stimulus) and *C* shows the end of the event when the body angle returns close to 180°.

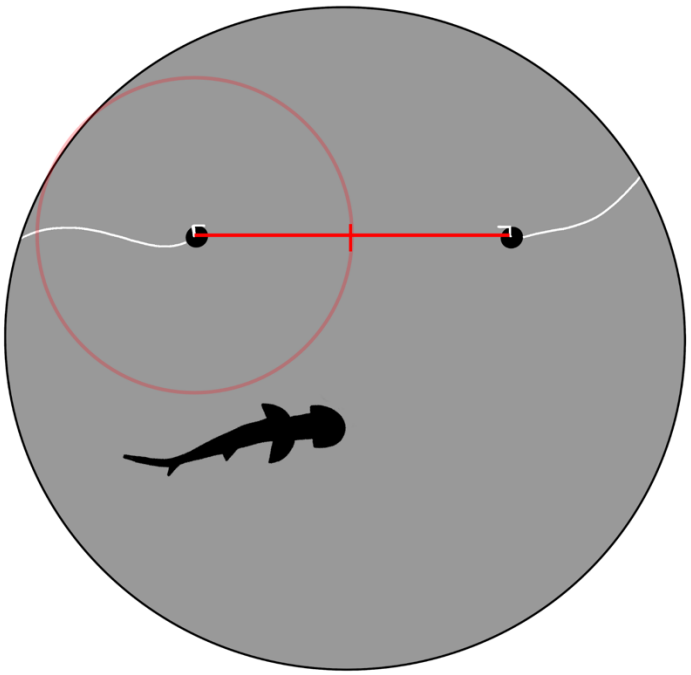


Figure IV.4. To define the injection radius, a circle was drawn around the injection site with a radius equal to half the distance between the two injection sites. Sharks were said to be within the “Injection Radius” when they were within that circle.

