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*University of Southern Mississippi*

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THE ELASMOBRANCH-MICROBE RELATIONSHIP: TRIMETHYLAMINE N-OXIDE SYNTHESIS, UREA HYDROLYSIS, AND MICROBE-OSMOLYTE INTERACTIONS IN THE ATLANTIC STINGRAY, DASYATIS SABINA

by

Kaitlin Kelly Doucette

A Thesis
Submitted to the Graduate School
and the Department of Coastal Sciences
at The University of Southern Mississippi
in Partial Fulfillment of the Requirements
for the Degree of Master of Science

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December 2016
ABSTRACT

THE ELASMOBRANCH-MICROBE RELATIONSHIP: TRIMETHYLAMINE N-OXIDE SYNTHESIS, UREA HYDROLYSIS, AND MICROBE-OSMOLYTE INTERACTIONS IN THE ATLANTIC STINGRAY, DASYATIS SABINA

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The elasmobranch osmoregulatory strategy is predicated on the accumulation of nitrogenous compounds, primarily urea and trimethylamine n-oxide (TMAO). Despite the abundance of these plasma osmolytes, it is notable that elasmobranchs appear to lack urease and TMA oxidase (Tmase), enzymes that scavenge urea-nitrogen and synthesize TMAO, respectively. However, urease and Tmase are found in many species of bacteria. Therefore, I hypothesized that intestinal bacteria are responsible for urease and Tmase activity in elasmobranchs. Absent dietary nitrogen sources, I evaluated the effects of reduced intestinal microbiota on osmoregulation in Atlantic stingray (Dasyatis sabina) in vivo. D. sabina were given daily broad-spectrum antibiotics per os and monitored for weight loss, plasma osmolality, amine metabolites, urea, and TMAO. qRT-PCR was used to determine the efficacy of antibiotics at reducing the intestinal microbial community and to quantify the hepatic expression of carbomoyl phosphate synthetase III (CPS III), the rate-limiting enzyme in urea production. Though antibiotics significantly reduced the bacterial community in the D. sabina gut, there was no significant change in plasma
osmolality, urea, or TMAO. However, amine metabolites changed significantly within control and antibiotic groups including benzoic acid, arginine, creatinine and L-citrulline. Also observed was significant down-regulation of CPS III expression, suggesting that urea production decreased in antibiotic-treated individuals. My findings suggest that elasmobranch osmoregulation is robust to microbiome perturbation and supports the hypothesis that plasma osmolytes are highly conserved in elasmobranchs. This is the first study to test the efficacy and subsequent effect of antibiotics on the elasmobranch intestinal microbiome and osmoregulation.
ACKNOWLEDGMENTS

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DEDICATION

To my family and friends, a sincere thank you for your encouragement, support, and humor. Thank you to my parents, Kathleen and Kevin, and my sister, Colleen, for helping me relocate to the Gulf Coast and their never-ending support from afar. To the wonderful community of graduate students at the Gulf Coast Research Lab, thank you for being fantastic classmates and even better friends. I will always cherish the years I spent in Ocean Springs, Mississippi. And finally, I would like to thank my loving grandparents, Mamo and Gramps, without whom I wouldn’t have spent so much time by the sea.
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CHAPTER I - INTRODUCTION

Osmoregulation is a critical physiological process in the aquatic environment whereby organisms constantly and intimately interact with water and dissolved solutes. Compared to our comprehensive understanding of teleost osmoregulatory strategy, a gap exists in our knowledge regarding several critical aspects of elasmobranch osmoregulation – primarily the synthesis, maintenance, and catabolism of several critical nitrogenous osmolytes. Similar to teleosts, elasmobranchs colonize diverse environments with salinities ranging from freshwater to hypersaline water (Hamlett 1999; Klimley and Oerdig 2013); however, elasmobranchs employ a unique osmoregulatory strategy to cope with the complexities of the aquatic environment. While teleosts and elasmobranchs both actively excrete Na\(^+\) and Cl\(^-\) (the dominant ions in seawater) via specialized chloride cells in the gills, through urinary excretion, and – in the case of elasmobranchs – use of the rectal gland, teleosts maintain hypo-osmotic plasma with respect to seawater (~360 milliosmoles) whereas elasmobranchs maintain plasma of equivalent osmolality to that of seawater (~1000 milliosmoles). Elasmobranchs maintain iso-osmotic – or slightly hyper-osmotic plasma – by accumulating high levels of nitrogenous compounds such as urea, trimethylamine \(n\)-oxide (TMAO), amino acids, sarcosine, and betaine (Robertson 1975; Pang \textit{et al.} 1977; Yancey and Somero 1979, 1980; Ballantyne \textit{et al.} 1987; Perlman and Goldstein 1988), which counteract the osmotic pressure of the seawater.
Of the total dissolved solutes in elasmobranch plasma, the importance of urea and TMAO to elasmobranch osmoregulation cannot be overstated. Urea and TMAO comprise roughly 60% of total plasma solutes: 40% and 20%, respectively (Goldstein and Funkhouser 1972; Ballantyne and Robinson 2010), which is notably more than any other dissolved solute in elasmobranch plasma. However, despite the importance of urea and TMAO to elasmobranch osmoregulation, our understanding of nitrogenous osmolyte synthesis, scavenging, and metabolism in these taxa is limited.

Urea

The importance of elasmobranch urea synthesis and retention is dictated by the osmotic demands of the aquatic environment. In marine elasmobranchs, urea is stored in blood plasma at extraordinarily high concentrations compared to other aquatic organisms (300-500mM) (Yancey 1994) and, as previously mentioned, constitutes a major proportion of osmolytes: typically, 40% of total blood solutes (Ballantyne and Robinson 2010). To a lesser extent, urea is synthesized and retained in certain freshwater stenohaline species such as the isolated *Dasyatis sabina* population in St. Johns River, Florida (Johnson and Snelson 1996), as well as species of *Himantura* in southeast Asia (Tam *et al.* 2003). Other freshwater stenohaline species such as *Potamotrygon* *spp.* however, have lost the ability to synthesize urea via the ornithine-urea cycle (O-UC) and no longer retain urea (Anderson 1991). Interestingly, it has been postulated that the ureosmotic strategy of elasmobranchs has contributed to the low penetrability of elasmobranchs into freshwater environments (Ballantyne and
Robinson 2010). Additionally, urea is energetically costly to produce via the O-UC (5 ATP per mole of urea) (Anderson 2001), which means urea must be highly conserved within tissues. For example, although urea represents 80-97% of elasmobranch nitrogenous waste (Anderson 2001; Kajimura et al. 2006), the total amount of excreted nitrogenous waste is significantly reduced by retention mechanisms in the gills and kidneys (Fines et al. 2001; Morgan et al. 2003; Kajimura et al. 2006). This indicates that, rather than constantly manufacturing large quantities of urea to account for large losses via excretion or diffusion, a majority of urea is retained.

As with many other aspects of physiology, elasmobranchs employ a unique method of urea synthesis. Urea is primarily synthesized via the O-UC (Figure 1) in the mitochondria of hepatocytes (Smith 1936; Casey and Anderson 1985; Perlman and Goldstein 1988; Mommsen and Walsh 1991) though recent studies found evidence that the O-UC also occurs in extrahepatic elasmobranch tissues such as skeletal muscle and the intestines (Tam et al. 2003; Steele et al. 2005). Briefly, in most vertebrate taxa, ammonia is converted to carbamoyl phosphate, which then enters the O-UC and – through a series of enzymatic reactions – is converted to ornithine (which re-enters the cycle) and urea. In this process in mammals and amphibians, carbamoyl phosphate synthetase I (CPS I) is the first enzyme in the cycle (Meijer et al. 1990; Campbell 1991). Most teleosts excrete nitrogen as ammonia thereby negating the necessity of a CPS enzyme (Anderson 1991). In contrast, the first enzyme in the elasmobranch O-UC is CPS III (Anderson 1991; Anderson 2001). Unlike CPS I, which uses ammonia as a
nitrogen-donating substrate, CPS III uses glutamine to synthesize carbamoyl phosphate (Perlman and Goldstein 1988; Ballantyne and Robinson 2010). Because of this, glutamine must first be synthesized from ammonia by glutamine synthetase (GSase), an accessory enzyme located in mitochondria (Casey and Anderson 1985). As such, glutamine is the limiting substrate for the O-UC in elasmobranchs and CPS III is the rate-limiting step in this cycle.

The importance of this enzymatic “check-point” is evident when considering the method by which urea-nitrogen is scavenged. Urea is hydrolyzed via urease to ammonia and carbon dioxide. If ammonia was used as a precursor, the catabolism of urea would directly feed back into the O-UC, thus prompting further synthesis of urea without additional regulation. Further, if a decrease in total urea concentration was advantageous, hydrolysis of urea would likely have little effect on urea concentrations; if ammonia was indeed the precursor to urea synthesis in elasmobranchs, the end-product of urea break-down would be the precursor to urea synthesis. Thus, having an intermediary amine – glutamine – and not ammonia as the substrate for CPS III allows urea to be recycled via urease hydrolysis.

Urea-nitrogen recycling would be highly beneficial given the nitrogen demands of elasmobranch physiology. Elasmobranchs are considered nitrogen-limited (Wood et al. 2005), thus a method of breaking down urea would allow elasmobranchs to re-use urea-nitrogen. It is worth noting that glutamine, which is synthesized from ammonia (the product of urea hydrolysis), can also be utilized as an oxidative substrate in numerous elasmobranch tissues (Ballantyne 1997).
Thus, glutamine produced via urea hydrolysis could be used as an oxidative fuel in elasmobranch tissue.

A method of recycling urea-nitrogen would also be advantageous given the varying osmotic demands of the aquatic environment, particularly for euryhaline species that can rapidly alter plasma osmolytes in response to environmental conditions. However, it is striking that these taxa appear to lack any mechanism for urea catabolism. According to the current literature, urease – the enzyme responsible for urea hydrolysis – has not been identified in elasmobranchs (Wood 1950; Simidu and Oisi 1951; Knight et al. 1988). Urease activity has, however, been identified in species of fungi, plants, invertebrates, and – perhaps most notably – bacteria. The existence of urease-positive bacterial species is compelling because of microbiological studies of elasmobranch tissues, which found multiple species of bacteria inhabiting healthy elasmobranch tissues (Grimes et al. 1985; Grimes et al. 1990; Mylniczenko et al. 2007). For elasmobranch hosts, these endogenous bacteria could confer the requisite urease activity that elasmobranchs require but do not inherently possess. Indeed, in vitro treatment of the livers of lemon shark (Negaprion brevirostris) and tiger shark (Galeocero cuvier) with both antibiotics and $^{14}$C-labeled urea yielded reduced urease activity to the point that it was effectively non-existent (Knight et al. 1988). Knight et al. (1988) further concluded that 65% of all bacteria taxa isolated from shark tissue using culture-based methods (Grimes et al. 1985) are urease-positive, meaning that these bacteria are capable of urea hydrolysis, in addition to those species that are able to utilize
urea as a sole energy source (e.g. *Vibrio damsela* and *V. charchariae* syn. *V. harveyi*) (Grimes *et al.* 1984).

