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Lauren Nicole Fuller

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DESCRIPTION AND ALLEVIATION OF THE STRESS RESPONSE IN ATLANTIC  
SHARPNOSE SHARKS (*Rhizoprionodon terraenovae*), WHITE-SPOTTED BAMBOO  
SHARKS (*Chiloscyllium plagiosum*), AND GOLDEN SHINERS (*Notemigonus crysoleucas*)

A Dissertation  
presented in partial fulfillment of requirements  
for the degree of Doctor of Philosophy  
in the Department of Biology  
The University of Mississippi

by

LAUREN N. FULLER

AUGUST 2019



## ABSTRACT

Sharks are an essential component of many marine ecosystems; however they have experienced population declines, mainly attributed to overfishing and capture of sharks as bycatch. Despite sharks often being released when captured as bycatch, the act of capture can result in a stress response which may cause severe physiological perturbations. Studies have investigated the physiological impacts of capture stress on elasmobranchs, but most have primarily focused on the secondary stress response. I conducted a series of studies to further describe the primary stress response through quantification of adrenocorticotrophic hormone (ACTH) while also investigating methods, such as sedation and limiting air exposure, to reduce the magnitude of the stress response to capture. Results implicated ACTH as a reliable indicator of the primary stress response in elasmobranchs, which I suggest as an additional measurement to go along with the suite of physiological stress indicators that are commonly measured in elasmobranchs. I also demonstrated the severe effect of air exposure on elasmobranch physiology and suggest that fishers limit sharks to no more than five minutes of air exposure during catch-and-release fishing. Lastly, I show the potential of iso-eugenol sedation at reducing levels of stress indicators. These studies have combined the disciplines of conservation and physiology in an effort to provide methods and results that can be utilized in conservation management of shark populations.

## LIST OF ABBREVIATIONS AND SYMBOLS

°C	Degrees Celsius
$\chi^2$	Test statistic in chi-squared statistical analysis
$\beta$	Slope
%	Percent
1 $\alpha$ -OHB	1alpha-hydroxycorticosterone
$\alpha$	Significance level
ACTH	Adrenocorticotrophic hormone
ANCOVA	Analysis of covariance
BD	Becton, Dickson and Company
cm	Centimeter
CO <sub>2</sub>	Carbon dioxide
CRH	Corticotropin-releasing hormone
df	Degrees of freedom
dL	Deciliter
DO	Dissolved oxygen (mg/L)
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
<i>F</i>	Test statistic in ANOVA
FDA	Food and Drug Administration

g	Gram
GAS	General adaptation syndrome
GOM	Gulf of Mexico
hr	Hour
HSD	Honestly significant difference
HPI	Hypothalamic-pituitary-interrenal axis
L	Liter
m	Meter
mg	Milligram
ml	Milliliter
mmol	Millimole
mOsm	Milliosmole
MS-222	Tricaine mesylate
NER	Nictitating eyelid reflex
O <sub>2</sub>	Oxygen
<i>P</i>	Probability value used in null hypothesis testing
pH	Measure of acidity; concentration of hydrogen ions
ppt	Parts per thousand
PVC	Polyvinyl chloride
rpm	Rounds per minute
SE	Standard error