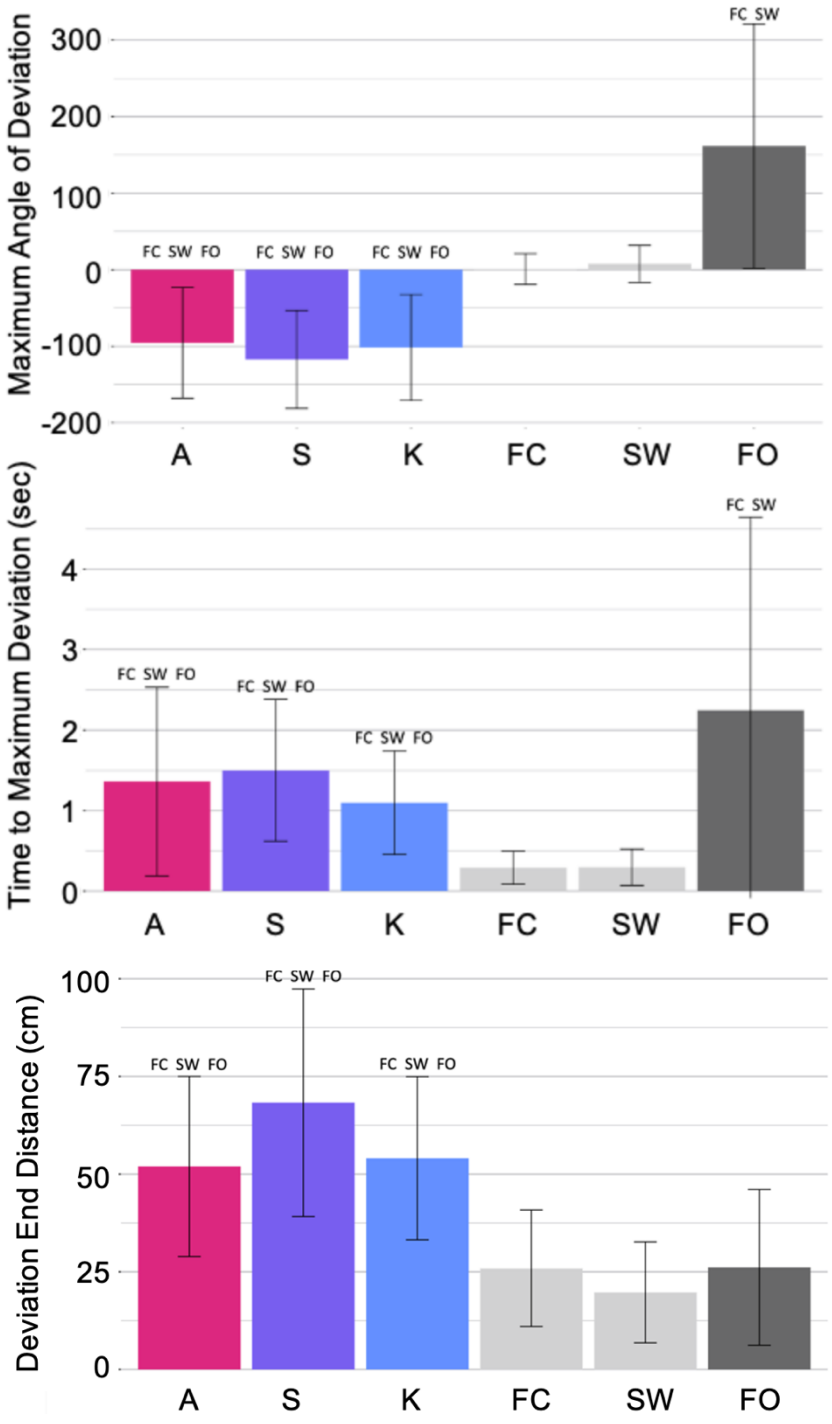
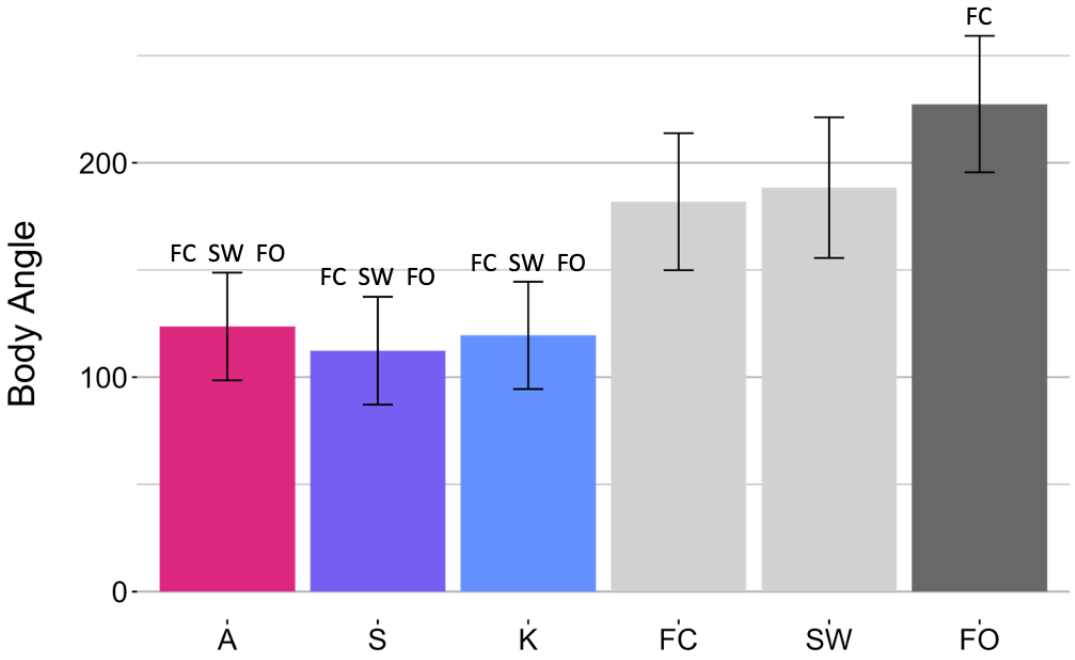


Figure IV.5. Mean and standard deviation for Maximum Angle of Deviation (**I**), Time to Maximum Deviation (**II**), and Deviation End Distance (**III**). A = *Aplysia*

ink, S = *Sepia* ink, *K* = *Kogia* ink, FC= food coloring, SW = seawater, and FO = food odor. Notations above the error bars connote significant difference from that stimuli (DunnTest p-val < 0.05).