The high proportion of urease-positive bacteria in elasmobranch tissues – tissues that contain high concentrations of urea – led to the hypothesis that bacteria are involved in regulating urea concentrations in elasmobranchs (Knight *et al.* 1988), specifically by hydrolyzing excess urea and recycling urea-nitrogen in the form of ammonia. Even without further evidence for the existence of bacteria within elasmobranch tissue, the bacterial urease hypothesis could certainly extend to the bacteria within the elasmobranch gut. Indeed, a study of urea flux within the intestine of spiny dogfish (*Squalus acanthias*) found an inverse relationship between urea and ammonia within the proximal and distal regions of the gut, which the authors postulated could be due to bacterial urea hydrolysis (Anderson *et al.* 2009). There is evidence for this kind of vertebrate-bacteria relationship in other taxa: studies of ruminants found that bacteria in the rumen were responsible for much of the urease-driven “nitrogen scavenging” during periods of low nitrogen inputs, which is a clear advantage over reliance on regular nitrogen input from the diet (Harmeyer and Martens, 1980). Additionally, the use of glutamine by CPS III in elasmobranch urea synthesis provides a key step in the regulation of this process. As mentioned previously, ammonium must first be converted to glutamine in order to re-enter the elasmobranch O-UC. Thus, CPS III provides a useful “check-point” for urea synthesis given that ammonia produced by microbial urease would not directly re-enter the elasmobranch O-UC.
If endogenous tissue or gut bacteria in elasmobranchs are involved in the hydrolysis of urea via urease, such a relationship will represent a novel vertebrate-microbe relationship. Even in ruminants, arguably the best example of microbial urea-nitrogen scavenging (UNS) with which to compare elasmobranchs, this proposed elasmobranch-microbe relationship is physiologically unique. Rumen bacteria hydrolyze urea-nitrogen to synthesize amino acids and proteins that the host can then reabsorb (Mobley and Hausinger 1989; Lapierre and Lobley 2001; Stewart and Smith 2005) and thus the relationship is predicated on an exchange of dietary nutrients. In contrast, elasmobranchs consume a nitrogen-rich diet that allows them to accumulate high levels of urea; thus the relationship between elasmobranchs and intestinal bacteria is predicated on osmoregulation. Both groups rely on this microbial process for nitrogen scavenging; the difference between ruminants and elasmobranchs is in how each group utilizes urea and the urea-derived nitrogen compounds. Arguably, in elasmobranchs, urea is an even more critical compound due to its accumulation as an abundant, important osmolyte. Because of the hypothesized importance of bacteria to the osmoregulatory urea cycle in elasmobranchs, further study is clearly warranted.

Trimethylamine n-oxide

In addition to the complexity of urea cycling, urea is also protein-toxic in high concentrations (Yancey et al. 1982; Yancey 1994, 2005). Given the exceptionally high urea concentration found in elasmobranch tissues, without compensatory mechanisms urea would have deleterious effects on normal
protein and enzymatic function. To counteract the effects of urea toxicity, methylamines such as TMAO are accumulated in tissues, which allows normal protein function and returns enzymatic $K_m$ values to pre-urea levels by strengthening the structure of water molecules surrounding enzymes and indirectly interacting with enzyme functional groups (Yancey and Somero 1979; Yancey et al. 1982; Zou et al. 2002; Qu and Bolen 2003). The ratio of urea to TMAO is relatively constant: normally between 3:1 and 2:1 in all species of elasmobranchs studied to date (Yancey and Somero 1980; Steele et al. 2005).

Although the presence and importance of TMAO has been well established in elasmobranchs (Goldstein and Dewitt-Harley 1973; Strom et al. 1979; Yancey and Somero 1979, 1980; Yancey 2001; Seibel and Walsh 2002), the mechanism by which TMAO is accumulated is still debated. In mammalian models, TMAO is synthesized from quaternary ammonium salts such as choline and carnitine, which are broken into trimethylamine (TMA) by bacteria in the gut (Al-Waiz et al. 1992; Wang et al. 2011; Koeth et al. 2013; Hartiala et al. 2014). This microbial-mammalian model appears to be consistent with the breakdown of choline to TMA in elasmobranchs (Bilinski 1960; Goldstein and Funkhouser 1972). TMA is then absorbed from the intestine by colonocytes – cells in the intestinal epithelium – and transported to the liver (Wang et al. 2011; Russell et al. 2013; Hartiala et al. 2014). In the liver, TMA is oxidized to TMAO by trimethylamine oxidase (Tmase) (Beatty 1938; Strom 1980; Seibel and Walsh 2002), an enzyme that specializes in TMA oxidation (Goldstein and DeWitt-Harley 1973), or an isoform of flavin-containing monooxygenase (FMO), which
oxidizes a broader range of tertiary amines (Ziegler and Mitchell 1972; Schlenk 1998). FMO isoform 3 (FMO3) is the hepatic isoform that catalyzes the monooxygenation of TMA in mammalian models (Lang et al. 1998). Traditionally, studies of FMOs have focused on mammalian models, however, recently FMOs have been identified in bacteria and several teleosts (Schlenk 1998), most notably the euryhaline teleost Oncorhynchus mykiss (Larsen and Schlenck 2001). In O. mykiss challenged with an increase in salinity, an increase in FMO expression was concurrent with TMAO accumulation in muscle tissue (Larsen and Schlenck 2001).

In elasmobranchs, however, there is conflicting and sporadic evidence as to the distribution and presence of Tmase or FMO3. Of elasmobranch species studied to date, only five species have demonstrated putative Tmase activity: S. acanthius (Schlenk and Li-Schlenk 1994), silky shark (Carcharhinus falciformis) (Schlenk and Li-Schlenk 1994), grey smooth-hound shark (Mustulus californicus) (Baker et al. 1963), nurse shark (Ginglymostoma cirratum) (Goldstein and Funkhouser 1972; Goldstein and DeWitt-Harley 1973), and lemon shark (Negaprion brevirostris) (Goldstein and DeWitt-Harley 1973). Additionally, these studies used in vitro and in vivo conversion of $^{14}$C-TMA-HCl and choline-methyl-$^{14}$C to $^{14}$C-TMAO as a proxy for Tmase or FMO3 activity: essentially, production of $^{14}$C-TMAO indicates a putative Tmase or FMO must exist in elasmobranch tissue. However, as all aforementioned studies did not employ antibiotics in vivo or in vitro, it would be impossible to elucidate whether the enzyme activity in elasmobranchs was of bacterial or elasmobranch origin. Other elasmobranch
species either have not been studied or were found to have no detectable levels of Tmase (Goldstein and DeWitt-Harley 1973; Seibel and Walsh 2002).

Due to conflicting evidence for Tmase or FMO in most elasmobranch species, it was theorized that TMAO may originate entirely from dietary inputs and is therefore accumulated and conserved (Seibel and Walsh 2002; Treberg and Dreidzic 2006; Treberg et al. 2006). Fasted S. acanthius maintained nearly constant plasma TMAO levels over a 41-day trial (Cohen et al. 1958; Wood et al. 2010) and fasted winter skate (Leucoraja ocellata) maintained constant plasma TMAO over a six-week period (Treberg and Driedzic 2006). During prolonged fasting, neither study detected appreciable levels of TMAO production. Compounded with low TMAO excretion rates, the authors concluded that TMAO must be highly conserved during between-meal periods. While TMAO is likely very well-conserved given its importance as an osmolyte, it seems unlikely that efficient retention is the only mechanism for regulating TMAO. For example, the ability to oxidize TMA has not yet been demonstrated in many species including Rajiformes, which includes the family Dasyatidae (Treberg et al. 2006), a finding that is particularly surprising given that Dasyatidae includes a number of euryhaline species. TMAO has, however, been shown to increase in D. sabina (Ferer 2007) and juvenile bull shark (Carcharhinus leucas) (Pillans et al. 2006) with increasing salinity even in the absence of dietary inputs. Thus, even though no mechanism of TMAO synthesis has been identified among the Dasyatidae stingrays, D. sabina can increase plasma TMAO by some currently unidentified
mechanism. As suspected by Seibel and Walsh (2006), this suggests that TMAO can be synthesized endogenously in elasmobranchs.

Despite the evidence for endogenous TMAO synthesis, the method of synthesis in elasmobranchs is still unknown. Treberg and Driedzic (2006) reported evidence of FMO activity but were unable to demonstrate TMA oxidation in vivo and in vitro in winter skate (Leucoraja ocellata). Goldstein and Funkhouser (1972) demonstrated conversion of $^{14}$C-TMA and $^{14}$C-choline to $^{14}$C-TMAO in nurse sharks in vivo through a putative Tmase or FMO enzyme. Given that conversion of choline to TMA, the precursor to TMAO, is only known to occur through microbial oxidation (Marzo and Curti 1997), the authors hypothesized that intravenously injected $^{14}$C-choline must cross the intestinal lumen; once in the gut, microbiota would convert $^{14}$C-choline to $^{14}$C-TMA, which could then be converted by Tmase in the liver to $^{14}$C-TMAO. However, because Goldstein and Funkhouser (1972) also demonstrated the conversion of $^{14}$C-choline to $^{14}$C-TMAO in liver homogenates in vitro, the authors assumed that their initial hypothesis regarding bacterial $^{14}$C-choline to $^{14}$C-TMA was incorrect. Based on evidence of symbiont bacteria living in liver tissue (Grimes et al. 1984, 1985; Knight et al. 1988), it is possible that bacteria within the liver are able to convert choline to TMA similar to bacteria within the gut. Additionally, experimental manipulations of choline consumption in mammals indicate that intestinal bacteria have a critical threshold for choline and thus are limited in their ability to synthesize TMA (Ziesel et al. 1989). In elasmobranchs, additional populations of endogenous bacteria within the liver could remediate the overload of choline in
the intestine: another possible benefit of symbiont microbes to the elasmobranch host. Given the conflicting evidence for elasmobranch Tmase and FMOs, exploring the potential for a bacterial role in TMAO synthesis via TMA oxidation within the gut and other elasmobranch tissues is clearly warranted.