*t* Test statistic for the t-test

YSI Yellow Springs Instrument

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## TABLE OF CONTENTS

Abstract.....	ii
List of abbreviations and symbols .....	iii
Acknowledgements.....	vi
List of tables.....	ix
List of figures.....	x
<b>Chapter 1: Introduction .....</b>	<b>1</b>
Background.....	1
Study site.....	6
Focal species .....	7
<b>Chapter 2: Circulating ACTH, lactate, and osmolality in relation to capture stress in Atlantic Sharpnose sharks (<i>Rhizoprionodon terraenovae</i>) .....</b>	<b>9</b>
Abstract.....	9
Introduction.....	10
Methods.....	13
Results.....	16
Discussion.....	23
Conclusions.....	28
<b>Chapter 3: The effects of air exposure on the stress response of blacktip sharks (<i>Carcharhinus limbatus</i>).....</b>	<b>30</b>
Abstract.....	30
Introduction.....	31
Methods.....	33
Results.....	35
Discussion.....	40
Conclusions.....	45
<b>Chapter 4: Effects of iso-eugenol on stress and respiration rates of Atlantic sharpnose sharks (<i>Rhizoprionodon terraenovae</i>), white-spotted bamboo sharks (<i>Chiloscyllium plagiosum</i>), and golden shiners (<i>Notemigonus crysoleucas</i>) .....</b>	<b>46</b>
Abstract.....	46
Introduction.....	47
Methods.....	49
Results.....	55
Discussion.....	65
Conclusions.....	70

**Chapter 5: Recommendations to managers, fishers, and scientists.....72**  
List of references.....75  
Appendix.....88  
Vita.....93

## LIST OF TABLES

### **Chapter 2**

Table 1. Description of release behavior rating .....	15
Table 2. Description of <i>R. terraenovae</i> utilized in this chapter .....	16

### **Chapter 3**

Table 3. Description of <i>C. limbatus</i> utilized in this chapter .....	35
---	----

### **Chapter 4**

Table 4. Description of <i>R. terraenovae</i> utilized in this chapter .....	55
Table 5. Description of <i>N. crysoleucas</i> utilized in this chapter .....	63

### **Appendix A**

Table A. Summary of statistical results .....	89
---	----

## LIST OF FIGURES

### Chapter 1

Fig. 1. Image of specimen collection site .....	7
---	---

### Chapter 2

Fig. 2. Comparison of ACTH ELISA standard curves .....	18
Fig. 3. Effect of time spent on line on ACTH .....	19
Fig. 4. Effect of temperature on ACTH .....	20
Fig. 5. Effect of time spent on line on lactate .....	21
Fig. 6. Effect of time spent on line on osmolality .....	22
Fig. 7. Effect of lactate on release behavior.....	23

### Chapter 3

Fig. 8. Difference in lactate across air exposure durations .....	37
Fig. 9. Difference in glucose across air exposure durations .....	38
Fig. 10. Difference in release behavior across air exposure durations .....	39
Fig. 11. Effect of air exposure on NER .....	40

### Chapter 4

Fig. 12. Image of respiration chamber for <i>R. terraenovae</i> .....	50
Fig. 13. Image of respiration chamber for <i>C. plagiosum</i> .....	53
Fig. 14. Change in lactate over time in <i>R. terraenovae</i> .....	57
Fig. 15. Difference in glucose across treatments in <i>R. terraenovae</i> .....	58
Fig. 16. Change in hematocrit over time in <i>R. terraenovae</i> .....	59
Fig. 17. Difference in respiration rate across treatments in <i>R. terraenovae</i> .....	60
Fig. 18. Effect of treatment on release behavior in <i>R. terraenovae</i> .....	61
Fig. 19. Difference in respiration rate across treatments in <i>C. plagiosum</i> .....	62
Fig. 20. Difference in ventilation rate across treatments in <i>C. plagiosum</i> .....	63
Fig. 21. Difference in respiration rate across treatments in <i>N. crysoleucas</i> .....	64
Fig. 22. Difference in late respiration rate across treatments in <i>N. crysoleucas</i> .....	65

## CHAPTER 1: BACKGROUND

A keystone species has been defined as a species that, if removed from the ecosystem, would cause dramatic trophic changes. As top predators in the world's oceans, some sharks can be considered keystone species, such that upon their removal, the ecosystem balance would be shifted. Some impacts of large sharks being removed have already been observed. Removal of great sharks (those that are top predators rather than meso-predators) from the oceans has the potential to result in a substantial trophic cascade and predator release on meso-predators (Myers et al., 2007; Ferretti et al., 2010). In the past few decades, there has been an increase in the number of anthropogenic stressors impacting sharks. The International Union for Conservation of Nature states that 25% of all shark and ray species are threatened with extinction. Stevens et al. (2000) reviewed the decline of sharks, skates, and rays and attributed it to overfishing and lack of regulations for bycatch from commercial fisheries. Many sharks caught as bycatch are kept to harvest their valuable fins, further perpetuating their decline. Of particular importance is the stress associated with capture and handling as a result of commercial fisheries.