I.


Body Angle
200
100
0
FC SW FO
FC SW FO
FC SW FO
FC
A
S
K
FC
SW
FO

II.

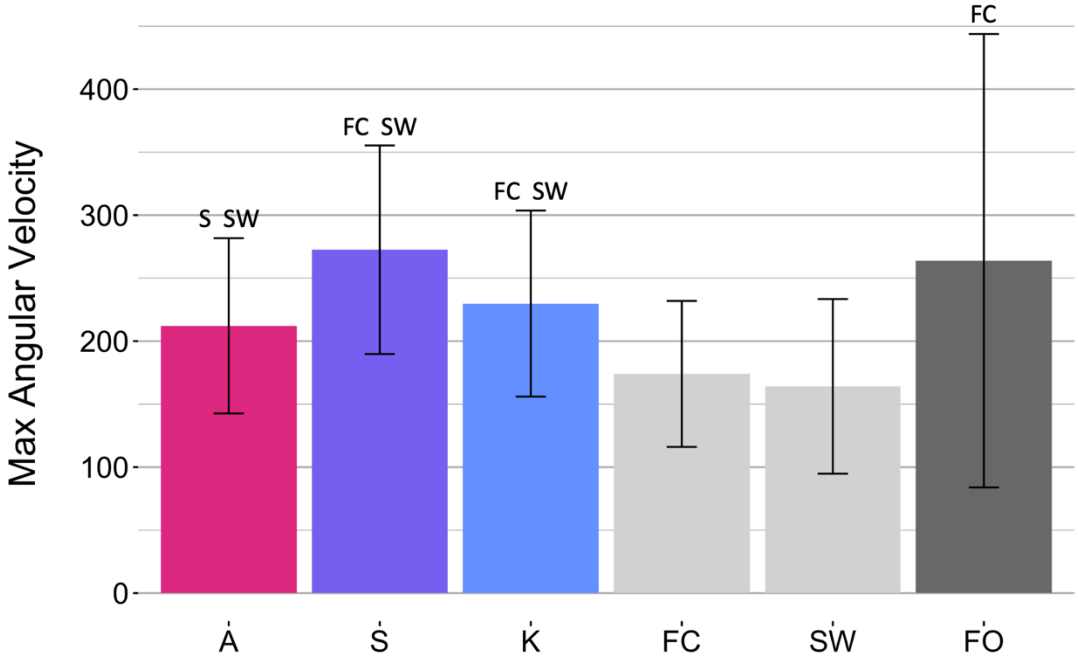

Max Angular Velocity
400
300
200
100
0
S SW
FC SW
FC SW
FC
A
S
K
FC
SW
FO

Figure IV.6. Mean and standard deviation for Body Angle (BA;**I**) and Maximum Angular Velocity (**II**). A = *Aplysia* ink, S = *Sepia* ink, *K* = *Kogia* ink, FC= food coloring, SW = seawater, and FO = food odor. Notations above the error bars connote significant difference from that stimuli (DunnTest p-val < 0.05)