Experimental Design

Exploring this novel relationship between elasmobranchs and their symbiont microbes will elucidate key physiological processes in elasmobranch fishes. Because elasmobranchs rely on protein-toxic urea as an osmoregulatory compound and, subsequently, TMAO to counteract the effects of urea toxicity, I would expect elasmobranchs to possess urease and Tmase. However, elasmobranchs do not appear to possess either of these enzymes, which indicates the existence of alternative mechanisms for urea and TMAO regulation. I hypothesize that intestinal microbes are responsible for the regulation of vital osmoregulatory metabolites in elasmobranchs. This would indicate that microbial nutrient cycling has profoundly influenced elasmobranch physiology, a finding that would substantially change the perception of elasmobranch-microbe relationships.

Establishing gnotobiotic individuals would be an ideal method to study the influence of tissue and gut bacteria on elasmobranch physiology; however, creation of gnotobiotic elasmobranchs is outside of the scope of this research. Gnotobiosis has been established in small mammalian, avian, and amphibian model organisms (Baker and Ferguson 1942; Reynier 1959; Gordan 1960) as well as a few key species of teleost, namely the zebrafish *Danio rerio* (Rawls et
However, the process of creating gnotobiotic individuals is time-consuming and expensive, requiring sterile birthing and rearing, sterile food and water, and a sterile tank environment (Reynier 1959) all of which is complicated by the reproductive strategies of elasmobranchs and the difficulty of rearing elasmobranchs that are born in captivity. Additionally, according to the current literature, there has been no successful generation or use of gnotobiosis in an elasmobranch model. For these reasons, gnotobiotic elasmobranchs were neither created nor obtained. Therefore, my goal throughout these experiments was instead to significantly reduce the elasmobranch microbial community using broad-spectrum antibiotics in vivo, an accessible alternative to gnotobiosis (Reikvam et al. 2011). Given a significant reduction in the intestinal microbiome, I then observed key osmolytes to determine if this reduction in microbiota caused an osmoregulatory or physiological response. Further, I chose to focus my efforts on reducing the intestinal microbiome and not the tissue microbiome; a study of antibiotic efficacy has never been published in elasmobranchs, thus delivery of targeted oral antibiotics was more likely to succeed at reducing the intestinal microbiome than antibiotics injected intravenously or intramuscularly. Additionally, intravenous antibiotic injection could cause confounding results given the lack of published studies of antibiotic effects on other aspects of elasmobranch physiology.

This study aims to answer these key hypotheses: (1) intestinal bacteria are responsible for the catabolism of urea via urease; and (2) intestinal bacteria are responsible for the production of TMAO. In summary, I investigated the
potential involvement of endogenous bacteria in the ureosmotic strategy of elasmobranch fishes *in vivo* by significantly reducing the intestinal bacterial community and observing subsequent changes in metabolites and mRNA expression of a key urea-cycle enzyme, CPS III. If bacteria are responsible for TMAO production, I would expect a decrease in TMAO concentrations in individuals treated with antibiotics. Additionally, if intestinal microbes are responsible for urea hydrolysis, I would expect an increase in urea concentrations or an increase in urea excretion, coupled with a decrease in urea production (i.e. a decrease in CPS III expression) in antibiotic-treated animals. This research will benefit future studies regarding the physiological importance of the elasmobranch microbiome and the use of antibiotics in captive elasmobranchs.

**Model Organism**

*Dasyatis sabina* is an ideal elasmobranch model for *in vivo* study because (1) there is a large body of literature on *D. sabina* physiology, (2) *D. sabina* are both eurythermal and euryhaline and can therefore tolerate a wide range of temperatures and salinities, (3) saltwater populations are well-established and readily available in the Gulf of Mexico, and (4) my lab is experienced with *D. sabina* husbandry and thus *D. sabina* are relatively easy to maintain in captivity. Given these unique characteristics, *D. sabina* is an appropriate as well as abundant model animal to draw conclusions regarding elasmobranch osmoregulatory strategy and the effects of antibiotics on the elasmobranch intestinal microbiome.
Figure 1. Elasmobranch ornithine-urea cycle.

The elasmobranch O-UC as depicted in a hepatic mitochondrion. Enzymes are displayed in pink, substrates in black, and membrane transport proteins in blue. Enzyme abbreviations are listed as follows: glutamine dehydrogenase (GDHase), glutamine synthetase (GSase), carbomoyl phosphate synthetase III (CPS III), ornithine transcarbamoylase (OTCase), argininosuccinate synthase (ASS), argininosuccinate lysate (ASL), and arginase (ARGase).
CHAPTER II – THE OSMOREGULATORY RESPONSE OF ATLANTIC STINGRAY, *DASYATIS SABINA*, TO INTESTINAL MICROBIOME REDUCTION

Introduction

Marine elasmobranchs employ a unique osmoregulatory strategy in which solutes – primarily nitrogenous compounds – are accumulated in the blood plasma and tissues to counteract dehydration due to the osmotic pressure of saltwater. The accumulation of nitrogenous compounds such as trimethylamine \( n \)-oxide (TMAO) and urea (Smith 1936; Pang *et al.* 1977; Yancey and Somero 1979; Ballantyne *et al.* 1987) results in plasma that is iso- or slightly hyper-osmotic with respect to saltwater. Of the total osmolytes present in elasmobranch plasma, urea and TMAO account for roughly 60% of these solutes (Goldstein and Funkhouser 1972; Ballantyne and Robinson 2010); thus, the importance of these compounds for osmoregulation cannot be overstated. Without constant maintenance of high concentrations of urea and TMAO marine elasmobranchs will dehydrate, which is detrimental to organismal function and overall health. Furthermore, without a method for conserving the nitrogen present in urea or synthesizing TMAO elasmobranchs are entirely reliant on dietary sources for these critical nitrogenous compounds. This poses a greater challenge for euryhaline elasmobranchs such as the Atlantic stingray (*Dasyatis sabina*), which travel freely between freshwater and saline environments and must rapidly alter plasma solutes in response to changing salinity.
However, despite the importance of these nitrogenous compounds to osmoregulation, our understanding of urea and TMAO metabolism in elasmobranchs is limited. Given the necessity for elasmobranchs to accumulate large quantities of urea and TMAO, nitrogen retention is the most effective and energetically efficient way for elasmobranchs to maintain osmolality, particularly given that elasmobranchs are nitrogen-limited (Wood et al. 2005). Urease, the enzyme responsible for hydrolysis of urea to ammonia, is a key step in urea-nitrogen scavenging (UNS) and is strikingly absent in elasmobranchs (Wood 1950; Simidu and Oisi 1951; Knight et al. 1988). Similarly, there is conflicting evidence for the presence of an elasmobranch-synthesized trimethylamine (TMA) oxidizing enzyme, TMA oxidase (Tmase) or flavin-containing monooxygenase 3 (FMO3), which is necessary for the production of TMAO (Goldstein and DeWitt-Harley 1973; Seibel and Walsh 2002).

There is, however, evidence of urease and Tmase activity in multiple bacterial taxa. Tmase or bacterial TMA monoxygenase (Tmm) has been conclusively found in *Methylocella silvestris* (Chen et al. 2011) and *Pseudomonas* strain P (Chandler 1983) – a species that was able to use TMA as a sole nitrogen source – as well as species of marine and coastal bacteria including *Ruegeria pomeroyi* and *Citreicella* sp. SE45 (Lidbury et al. 2015), which have critical roles in ocean nitrogen cycles through the remineralization of nitrogen in the form of ammonia. Additionally, homologous sequences of Tmm have been identified in many marine bacterial taxa (Chen et al. 2011). Urease is
not only common among bacteria (Mobley and Hausinger 1989), but has been identified in bacteria within the elasmobranch microbiome (Grimes et al. 1984, 1985; Knight et al. 1988; Youngren-Grimes 1990) in addition to the intestinal microbiome of other vertebrates (Wozny et al. 1977; Suzuki et al. 1979; Robinson et al. 1981).

Because urease and Tmase or Tmm have yet to been identified in elasmobranchs but are readily found in bacteria, I hypothesize that endogenous microbes are primarily responsible for urease and TMA oxidation activity. Given the importance of urea and TMAO to elasmobranch osmoregulation, if bacteria are involved in elasmobranch osmolyte metabolism this will represent a novel relationship previously unseen between aquatic vertebrates and microbes. Further, this would suggest that elasmobranchs are dependent upon endogenous microbes for key osmolyte synthesis and scavenging.

To test this microbe-osmolyte hypothesis in elasmobranchs, I administered a broad-spectrum antibiotic cocktail to captive D. sabina and quantified the resulting changes in osmolyte and metabolite concentrations in a "bacteria-reduced" state over the course of two weeks. If bacteria were responsible for urease activity (i.e. hydrolysis of urea to ammonia), I expected to observe a cessation of urease activity and either an increase in plasma urea concentration or an increase in expelled urea in antibiotic-treated (+AB) individuals. I also explored the effect of bacterial population reduction on the urea cycle by quantifying relative gene expression of the rate-limiting enzyme in the
liver ornithine-urea cycle (O-UC), carbomoyl phosphate synthetase III (CPS III) (Casey and Anderson 1985). If reduction in urea recycling indeed caused an increase in urea concentration or excretion, I hypothesized that urea production would be down-regulated in order to conserve nitrogen in animals with reduced intestinal microbiota. Additionally, if bacteria were responsible for TMAO production given no dietary input, I expected a cessation of TMA oxidase activity and thus a termination of TMAO production in +AB individuals.