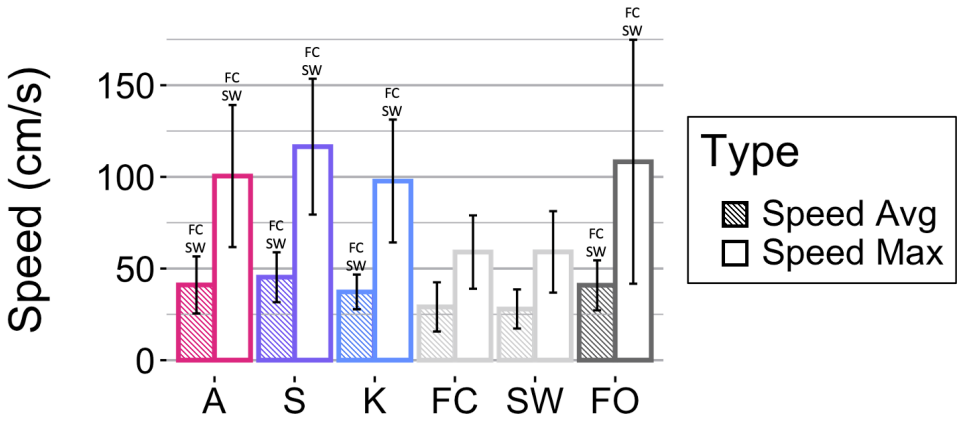


Figure IV.7. Mean and standard deviation for average and maximum speed. A = *Aplysia* ink, S = *Sepia* ink, *K* = *Kogia* ink, FC= food coloring, SW = seawater, and FO = food odor. Notations above the error bars connote significant difference from that stimuli (DunnTest p-val < 0.05)

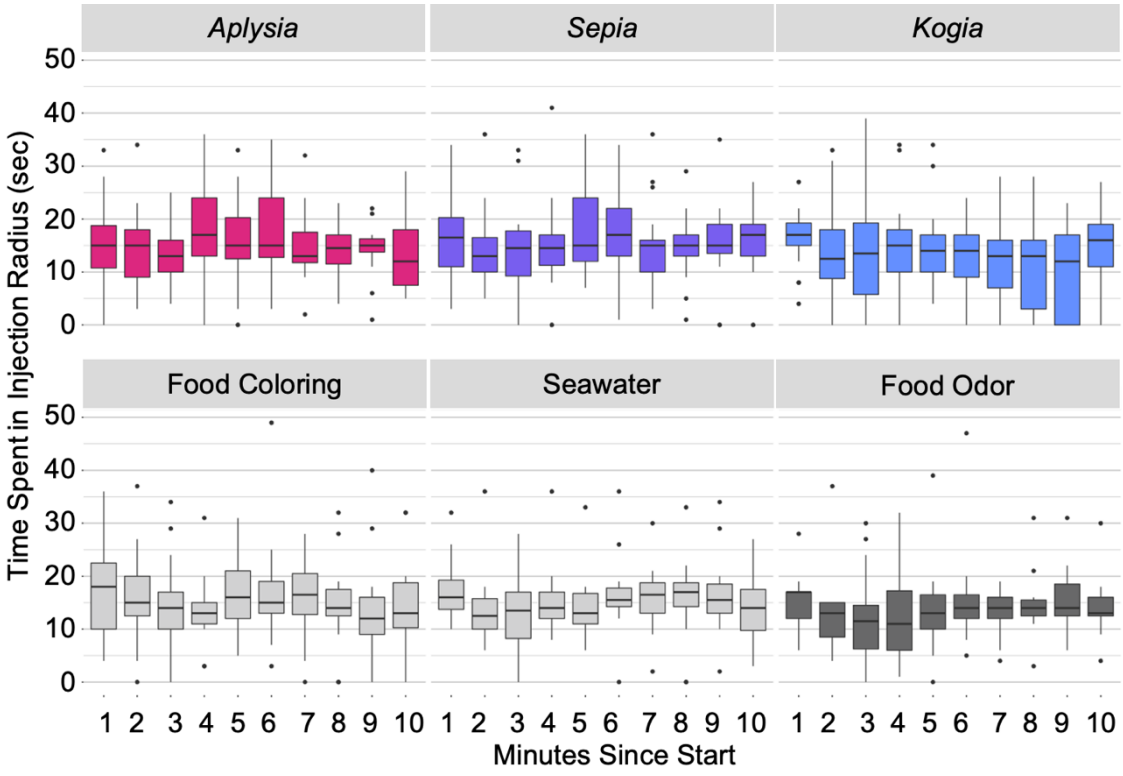


Figure IV.8. Time spent in the Injection Radius by stimuli for the 10 minutes following injection. There was no significant difference between stimuli at any time period and no significant trends over time. (Kruskal-Wallis; $p>0.05$)

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CHAPTER V

CONCLUSIONS

Inking is an antipredation strategy that has evolved at least three separate times in evolutionary history. Investigations into ink function have typically focused on one type of ink, neglecting the convergent evolution aspect of this unusual predator defense. This dissertation approached the study of ink from a comparative approach, comparing the chemistry and function of ink across three diverse invertebrate and vertebrate taxa: *Aplysia*, *Sepia*, and *Kogia*. This work also investigated ink through the lens of the form-function paradigm. Chapters 2 and 3 focused on the form, describing the chemical composition of each ink and the morphology of the target sensory system- the shark olfactory organ. Chapter 4 focused on the behavioral response by a typical predator and quantified the changes in locomotion kinematics elicited from the interaction between the ink chemical cocktail and the shark olfactory structures.

In addition to being novel for its comparative approach, this project is the first to collect ink data on *Kogia* or any inking vertebrate. Before this research, no data on either the chemistry nor the function of *Kogia* ink had been published. *Kogia* research as a whole is sparse, due to their solitary, open ocean lifestyle (Caldwell and Caldwell, 1989). What we do know of *Kogia* has come from a few anecdotal records (Scott and Cordaro, 1987), observations during their limited stays in captivity (Manire et al., 2004), and necropsy reports (Bossart et al.,

2007; Brentano and Petry, 2020; Long, 1991). This data adds significantly to our current knowledge of *Kogia*.

This dissertation contributes to our current understanding of shark olfactory morphology (Chapter 3). My research is the first to produce scanning electron microscopy images of bonnethead shark olfactory lamellae and to track the axon projection of the olfactory receptor neurons through the lamellae of any shark. Recently, the sphyrnid olfactory organ's fluid dynamics has become a topic of interest. New work has elucidated the patterns of water velocity within the hammerhead olfactory organ, leading to hypotheses regarding differential sensitivities within the organ (e.g Abel et al., 2010; Rygg et al., 2013), I reported differences in both the sensory epithelial surface area and degree of secondary folding within the bonnethead olfactory organ. I found that medial areas of the rosette had less sensory area and less secondary folding than those more centrally or laterally located. This correlates with published modeled fluid velocities, which are near-stagnant and recirculating in the medial part of the rosette. While morphometrics like these are only proxies for sensitivity, these data lay the groundwork for future experimentation into differential sensitivity within the rosette of bonnethead sharks.

This research supports the role of ink as a chemical deterrent. In Chapter 2, I report high concentrations of free amino acids which are within the ranges of sensitivities for lobsters, bony fish, and elasmobranchs- relevant predators for *Aplysia Sepia,* and *Kogia.* In Chapter 4, I used kinematic analyses to

demonstrate the aversive response of free-swimming bonnethead sharks to ink. All three inks caused sharks to divert from their original swimming paths and turn away from the ink cloud. This response was significantly different from both food coloring and seawater, suggesting that neither color nor mechanosensory stimulation was causing these aversive responses. Instead, I conclude that ink was acting as a chemical deterrent. Additionally, I suggest that these three inks do not have phagomimetic effects on sharks. The responses of sharks to food odor were completely opposite to that of ink- they were attracted instead of repelled.

Furthermore, the chemical data reported in Chapter 2 supports the current evolutionary hypotheses for ink evolution: co-option of antimicrobial compounds and excretory structures. I report high amounts of D-form free amino acids in all three inks. D-amino acids have been shown to strengthen the efficacy of antimicrobial peptides and prevent the production of bacterial biofilms. Additionally, D-amino acids are found in the gut microbiome of metazoans. Both of these connections link the D-amino acids in ink with the hypothesized evolutionary ties of ink to antimicrobial function and excretory pathways.