From the literature, in vivo studies of UNS using bacterial knockouts have primarily focused on ruminants and their symbiont bacteria (Visek 1978) and this topic has never been explored in an elasmobranch model. Additionally, bacteria may colonize elasmobranchs tissues and thus the viability of creating a true bacterial knockout is complex. This hypothesis is predicated on previous studies by Grimes et al. (1984, 1985) and Mylniczenko et al. (2007), who found evidence of endogenous bacteria residing in tissues and blood, respectively. Because I can more readily guarantee the efficacy of antibiotics on intestinal bacteria, in vivo study will focus on the effects of reducing bacteria in the intestines only. The effect of intestinal bacterial reduction on TMA oxidation or urease activity in elasmobranchs has never been studied in vivo. This experiment seeks to determine the relationship between elasmobranchs and gut microbiota in the absence of exogenous nitrogen inputs by reducing resident bacteria and quantifying subsequent impacts on osmolyte concentration and O-UC enzyme gene expression.
Materials and Methods

*In Silico Analysis of Urease and Tmase/FMO Nucleotide and Protein Sequences*

Before exploring the possibility of bacterial urease activity in elasmobranchs, the absence of an elasmobranch-synthesized urease enzyme had to first be verified. Urease protein and nucleotide FASTA sequences (Table A1) were aligned using the National Center for Biotechnology Information (NCBI) Protein or Nucleotide Basic Local Alignment Search Tool, BLASTp 2.5.0 and BLASTn 2.5.0, respectively (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al. 1997) and SkateBLAST (http://skatebase.org) (Wang et al. 2012; Wyffels et al. 2014). Nucleotide and protein databases were searched for urease sequences using the available NCBI taxonomic tree to first identify known elasmobranch, chordate, and vertebrate urease sequences. Following this initial search, urease sequences were selected from species of bacteria, fungi, and plants: the most abundant urease-positive taxa. Using NCBI BLASTp and BLASTn, urease protein and nucleotide sequences were aligned to elasmobranch sequences (BLAST taxa ID: 7778) using the blastn algorithm for “somewhat similar sequences”. In SkateBLAST, urease sequences were aligned to the available *Batoidea* genome data from the little skate (*Leucoraja erinacea*); transcriptome data from *L. erinacea*, small-spotted cat shark (*Scyliorhinus canicula*); and the holocephalan elephant shark (*Callorhinus milii*); in addition to mitochondrial genomes and genes from *L. erinacea*, the thorny skate (*Amblyraja radiata*), and the ocellate spot skate (*Okamejei kenojei*).
This same procedure was used to identify an elasmobranch-synthesized Tmase enzyme, however, this search was instead directed at FMOs after searches for Tmase-specific nucleotide and protein sequences were unsuccessful. Instead, nucleotide and protein sequences for FMO – the vertebrate hepatic enzyme that monooxygenates compounds including TMA (Lang et al. 1998) – were collected from various vertebrates (Table A2) and aligned to known elasmobranch sequences using methods identical to those used in urease alignments. mRNA sequences were aligned using NCBI tBLASTn 2.5.0 (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al. 1997).

Animal Collection and Holding

*D. sabina* (n=16) were caught via trawl and hook-and-line in the Mississippi Sound by the University of Southern Mississippi (USM) Center for Fisheries Research and Development and the USM Marine Education Center, or donated by local shrimp fisherman. *D. sabina* selection was not based on size or sex because osmoregulatory strategies should be consistent across both sexes; however, only mature individuals were selected because the rigors of the experimental methods might negatively affect smaller individuals disproportionately. Animals were treated for parasitic copepods for three days in a 500-gallon tank dosed with 12.5 mL Dimilin® (Diflubenzuron N-[(4-Chlorophenyl) amino] carbonyl)-2, 6-difluorobenzamide) prior to transport to the Gulf Coast Research Laboratory’s Marine Experimental Research Laboratory (MERL), an enclosed, temperature-controlled facility (23-25°C) with a diurnal
photoperiod that simulates natural conditions. Holding tanks were maintained at 20 ppt using Crystal Sea Marinemix® (Marine Enterprises International, Baltimore, MD, USA) due to the capture conditions of the experimental *D. sabina*; the salinity in the Mississippi Sound during the sampling period was ≤20 ppt. Tank temperature, water quality, and pH were monitored daily to ensure uniformity for the duration of the experiment. Water changes occurred in response to elevated nutrients and pH. Animals underwent a general health assessment and were allowed to acclimate to tank conditions for two weeks prior to the start of the experiment. During acclimation, all animals were fed a diet of locally caught shrimp twice daily until sated. Any uneaten food was removed from the tanks within 15 minutes of feeding. Following the two-week acclimation period, animals fasted for the duration of the experiment to control for dietary inputs of nitrogen and ensure that all nitrogenous osmolytes were synthesized endogenously.

**Experimental Design**

Experiments were carried out in accordance with the USM Animal Care and Use Committee (IACUC protocol #15101510). At the MERL, animals were divided into one of 18 tanks within three stand-alone recirculating systems (Figure 2). Each of these three systems included a head tank, where a bio-filter broke down harmful ammonium to nitrate, and six holding tanks. Water was pumped from the head tank into each holding tank and then returned to the head tank. Because water was treated by the bio-filter before flowing back to individual
Figure 2. Animal holding schematic

Schematic of the *in vivo* experimental design with antibiotic (+AB) and control tanks randomly distributed among three stand-alone recirculating systems.
tanks and each tank housed a single animal, each holding tank was treated as a singular unit. Male (n=6) and female (n=11) *D. sabina* were distributed to randomly assigned control (n=8) and experimental tanks (n=8). Every 24 hours, animals were removed from their holding tanks and placed on a wet cloth on top of a flat surface for subsequent weighing, blood draws, and antibiotic (+AB) or placebo (control) dosing. Animals were weighed (kg) and blood was drawn from each individual prior to the dosing on day one and again on day seven and day 14. Disc width was also measured on day 14. According to tank assignment, animals were dosed with antibiotics or a placebo and then placed back in their respective holding tanks. Time spent out of water was limited to two minutes.

At a minimum, 0.2 mL of blood was drawn from the dorsal wing using a 1mL syringe and 22-gauge needle and transferred to a heparinized vacutainer. Blood samples were then transferred into a 1.5 mL micro-centrifuge tube and centrifuged at 5,000 rpm for 5 minutes to separate plasma from the cellular blood components. Plasma supernatant was transferred into a separate tube, immediately frozen on dry ice, and stored at -80°C until further analysis.

Immediately following blood draw on day 14, animals were euthanized in MS-222 (Tricaine methanesulfonate). Animals were necropsied with autoclave-sterilized tools, which were cleaned with 95% ethanol between samples. Liver samples (≤5 mm section) were collected immediately after sacrifice, flash-frozen on dry ice, and stored at -80°C until further analysis. Whole intestines were removed, rapidly divided into three distinct sections (duodenum, spiral valve, and
colon) using autoclave-sterilized hemostats, and placed in separate sterile Petri dishes. The lumen-side of each section was scraped using a laboratory spatula, placed into individual 1.5 mL microcentrifuge tubes, and immediately frozen on dry ice. Intestinal scrapes were stored at -80°C until further analysis.

Antibiotics

Because there are no published studies on antibiotic efficacy in elasmobranchs, antibiotics were selected in consultation with Dr. Natalie Mylniczenko, DVM and recent studies of the intestinal microbiome in mouse models (Reikvam et al. 2011; Hill et al. 2010). Our protocol closely follows the methods of Reikvam et al. (2011) with the exception of metronadizole, which was eliminated because I was unable to achieve complete dissolution in water. Additionally, this mixture served as a broad-spectrum antibiotic because each chosen antibiotic targeted different bacterial groups: neomycin is an aminoglycoside that affects gram-negative bacteria, ampicillin affects gram-negative and gram-positive bacteria by disrupting the ability of bacteria to construct cell walls, and vancomycin affects gram-positive bacteria that are unresponsive to ampicillin through a similar mechanism of action. Given that species of bacteria from either group synthesize urease or Tmase enzymes, a broad-spectrum approach to microbiome reduction was selected to reduce the community of microbiota within the elasmobranch gut without solely targeting gram-negative or gram-positive bacteria. Control animals received a placebo dose containing only autoclave-sterilized de-ionized (DI) water.
The antibiotic cocktail was made fresh daily and consisted of 100 mg · kg\(^{-1}\) animal weight Neomycin sulfate (Calbiochem), 100 mg · kg\(^{-1}\) animal weight Ampicillin sodium salt (Alfa Aesar), and 50 mg · kg\(^{-1}\) animal weight Vancomycin hydrochloride (VWR) dissolved in autoclave-sterilized DI water and administered in volumes of 1 mL · kg\(^{-1}\) animal weight. Dosages were administered via oral gavage inserted to an area between the gill slits and the stomach to maximize antibiotic absorption to the intestine. Separate 14-gauge gavage needles and 3 mL sterile syringes were used for control and antibiotic groups with care taken to remove all air from the syringe prior to administration. Gavage needles were thoroughly cleaned with 95% ethanol between doses and stored in 95% ethanol when not in use.

**Plasma Osmolality and Osmolytes**

Blood plasma urea (mmol/L), TMAO (mmol/L), and osmolality (mg/kg) were measured using samples collected from each individual at day one, seven, and 14. Aliquots containing 10 μL of plasma were diluted to 1:25 and urea was quantified via colorometric assay (Quantichrom Urea Assay Kit, Biosystems Systems). TMAO was quantified spectrophotometrically (Molecular Devices SpectraMax M2) with aliquots of 15 μL of plasma following the iron-sulfate method (Wekell and Barnett 1991) modified by Raymond (1998) for small plasma volumes. Because TMA is negligible in elasmobranch plasma (Sulikowski *et al.* 2003), TMA was not quantified. Osmolality was measured in 10 μL of plasma.
from each sample using a Wescor VAPRO Vapor Pressure Osmometer (Model No. 5520).

Aliquots of 20 μL plasma from a subset of control (n=4) and +AB (n=6) individuals at day one and day 14 were sent to the West Coast Metabolomics Center (University of California, Davis) for untargeted metabolomic analysis of biogenic amines using published techniques for HILIC-ESI QTOF tandem mass-spectrometry (Feihn et al. 2011).

**DNA Extraction**

DNA was extracted from all frozen gut scrape samples using the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA). Following kit instructions for tissue DNA extraction, 20 mg of scraped tissue was added to 180 μL of Buffer ATL with 20 μL proteinase K. Samples were vortexed and incubated at 56ºC for 30 minutes before proceeding with spin column purification as outlined in the manufacturer’s protocol. Extracted DNA was quantified and verified for purity spectrophotometrically using a Thermofisher Nanodrop (ND-2000). DNA was then frozen at -80ºC until further analysis.