This work could be further confirmed with electrophysiological experiments, testing the response of the shark olfactory system to ink. Confirmation that the olfactory system is able to detect ink would strengthen the evidence for ink acting as a chemical deterrent and explain the

neurophysiological basis for the aversion behaviors reported in Chapter 4. Elasmobranch sensitivity to amino acids is well documents and their detection thresholds are much lower than the concentration of free amino acids that we report for all three inks in Chapter 2. Additionally, testing the shark olfactory system's response to fractionated ink would help parse out the active chemical components which drive the observed deterrent response. This type of experimentation can also be used to test our hypotheses about decreased sensitivity in the medial section of the olfactory rosette.

Since ink is a chemically mediated defense, it is subject to alteration when exposed to changing ocean conditions. The predicted increase in oceanic CO_2 absorption leads to reduced seawater pH also known as ocean acidification. Ocean acidification can affect animal's abilities to not only perceive sensory cues, but also make decisions based on these cues (Briffa et al., 2012). Ocean acidification decreases the ability for prey species to detect, respond to, and learn chemical predator cues (Dixson et al., 2010; Domenici et al., 2012; Ferrari et al., 2011a; Ferrari et al., 2011b; Ferrari et al., 2012; Munday et al., 2010; Munday et al., 2013; Munday et al., 2014).

In addition to the evaluation of prey response, recent research has addressed the threat of ocean acidification from the predators' perspective. While most of this research has been conducted on teleosts (Cripps et al., 2011; Ferrari et al., 2011b), Dixson et al. (2015) investigated spiny dogfish (*Squalus acanthias*) odor tracking ability and feeding behavior under present day oceanic

CO_2 levels versus CO_2 predicted for 2100. Under acidified ocean conditions, dogfish switched from being attracted to prey odor to avoiding it; this effect is also reported in teleosts (Cripps et al., 2011). The sharks exposed to increased CO_2 levels also spent 45% less time in the odor stimulus plume and attacked less aggressively than the control group.

Nilsson et al. (2012) linked effects like these to GABBA-A, the main inhibitory neurotransmitter in the vertebrate brain. Normally an opening of a GABBA-A receptor causes inflow of Cl^-, leading to the inhibition of the neuron. Marine fish fight acid-base balance by accumulating HCO_3 and reducing Cl^-. Exposure to ocean acidification conditions leads to a reversal of neural function, causing an excitatory response when GABA-A receptor is activated. The abnormal olfactory preferences and loss of behavioral lateralization associated with ocean acidification were reversed when fish were treated with an antagonist of the GABA-A receptor.

Predators' impaired ability to detect prey olfactory cues may also be correlated with a shift in response intensity to food stimuli. This could result in a decrease in predator activity and a reduced ability to respond to fluctuations in food availability (Dixson et al., 2015). Although the impairment of predators may be superficially beneficial to prey species, the deleterious effects of ocean acidification on prey are greater than on predators. This magnitude of difference makes it unlikely that the alleviation of predation pressure is enough to compensate for the effects of ocean acidification on prey mortality (Cripps et al.,

2011). Even so, reduced predation leads to increased competition, limiting the amount of resources available. Moreover, studies have shown that reduced olfactory ability is compensated for by increases in activity (Cripps et al., 2011, Munday et al., 2010). This can increase the energetic demand and feeding rates in affected fishes. Lowered pH also negatively affects the ability for marine species to detect olfactory cues associated with larval settlement and homing behavior (Devine et al., 2012a; Devine et al., 2012b; Munday et al., 2009). While most of these studies have been conducted on tropical species, Jutfelt et al. (2013) also reported a connection between ocean acidification and deleterious effects on olfactory related behavior, lateralism, and learning in the three-spined stickleback (*Gasterosteus aculeatus*), a temperate species known to be tolerant to other environmental stressors. This indicates that ocean acidification has a damaging behavioral, olfactory, and cognitive effect on chemical cue detection, including chemically mediated defenses like inking. Therefore, it is important to fully understand the underlying mechanisms of these chemically based predator-prey interactions at present day ocean conditions.

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