**RNA Extraction and cDNA Synthesis**

Because urea is primarily synthesized in the liver, hepatic tissue was selected as the target tissue for CPS III expression (the rate limiting enzyme in the O-UC). RNA was extracted from 25 mg liver tissue per sample, which was homogenized manually with microcentrifuge tube pestles in 500 μL TRIZol reagent (Invitrogen Corp., Carlsbad, CA). RNA was then extracted using phenol-
chloroform following the manufacturer’s protocol for RNA isolation. Following isolation, 10 μg RNA per sample was purified and concentrated using Zymo Clean and Concentrator columns with DNase I treatment (Zymo Research, Orange, CA). The sample with the lowest RNA yield was used to determine the maximum amount of RNA used for cDNA synthesis, therefore, 0.54 μg purified and DNase-treated RNA from each sample was used to synthesize cDNA. Per reaction, 0.54μg RNA was combined with 0.5 μg random primers adjusted to a final volume of 5 μL. RNA and primers were heated to 70°C for 5 minutes, then placed on ice until addition of reverse transcription (RT) mix. RT mix consisted of 4 μL 5X Go Script Reaction Buffer, 4 μL MgCl₂, 1 μL PCR nucleotide mix, 0.5 μL Recombinant RNasin Ribonucleotide Inhibitor, 1 μL GoScript Reverse Transcriptase, and 4.5 μL nuclease-free water per reaction (Promega, Madison, WI, cat. no. A5000). For each reaction, 15 μL of RT mix was combined with 5 μL of RNA and random primers. RT reactions were carried out as follows: 5 minutes annealing at 25°C, 60 minutes extension at 42°C, followed by reverse transcription inactivation at 70°C for 15 minutes. Liver cDNA was stored at -20°C until further analysis.

qRT-PCR

Quantitative real-time PCR (qRT-PCR) was performed using PowerUp SYBR Green (Applied Biosystems) to determine the efficacy of antibiotics at reducing the microbiome in +AB individuals and the expression of CPS III in liver tissue. From each gut scrape DNA or liver cDNA sample, 2 μL of DNA or cDNA
was added to 18 μL PowerUp SYBR Green master mix (A25742, Applied Biosystems), which included 10 μL of PowerUp SYBR Green master mix, 1 μL of forward primer at 5 μM, 1 μL of reverse primer at 5 μM, and 6 μL of nuclease-free water per reaction. Relative bacterial quantity was assessed in gut scrape DNA using a bacterial target gene (16S V2 region) and a host control gene (fish actin) (Table A3) following similar protocols in mice (Reikvam et al. 2011). CPS III gene expression was assessed in liver cDNA using CPS III primer sequences identified by the Evans laboratory (unpublished data) and 18S as a control gene (Table A4). Each reaction was run in triplicate to verify reading accuracy on MicroAmp Fast Optical 96-well reaction plates (Applied Biosystems) sealed with optical adhesive film (VWR). qRT-PCR conditions were performed and analyzed using a 7500 Fast Real-Time PCR System (Applied Biosystems) and proceeded as follows: an initial denaturation cycle at 50°C for 2 minutes and 95°C for 2 minutes, 40 cycles at 95°C for 15 seconds and 60°C for 1 minute, followed by a dissociation curve.

Statistical Analysis

After assessment for homogeneity and normality of variance, changes in measured osmolytes and animal weight between groups (control and +AB) and between time points (day one, seven and 14) were assessed using a two-factor repeated-measures analysis of variance (ANOVA). Any analysis yielding a p-value <0.05 was considered statistically significant. A post-hoc t-test for paired
two-sample means was performed on all ANOVA tests for which the null hypothesis was rejected.

qRT-PCR results for liver tissue CPS III expression and gut microbiome reduction were analyzed first with an $F$-test to determine equality of variance between control and antibiotic groups. After verification of variance equality, $t$-tests of $\Delta$Ct values (Yuan et al. 2006) were used to determine significant expression differences between control and +AB groups.

Because of the unbalanced number of individuals in control (n=4) and +AB (n=6) groups, an ANOVA could not be performed on the metabolomics data. Instead, both groups were analyzed separately using paired analyses to detect significant differences between plasma metabolites at day one and day 14. Variables that were significantly different in the control group were considered to be caused by starvation and variables that were significant only in the +AB group were considered to be a result of antibiotics and/or a reduction in the host microbiome. Using the peak intensity table generated by the West Coast Metabolomics Center, metabolites were filtered based on interquartile range to eliminate metabolites unlikely to provide significant results. Metabolomics data was then normalized by sum to reduce concentration-related differences and Pareto-scaled (mean-centered and divided by the square root of the standard deviation of each metabolite) to compare all metabolites regardless of absolute value. Filtered and normalized values were analyzed using MetaboAnalyst 3.0 (http://www.metaboanalyst.ca; Xia et al. 2015), a website that allows users to
interrogate metabolomics data for significantly different metabolites using $t$-tests, calculate fold-change of metabolites, and generate volcano plots. In this study, I used paired tests for all analyses with a fold change threshold of 2.0 and $\alpha = 0.05$ representing statistically significance.

Results

**In Silico Analysis of Urease and FMO Nucleotide and Protein Sequences**

All alignments for urease nucleotide or protein within known elasmobranch sequences yielded no meaningful alignment results even with the broadest and most forgiving search parameters. All positive hits for elasmobranch nucleotide or protein sequences in either database had 0-1% query cover with all aligned sequences (Table A1) and were short fragments of <40 base pairs per alignment or <20 amino acids. Additionally, all positive hits had <63% identity, which is significant given the average full urease sequence length is >5,000 base pairs and >500 amino acids. This was true of all urease nucleotide and protein sequences interrogated with all available elasmobranch genomes, transcriptomes, and mitochondrial genome sequences.

Search for an elasmobranch-synthesized FMO yielded promising, though inconclusive, results. SkateBLAST alignments of the holocephalan *C. milii* NCBI-predicted FMO mRNA (NCBI Ref. Seq. XP_007887552.1) with the *S. canicula* transcriptome identified high-identity, low-query cover fragments (<100 bp fragments with $\leq$71% similarity). The predicted FMO protein sequence from *C. milii* did not, however, produce significant alignment results with any
elasmobranch sequences in either NCBI or SkateBASE. To identify the likelihood that the NCBI-predicted *C. milli* FMO sequence is indeed an FMO, *C. milii* sequences were aligned with the sequences of other vertebrate FMOs. *C. milli* FMO mRNA had high-identity, low-query cover alignment with African clawed frog (*Xenopus laevis*) predicted FMO sequence (5% query cover with 75% identity). Additionally, the predicted *C. milii* FMO protein sequence aligned with three predicted FMO protein sequences from the West Indian Ocean coelacanth (*Latimeria chalumnae*) (58% identity with 98% query cover), tongue sole (*Cynoglossus semilaevis*) (48% identity with 97% query cover), *D. rerio* (48% identity with 98% query cover) as well as the *O. mykiss* FMO protein (37% identity with 86% query cover). Further, alignment of the *O. mykiss* FMO nucleotide sequence produced high-identity, low-query cover alignments with the *L. erinacea* transcriptome (<100 bp fragments with ≤93% similarity). All broad FMO nucleotide and protein queries within the NCBI database returned no significant results and positive results were limited to short fragments of <40 base pairs or <20 amino acids per alignment.  

**Antibiotic Efficacy**

Efficacy of antibiotics was measured based on relative quantification of the V2 region of 16S rRNA present in tissue scrapes from three different regions of the intestinal tract: duodenum, spiral valve, and colon. Following the methods outlined by Yuan *et al.* (1998), ΔCt values were compared between +AB and control groups to determine significant changes in relative expression of 16S
rRNA. Fold change was calculated using $2^{-\Delta \Delta Ct}$ values. Using an antibiotic cocktail of neomycin, vancomycin, and ampicillin, the total bacterial load in *D. sabina* was significantly reduced in the spiral valve ($F$-test: $p=0.80$; *t*-test assuming equal variance: *p*-value=$0.03$) (Figure 3) and the colon ($F$-test: $p=0.62$; *t*-test assuming equal variance: *p*-value=$0.003$) (Figure 3). The bacterial community in the duodenum was the only region of the intestine in which antibiotics did not significantly reduce the total bacterial load ($F$-test: $p=0.76$; *t*-test assuming equal variance: *p*-value=$0.15$) (Figure 3).

**Figure 3. Efficacy of antibiotics in *D. sabina* intestine**

Note: 16S rRNA (V2 region) mRNA fold changes (control:antibiotic) in *D. sabina* duodenum, spiral valve, and colon in control and +AB animals. Values marked with an asterisk (*) or two asterisks (**) are significantly different from control animals (*t*-test, *p*<0.05 and *p*<0.01, respectively).
Animal Weight and Plasma Osmolytes

Plasma osmolytes did not change significantly over the course of the experiment, nor was there a significant difference between *D. sabina* dosed with antibiotics and a placebo. Plasma osmolality (two-factor repeated-measures ANOVA, F=0.48, p=0.22) (Figure 4), urea concentration (two-factor repeated-measures ANOVA, F=0.63, p=0.43) (Figure 5), and TMAO concentration (two-factor repeated-measures ANOVA, F=0.63, p=0.44) (Figure 6) were not significantly altered by antibiotics or the two-week experiment period. However, animal weight did significantly change over two weeks (two-factor repeated-measures ANOVA, F=4.04, p=0.03) (Figure 7). Using a *post hoc* t-test for paired two-sample means, *D. sabina* weight was significantly different between day one and day 14 in both control (p=0.03) and antibiotic (p<0.001) groups.

![Figure 4. Plasma osmolality](image)

Note: *D. sabina* blood plasma osmolality (mOsm) over two-weeks of *in vivo* antibiotic or placebo (control) dosage.
Figure 5. Plasma urea concentration

*Note:* *D. sabina* blood plasma urea (mM) over two-weeks of *in vivo* antibiotic or placebo (control) dosage.

Figure 6. Plasma TMAO concentration

*Note:* *D. sabina* blood plasma TMAO (mM) over two-weeks of *in vivo* antibiotic or placebo (control) dosage.
Figure 7. Animal weight

Note: *D. sabina* weight (kg) over two-weeks of *in vivo* control and +AB animals.

**Metabolomics**

Of the 477 metabolites measured in *D. sabina* plasma between the beginning and end of the two-week *in vivo* experiment, 32 metabolites significantly changed among control *D. sabina* (paired *t*-test, *p*<0.05) (Figure 8) (Table 1) and 35 metabolites among antibiotic-treated *D. sabina* (paired *t*-test, *p*<0.05) (Figure 9) (Table 2). Within the control group, 27 significantly changed metabolites were unique to the control group and 30 were unique to the antibiotic group. However, many of these metabolites are unidentified thus no further conclusions can be drawn about their importance to overall elasmobranch physiology and metabolism at this time; the remainder of this analysis will focus on known metabolites. Creatinine and L-citrulline changed significantly in control *D. sabina* plasma: creatinine abundance increased (*t*<sub>3</sub>=3.76, *p*=0.03) and L-
citrulline decreased \( (t_3=-3.72, p=0.03) \). In the antibiotic group, there was a significant increase in 1,2-diamino-2-methylpropane \( (t_5=3.63, p=0.02) \) and a significant decrease in arginine \( (t_5=-2.63, p=0.05) \). Benzoic acid is another metabolite of note, which is the only identified metabolite that significantly increased in \( D. \) sabina regardless of antibiotic dosage. Further, +AB \( D. \) sabina experienced a greater increase (4,049-fold change ± 3,163) in benzoic acid than control individuals (800-fold change ± 421).
**Figure 8.** Heatmap of significant metabolites in control *D. sabina*

Note: *D. sabina* control (n=4) metabolites that changed significantly (p<0.05) between day one (under the red bar) and day 14 (under the green bar).

Table 1

**Significant metabolites in control *D. sabina***

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>t stat</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.40_266.18*</td>
<td>8.83</td>
<td>0.003</td>
</tr>
<tr>
<td>2.60_122.07*</td>
<td>8.30</td>
<td>0.004</td>
</tr>
<tr>
<td>1.04_166.09*</td>
<td>7.41</td>
<td>0.005</td>
</tr>
<tr>
<td>8.53_151.05*</td>
<td>7.28</td>
<td>0.005</td>
</tr>
<tr>
<td>4.72_227.12*</td>
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<tr>
<td>8.20_236.14*</td>
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<td>0.009</td>
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<tr>
<td>7.96_261.14*</td>
<td>5.13</td>
<td>0.014</td>
</tr>
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<td>0.021</td>
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<td>-4.44</td>
<td>0.021</td>
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<tr>
<td>9.88_265.02*</td>
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<td>0.021</td>
</tr>
<tr>
<td>9.46_170.09*</td>
<td>4.39</td>
<td>0.022</td>
</tr>
<tr>
<td>8.59_190.12</td>
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<td>0.025</td>
</tr>
</tbody>
</table>

Note: significant metabolites unique to the control group are marked with an asterisk (*).
Table 1 (continued).

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<thead>
<tr>
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<th>Value 2</th>
<th>Value 3</th>
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<tr>
<td>9.39_351.24*</td>
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<tr>
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<tr>
<td>6.88_120.08*</td>
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</tr>
<tr>
<td>L-Citrulline*</td>
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<td>8.52_84.04_8.52_101.07*</td>
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<td>0.039</td>
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<tr>
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</tr>
<tr>
<td>6.88_160.10*</td>
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<td>Benzoic acid, 3-amino-MH</td>
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<td>9.50_177.12*</td>
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<tr>
<td>1.44_583.26*</td>
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<tr>
<td>4.75_172.12*</td>
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<td>0.049</td>
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</tbody>
</table>

Note: significant metabolites unique to the control group are marked with an asterisk (*).
Figure 9. Heatmap of significant metabolites in +AB D. sabina

Heatmap of +AB D. sabina (n=6) blood plasma metabolites that changed significantly (p<0.05) between day one (under the red bar) and day 14 (under the green bar).
Table 2

significant metabolites in +AB D. sabina

<table>
<thead>
<tr>
<th>Metabolite</th>
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<th>p value</th>
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<tr>
<td>9.39_523.34*</td>
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<td>7.34_215.06*</td>
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<td>6.19_537.17*</td>
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<td>8.80_203.15*</td>
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<td>9.34_772.37*</td>
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<td>0.013</td>
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<tr>
<td>1.32_151.14*</td>
<td>3.75</td>
<td>0.013</td>
</tr>
<tr>
<td>1,2-Diamino-2-methylpropane MH_noMSMS*</td>
<td>3.63</td>
<td>0.015</td>
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<tr>
<td>9.39_349.23*</td>
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<tr>
<td>9.39_175.24*</td>
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<td>1.64_115.09*</td>
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<td>1.43_603.34*</td>
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<tr>
<td>7.97_176.13*</td>
<td>-3.38</td>
<td>0.020</td>
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</table>

Note: significant metabolites unique to the +AB group are marked with an asterisk (*).
Table 2 (continued).

<table>
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<tr>
<th>Compound</th>
<th>ΔCt</th>
<th>p-value</th>
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<tr>
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</tr>
<tr>
<td>6.19_559.15*</td>
<td>-3.28</td>
<td>0.022</td>
</tr>
<tr>
<td>8.67_116.03*</td>
<td>-3.25</td>
<td>0.023</td>
</tr>
<tr>
<td>Benzoic acid, 3-amino-MH</td>
<td>3.12</td>
<td>0.026</td>
</tr>
<tr>
<td>8.03_356.08*</td>
<td>-2.99</td>
<td>0.030</td>
</tr>
<tr>
<td>1.43_311.20*</td>
<td>2.95</td>
<td>0.032</td>
</tr>
<tr>
<td>8.75_159.08_8.75_176.10*</td>
<td>-2.88</td>
<td>0.034</td>
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<tr>
<td>9.73_257.68*</td>
<td>2.85</td>
<td>0.036</td>
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<tr>
<td>9.14_189.16*</td>
<td>-2.78</td>
<td>0.039</td>
</tr>
<tr>
<td>1.45_305.16*</td>
<td>2.77</td>
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<tr>
<td>1.43_589.33*</td>
<td>2.76</td>
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<td>9.04_369.25</td>
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<td>0.046</td>
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<td>7.84_231.17*</td>
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<td>0.046</td>
</tr>
<tr>
<td>Arginine MH*</td>
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<td>0.047</td>
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<td>0.048</td>
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<tr>
<td>8.59_190.12</td>
<td>2.60</td>
<td>0.048</td>
</tr>
</tbody>
</table>

Note: significant metabolites unique to the +AB group are marked with an asterisk (*).

*CPS III Expression in Dasyatis sabina Liver*

Relative expression of CPS III was quantified in liver cDNA using ΔCt values between control and antibiotic groups (Yuan et al. 1998). Fold change
results were calculated using $2^{-\Delta\Delta Ct}$ values. Expression of CPS III in the liver was significantly reduced in *D. sabina* dosed with antibiotics (*F*-test: $p=0.28$; *t*-test: $p=0.02$) (Figure 10).

![Figure 10. Liver CPS III expression](image)

Expression fold change of CPS III mRNA in *D. sabina* liver tissue in control and +AB animals. Values marked with an asterisk (*) are significantly different from control animals (*t*-test, $p<0.05$).

**Discussion**

This is the first study to demonstrate an effective method of reducing the intestinal microbiome and to subsequently explore the effect of an altered gut microbiome on osmoregulation *in vivo*. Additionally, I provide the first evidence that urease and Tmase or an FMO may be absent in elasmobranchs using an *in silico* analysis. This is also, according to current literature, one of only three examples of a metabolomic approach to evaluate elasmobranch health and physiology: the whale shark (*Rhincodon typus*) metabolome was published by
Dove et al. (2012) and a preliminary metabolomic study on the influence of air exposure on *D. sabina* was published by Lambert (2014). Given the efficacy of the antibiotics used in this study, if there is a link between the metabolic processes of gut bacteria and key osmolytes in elasmobranchs I would expect a reduction in the elasmobranch intestinal bacterial assemblage to effect osmolyte concentrations. The use of starved *D. sabina* is particularly compelling because in the absence of dietary nitrogen input, all osmolytes must be synthesized, maintained, and catabolized endogenously.

**Antibiotic Efficacy**

Using an antibiotic concoction of neomycin, vancomycin, and ampicillin, I demonstrated a significant reduction in spiral valve and colon microbiota in +AB *D. sabina*. While I did not observe a significant reduction in the bacterial load in the duodenum, the abundance of bacteria in the duodenum is considerably lower than that of the distal portion of the intestine (Gorbach 1996; O'Hara and Shanahan 2006). Interestingly, the number of bacteria in the duodenum (10\(^1\) - 10\(^3\) viable cells · mL\(^{-1}\)) is roughly equivalent to the number of bacteria found in the stomach (Gorbach 1996; O'Hara and Shanahan 2006) and is thought to be due at least in part to the proximity of the proximal intestine to the highly acidic stomach environment. In comparison, the number of bacteria found in the distal portions of the gut and the colon is between 10\(^{11}\) - 10\(^{12}\) viable cells · mL\(^{-1}\) (Gorbach 1996; O'Hara and Shanahan 2006). As such, I considered these
antibiotics effective at reducing the overall microbiome of the elasmobranch gastrointestinal tract.

Though antibiotic-treated animals did lose a significant amount of weight over two weeks, they did not proportionally lose more weight than the control group. Thus, weight loss is likely solely due to food restriction and not compounded by the use of antibiotics. This is an important observation when considering the implementation of antibiotics *per os* in captive elasmobranchs. This study is the first published demonstration of antibiotic efficacy in elasmobranchs and indicates that this combination, dosage, and method of antibiotic delivery could be used in future investigations of microbiome-reduced *D. sabina*.

**Osmolytes and Osmolyte Metabolism**

Before beginning *in vivo* study, I explored the possibility of an elasmobranch-synthesized urease enzyme *in silico* by aligning urease sequences (Table A1) to known elasmobranch genome databases. Despite utilizing the broadest search parameters, no significantly homologous nucleotide or protein sequence was identified within available elasmobranch genomes. This analysis will remain somewhat incomplete until an entire elasmobranch genome is published, however, the absence of urease in elasmobranchs is not surprising given that a urease enzyme is not found in other vertebrate taxa. Therefore, any urease activity in elasmobranchs is unlikely to be the result of an elasmobranch-synthesized enzyme. Instead, I propose that any urease activity in
elasmobranchs is due to the presence of microbial enzymes. This assumption does not come without precedence: in the gut of ruminants, bacteria in the rumen recycle urea-nitrogen into ammonia, which can then be used as a nitrogen source by other rumen bacteria (Bryant 1959; Mobley and Hausinger 1989; Lapierre and Lobley 2011). Additionally, antibiotics have been shown to interrupt microbial UNSg in the rumen (Prescott 1953; Muscher et al. 2010).

Despite a significant reduction in bacterial populations I did not observe a significant increase in plasma urea concentrations over time. This could be due to a number of compensatory factors, including an increase in urea excretion, a decrease in urea production, or a combination of these two factors. I did not quantify urea excretion given (1) the complexity of urine dilution in the aquatic environment and (2) to avoid trauma caused by catheterization in vivo, however, an increase in urea excretion could explain the consistent urea concentrations found in antibiotic-treated D. sabina. Due to the high levels of urea found in the proximal portion of the elasmobranch gut (Anderson et al. 2009), it is possible that – in the absence of urease – urea cannot be converted to ammonia, which cannot be transported out of the intestine via ammonia transporter, and is excreted as a consequence to maintain steady plasma concentrations. Alternatively, expression of urea transporters in the proximal intestine could be down-regulated to conserve urea, which may be particularly important in starved animals. Determining the expression of urea and ammonia transporters in the
duodenum, spiral valve, and colon of antibiotic-treated animals would be an interesting area of future study.

It is also possible that urea was not transported to the intestine due to a shift in concentration gradient. Studies in ruminants demonstrated a significant decrease in urea diffusion into the rumen when the microbial load was reduced, indicating that microbial urea metabolism may provide a urea-diffusion concentration gradient that is interrupted during antibiotic treatment (Houpt and Houpt 1968; Cheng and Wallace 1979). Additionally, during times of low protein consumption, ruminant urea excretion via urine decreased disproportionally – an 84% decrease in urea excretion with only a 57% reduction in protein (Tebot et al. 2002) – due to increased urea reabsorption in the kidneys (Ergene and Pickering 1978; Leng et al. 1985). This urea-nitrogen balance between host and microbe might also modulate the response of *D. sabina* to decreased dietary nitrogen inputs and possibly the effect of antibiotics: the “critical threshold” of the elasmobranch microbiome could be lower than even the significant bacterial reduction demonstrated by this study. Thus, *D. sabina* could have maintained homeostatic osmolyte concentrations through compensatory mechanisms by both the host and the microbiome.

This regulation and return to homeostatic conditions by the host is supported by the quantification of CPS III mRNA abundance in the liver of control and +AB *D. sabina*. I found that CPS III mRNA was significantly less abundant in +AB animals, which, given that CPS III activity is a proxy for the rate of urea
synthesis by the O-UC, implies a decrease in hepatic urea synthesis. This could also be explained by a decrease in UNSg via microbial urease, which caused an accumulation of urea-nitrogen in elasmobranch tissue and thus, a reduction in requisite urea production. Indeed, in little skate (Raja erinacea) exposed to decreased salinity, a decrease in hepatic CPS III activity coincided with an increase in urea excretion due to a salinity-driven decrease in required urea concentration (Steele et al. 2005). It is also possible that combination of both mechanisms (i.e. an increase in urea excretion and a decrease in urea production) are involved: urea could initially accumulate leading to an increase in urea excretion which could subsequently be counteracted by a decrease in urea production.

The existence of an elasmobranch-synthesized Tmase or FMO was also explored using an in silico analysis with the sequences outlined in Table A2. FMO has been identified genetically in the euryhaline teleost O. mykiss, however, this enzyme has not been identified in elasmobranch taxa using modern genetic techniques. Instead, expression of putative FMO has been passively identified by precursor conversion to TMAO in vitro (Baker et al. 1963; Goldstein and Funkhouser 1972; Goldstein and Dewitt-Harley 1973). As I proposed, given the absence of antibiotics in the incubation media in these studies, TMA oxidation could be due to bacterial enzymes that are still active in vitro and not the result of elasmobranch FMO3 activity. In silico, I found possible evidence of an elasmobranch-synthesized FMO protein. Though high-identity
sequences were relatively short fragments (<100bp), it is certainly possible that these fragments are part of an elasmobranch FMO. However, even with successful cloning of the elasmobranch FMO gene, further verification would be required in order to ensure that this FMO can, in fact, synthesize TMAO: an in-depth procedure that is outside of the scope of this research. However, these findings *in silico* suggest that existence of an elasmobranch FMO is possible and, given that the FMO3 isoform synthesizes TMAO in other vertebrates, could indicate endogenous TMAO production in elasmobranchs. The existence of this elasmobranch FMO clearly warrants further investigation.

Similar to urea, TMAO did not change significantly in *D. sabina* over two weeks given antibiotics and lack of food, although – as in the case of urea – there are a number of explanations for this finding. Elasmobranchs could be robust to perturbations in the microbiome or antibiotics did not significantly reduce specific portions of the bacterial assemblage, namely urease- and Tmase-positive bacteria (i.e. the proposed “critical threshold” of the elasmobranch microbiome). If this “critical threshold” of bacteria is required for biologically-appreciable Tmase function, it is possible that I did not sufficiently reduce Tmase-positive microbiota in order to see a reduction in enzyme activity. Also, given the evidence of bacterial communities endogenous to elasmobranch tissues such as liver, kidney, muscle, (Grimes *et al.* 1984, 1985) and blood (Mylniczenko *et al.* 2007) bacteria within tissues could provide the requisite urease and Tmase activity in the event of intestinal microbiome perturbations. It
is also possible that +AB individuals had already reached a steady-state of urea and TMAO excretion and retention after day seven, in which case I would not have observed any significant difference in osmolyte concentrations. It is surprising that this steady-state continued for 14 days in starved animals given antibiotics, however, Wood et al. (2010) found that fasted S. acanthias maintained plasma urea over 56 days at the expense of muscle nitrogen. The effects of antibiotics immediately following administration in addition to conducting experiments longer than 14 days should be explored in future research. Additionally, salinity challenges presented to antibiotic-treated D. sabina could be used to test the ability of microbiome-reduced individuals to respond to increased salinity (e.g. increased pressure to elevate plasma concentrations of both urea and TMAO).

**Metabolites**

From metabolomic analysis, I found a significant increase in benzoic acid in both control and +AB animals over the course of two weeks. Benzoic acid is found in berries and food preservatives – an unlikely source for elasmobranchs – and is produced by phenylalanine and plant polyphenol metabolism in gut bacteria. Phenylalanine metabolism seems an unlikely source of benzoic acid given the lack of food and the low abundance of phenylalanine (<0.3mM) in elasmobranch tissues to date (Ballantyne 1997). An increase in plant polyphenols also seems an unlikely source given the absence of dietary inputs, however, there is evidence of varying concentrations of polyphenols in ray liver
oil (Sellami et al. 2014). Degradation of polyphenols and other components of the liver during periods of starvation could account for the substantial increase in benzoic acid concentration. As an interesting aside, an increase in polyphenol intake has been correlated with an improvement in non-alcoholic fatty liver disease (Rodriguez-Ramiro et al. 2015). Polyphenols may play an interesting role in elasmobranchs given the high abundance of lipids in the liver (Hayashi and Kishimura 2000; Sellami et al. 2014). Additionally, an increase in benzoic acid could occur due to shifts in gut microbiota community composition toward bacteria that are more efficient at degrading polyphenols (Ktsoyan et al. 2011). Benzoic acid is also the intermediary to many secondary metabolites, which could indicate that metabolic activity is not proceeding efficiently in starved animals. Additionally, hippurate formation – the excreted conjugate of benzoic acid and glycine – is limited to the availability of glycine, a protein precursor that could be limited by starvation conditions and thus limit the excretion of benzoic acid.

In control animals, creatinine increased and L-citrulline decreased significantly. An increase in creatinine could be due to the breakdown of creatine in muscles due to starvation, however, this same increase was not seen in antibiotic animals that also experience starvation. Among its many functions, L-citrulline is a substrate in the O-UC that is synthesized from carbamoyl phosphate and ornithine. L-citrulline is also associated with intestinal functionality: in humans, a decrease in citrulline is indicative of a decrease in
nutrient absorption from the intestine and intestinal failure and could also be indicative of late-stage renal disease or dysfunction (Crenn et al. 2000) as creatinine is cannot be eliminated from the blood. Interestingly, in human subjects with endotoxemia – the presence of endotoxins produced from gram-negative bacteria in the blood – during calorie restriction caused a decrease in citrulline (Poeze et al. 2010). This could indicate that control animals are immuno-compromised by the in vivo conditions, which is further exacerbated by calorie restriction allowing otherwise commensal bacteria to proliferate at the detriment of the elasmobranch host. Interestingly, this same decrease was not observed in fasted S. acanthias (Wood et al. 2010) even after 56 days, however, this could be due to the low concentrations of citrulline present in elasmobranch plasma even pre-starvation.

In D. sabina treated with antibiotics, I observed a significant increase in 1,2-diamino-2-methylpropane and a decrease in arginine. Though not much information is available regarding 1,2-diamino-2-methylpropane, it may be an intermediate of amine synthesis. A significant increase could indicate that amine synthesis is not proceeding efficiently and to completion. Arginine is a substrate in the synthesis of urea and ornithine in the O-UC and is critical for ammonia detoxification. Depletion could indicate that arginine was consumed during the process of urea generation, however, this contradicts my earlier finding that CPS III – the rate limiting O-UC enzyme – is down-regulated. Possibly, prolonged starvation and the absence of UNSg from intestinal bacteria caused an increase
in urea production over time, but arginine stores were exhausted by day 14. Starvation conditions seem a likely explanation as Wood et al. (2010) found a significant decrease in arginine in fasted *S. acanthias* after five days. Possibly, this increase was not observed in control animals due to increased nitrogen loss in antibiotic-treated animals.

**Conclusions**

This study provides compelling future directions for microbiome and metabolomic studies in elasmobranchs. Given the evidence for UNS in other vertebrates and in elasmobranch liver tissue, this is the first study of elasmobranch microbial urease activity *in vivo* and the only exploration of the elasmobranch amine metabolome. Despite compelling evidence for microbial urea metabolism in other vertebrate taxa and evidence of TMAO synthesis in starved elasmobranchs given the conflicting evidence for an elasmobranch-synthesized Tmase enzyme, the observations of this study suggest that the elasmobranch osmoregulatory strategy is robust to perturbations of the microbiome. I postulate that this is due to compensatory mechanisms in elasmobranch hosts, primarily osmolyte conservation. I also demonstrated significant changes in 32 and 36 amine metabolites in control and antibiotic, respectively, during starvation conditions and antibiotic treatment. The association between bacterial metabolites and elasmobranch osmolytes represents a unique relationship that warrants in-depth future study.
Table A1.

**Urease sequences queried**

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<th>Organism</th>
<th>Accession number</th>
<th>Nucleotide</th>
<th>Protein</th>
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<td><em>Agaricus bisporus</em> f</td>
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<tr>
<td><em>Arthroderma benhamiae</em> f</td>
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<tr>
<td><em>Bacillus subtilis</em> b</td>
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<tr>
<td><em>Brucella abortus</em> b</td>
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<td>—</td>
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<td><em>Campylobacter pylori</em> b</td>
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<td>P69996.1</td>
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<tr>
<td><em>Coccidiodes posadasii</em> f</td>
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<td><em>Coccidioides immitis</em> f</td>
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<tr>
<td><em>Corynebacterium glutamicum</em> b</td>
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<tr>
<td><em>Cryptococcus neoformans</em> var.</td>
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<td><em>grubii</em> f</td>
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<td><em>Enterobacter aerogenes</em> b</td>
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<td>P18316.1</td>
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<td><em>Escherichia coli</em> (O157:H7) b</td>
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<td><em>Fission yeast</em> (Saccharomyces</td>
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<tr>
<td><em>pombe</em>) f</td>
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</tbody>
</table>

Note: Urease nucleotide and protein sequences used for *in silico* identification of an elasmobranch-synthesized urease.

Subscripts indicate taxonomic kingdom: bacteria (b), fungi (f), and plant (p).
Table A1 (continued).

<table>
<thead>
<tr>
<th>Organism</th>
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<th>Protein Accession Number</th>
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<tr>
<td>Heliobacter pylori&lt;sup&gt;b&lt;/sup&gt;</td>
<td>AB018202.1</td>
<td>P14916.2</td>
</tr>
<tr>
<td>Jack bean (Canavalia ensiformis)&lt;sup&gt;p&lt;/sup&gt;</td>
<td>M65260.1</td>
<td>P07374.3</td>
</tr>
<tr>
<td>Klebsiella pneumoniae&lt;sup&gt;b&lt;/sup&gt;</td>
<td>L07039.1</td>
<td></td>
</tr>
<tr>
<td>Mouse-ear cress (Arabidopsis thaliana)&lt;sup&gt;p&lt;/sup&gt;</td>
<td>NM_105422.4</td>
<td>Q9SR52.1</td>
</tr>
<tr>
<td>Mycobacterium tuberculosis&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td>P9WFF1.1</td>
</tr>
<tr>
<td>Neosartorya fumigate&lt;sup&gt;f&lt;/sup&gt;</td>
<td></td>
<td>Q6A3P9.2</td>
</tr>
<tr>
<td>Pleurotus ostreatus&lt;sup&gt;f&lt;/sup&gt;</td>
<td>KF312589.1</td>
<td></td>
</tr>
<tr>
<td>Prochlorococcus marinus sub.</td>
<td></td>
<td>Q9L664.1</td>
</tr>
<tr>
<td>Pastoris&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td>P17086.2</td>
</tr>
<tr>
<td>Proteus mirabilis&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td>P17086.2</td>
</tr>
<tr>
<td>Pseudogymnoascus pannorum&lt;sup&gt;f&lt;/sup&gt;</td>
<td>KP195073.1</td>
<td></td>
</tr>
<tr>
<td>Pseusogymnoascus destructans&lt;sup&gt;f&lt;/sup&gt;</td>
<td>KP195072.1</td>
<td></td>
</tr>
<tr>
<td>Rhizoctonia solani&lt;sup&gt;f&lt;/sup&gt;</td>
<td>CAOJ01012310.1</td>
<td></td>
</tr>
<tr>
<td>Rice (Oryza sativa sub. Indica)&lt;sup&gt;p&lt;/sup&gt;</td>
<td></td>
<td>E0ZS48.1</td>
</tr>
<tr>
<td>Solanum tuberosum&lt;sup&gt;p&lt;/sup&gt;</td>
<td>AJ308543.1,</td>
<td>AJ308544.1</td>
</tr>
<tr>
<td>Sporosarcina pasteurii&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td>P41020.1</td>
</tr>
<tr>
<td>Staphylococcus saprophyticus&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td>Q4A0J5.3</td>
</tr>
</tbody>
</table>

Note: Urease nucleotide and protein sequences used for in silico identification of an elasmobranch-synthesized urease.

Subscripts indicate taxonomic kingdom: bacteria (b), fungi (f), and plant(p).
Table A1 (continued).

<table>
<thead>
<tr>
<th>Organism</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Streptococcus salivarius</em></td>
<td>P50047.3</td>
</tr>
<tr>
<td><em>Uncultured rumen bacterium clone</em></td>
<td></td>
</tr>
<tr>
<td>SU134</td>
<td></td>
</tr>
<tr>
<td>SU75</td>
<td></td>
</tr>
<tr>
<td><em>Ureaplasma urealyticum</em></td>
<td></td>
</tr>
<tr>
<td><em>Vibrio parahaemolyticus</em></td>
<td></td>
</tr>
<tr>
<td><em>Yersinia enterocolitica</em></td>
<td></td>
</tr>
</tbody>
</table>

Note: Urease nucleotide and protein sequences used for in silico identification of an elasmobranch-synthesized urease. Subscripts indicate taxonomic kingdom: bacteria (b), fungi (f), and plant(p).

Table A2.

**FMO sequences queried**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Xenopus laevis</em></td>
<td>XM_018253772.1</td>
</tr>
<tr>
<td><em>Xenopus tropicalis</em></td>
<td></td>
</tr>
<tr>
<td><em>Oncorhynchus mykiss</em></td>
<td>EU519462.1</td>
</tr>
<tr>
<td><em>Gallus gallus</em></td>
<td></td>
</tr>
<tr>
<td><em>Sus scrofa</em></td>
<td></td>
</tr>
<tr>
<td><em>Callorhinchus milii</em></td>
<td>XM_007889361.1</td>
</tr>
</tbody>
</table>

Note: FMO nucleotide and protein sequences used for in silico identification of an elasmobranch-synthesized FMO. Taxa marked with an asterisk (*) are NCBI predicted FMO sequences. Accession numbers marked "p" indicates partial FMO sequences.
Table A2 (continued).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession number(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Latimeria chalumnae</em></td>
<td>XP_014352628.1</td>
</tr>
<tr>
<td><em>Danio rerio</em></td>
<td>XP_001332968.4</td>
</tr>
<tr>
<td><em>Cynoglossus semilaevis</em></td>
<td>XP_008331640.1</td>
</tr>
<tr>
<td><em>Homo sapiens</em></td>
<td>AH006707.2</td>
</tr>
<tr>
<td>Mus musculus</td>
<td>BAA03745.1</td>
</tr>
<tr>
<td><em>Pan troglodytes</em></td>
<td>AH012919.2</td>
</tr>
<tr>
<td><em>Bos Taurus</em></td>
<td>AF488421.1^p</td>
</tr>
</tbody>
</table>

Note: FMO nucleotide and protein sequences used for in silico identification of an elasmobranch-synthesized FMO. Taxa marked with an asterisk (*) are NCBI predicted FMO sequences. Accession numbers marked "^p" indicates partial FMO sequences.

Table A3.

Antibiotic efficacy qRT-PCR primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA V2 region</td>
<td>Forward</td>
<td>5’-AGYGGCGIACGGGTGAGTAA-3’</td>
</tr>
<tr>
<td>16S rRNA V2 region</td>
<td>Reverse</td>
<td>5’-CYIACTGCTGCCTCCCCGTAG-3’</td>
</tr>
<tr>
<td>Fish actin</td>
<td>Forward</td>
<td>5’-TCGTCACTGGACTCTGGTGATGG-3’</td>
</tr>
<tr>
<td>Fish actin</td>
<td>Reverse</td>
<td>5’-CTCCTGCTCAAGTCCAGTG-3’</td>
</tr>
</tbody>
</table>

Note: qRT-PCR primers for 16S quantification of bacteria in *D. sabina* gut scrapes from control and antibiotic-dosed animals (*Reikvam et al. 2011*).
Table A4.

Liver CPS III qRT-PCR primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPS III</td>
<td>Forward</td>
<td>5’-ATATCCTGTGGCCTTTGACG-3’</td>
</tr>
<tr>
<td>CPS III</td>
<td>Reverse</td>
<td>5’-GAAAACTGCGATCAAGGTGGT-3’</td>
</tr>
<tr>
<td>18S</td>
<td>Forward</td>
<td>5’-GTTAATTCCGATAACGAACGAGACTC-3’</td>
</tr>
<tr>
<td>18S</td>
<td>Reverse</td>
<td>5’-ACAGACCTGTTATGGCTCAATCTCGTG-3’</td>
</tr>
</tbody>
</table>

Note: qRT-PCR primers for quantification of CPS III expression in D. sabina liver tissue from control and antibiotic-dosed animals.
APPENDIX B – IACUC Approval Letter

NOTICE OF COMMITTEE ACTION

The proposal noted below was reviewed and approved by The University of Southern Mississippi Institutional Animal Care and Use Committee (IACUC) in accordance with regulations by the United States Department of Agriculture and the Public Health Service Office of Laboratory Animal Welfare. The project expiration date is noted below. If for some reason the project is not completed by the end of the approval period, your protocol must be reactivated (a new protocol must be submitted and approved) before further work involving the use of animals can be done.

Any significant changes should be brought to the attention of the committee at the earliest possible time. If you should have any questions, please contact me.

PROTOCOL NUMBER: 15101510 (Replaces 13121201)
PROJECT TITLE: Initial Characterization of the Elasmobranch Microbiome and Potential Roles in Host Physiology
PROPOSED PROJECT DATES: 10/2015 - 09/2018
PROJECT TYPE: Renewal
PRINCIPAL INVESTIGATOR(S): Andrew Evans
DEPARTMENT: Coastal Sciences
FUNDING AGENCY/SPONSOR: N/A
IACUC COMMITTEE ACTION: Full Committee Approval
PROTOCOL EXPIRATION DATE: September 30, 2018

Frank Moore, PhD
IACUC Chair

Date 10/01/2015
REFERENCES


Chandler SR. 1983. The utilization of methylamine-nitrogen by the methazotrophic bacterium *Pseudomonad P*. [dissertation] [Berkshire (UK)] University of Reading.


Ferer EJ. 2007. Salinity effect on urea and TMAO levels in blood plasma of atlantic stingray, Dasyatis sabina [thesis]. [Pensacola (FL)]: University of West Florida.


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