To define a concept such as stress, it would be more useful to first define a stressor. A stressor is anything that threatens to disrupt the homeostasis of an organism. It can be physical (injury or temperature change), chemical (changes in O<sub>2</sub> or CO<sub>2</sub> concentration in the environment), or perceived (predatory or competition) (Moyle and Cech, 2004), but all will

initiate a similar cascade of responses, indicating that the stress response is nonspecific (Selye, 1973). The general adaptation syndrome (GAS), also known as a stress response, consists of three stages: (1) alarm stage, (2) resistance stage, and (3) fatigue stage (Selye, 1951). When a stimulus is perceived as a stressor, the alarm stage (or primary response) is generated by the central nervous system, involving release of corticotropin releasing hormone (CRH) from the hypothalamus. This activates the pituitary, which releases adrenocorticotrophic hormone (ACTH) to stimulate the adrenal gland. When activated, the adrenal medulla releases catecholamines and the adrenal cortex releases glucocorticoids. Glucocorticoids are species-specific; fish and some mammals typically produce cortisol, while amphibians, reptiles, birds, and some mammals typically produce corticosterone. The circulating catecholamines and glucocorticoids prepare the body for a 'fight or flight' response. The persistence of the stressor causes the resistance stage (or secondary response) where the animal is mobilizing energy stores in an attempt to escape the threat. This can involve (1.) an increase in plasma lactate, a byproduct of the anaerobic metabolism of glucose, (2.) an increase in glucose due to glycogenolysis in the liver stimulated by glucocorticoids, (3.) alterations in hematocrit levels due to the disruption of osmosis, and 4.) a decrease in pH as a result of lactate accumulation (Hoffmayer and Parsons, 2001). The effects of a prolonged state of fight or flight on the body will cause the fatigue stage (or tertiary response) and possibly an emergency life history stage. During the fatigue stage, the animal has prolonged mobilization of energy reserves from the periphery and can involve decreased growth, reproduction, disease resistance, and overall survival, due to energy and resources being directed elsewhere for immediate survival (Wendelaar Bonga, 1997; Skomal and Mandelman, 2012). The emergency life history stage is when a stressor has sustained effects on the organism through

alterations of their normal behaviors to those that will allow for the survival of the organism (Wingfield et al., 1998).

Similar to a stress response, allostasis has been defined as “an adaptive process for actively maintaining stability through change” (Korte et al., 2005). Mediators, such as glucocorticoids and catecholamines, work to maintain stability through their effects of energy mobilization, increased heart rate, and increased respiratory rate. An allostatic load can occur when the stress response is activated too often or for unnecessary reasons, and over time it can cause damage to the organism (McEwen and Wingfield, 2003). Depending upon previous experiences, individuals may have different allostatic loads, affecting how they respond to future stress events which can result in individual variation in the stress response.

Human population size has been and continues to rise, increasing the chance of human-animal interactions. Humans can inflict a variety of anthropogenic stressors on animals, which often negatively impact their populations. Biodiversity is most threatened by these anthropogenic factors: 1.) habitat loss and fragmentation, 2.) overexploitation, 3.) spread of invasive species and diseases, 4.) pollution, and 5.) climate change (Soule, 1991). For example, Steller’s sea lion (*Eumetopias jubatus*) populations have been declining since the 1960’s mainly due to anthropogenic stressors such as competition with humans for food, bycatch, and hunting (Atkinson et al., 2008). Similarly, amphibian populations have declined due to the spread of chytridiomycosis, a fungal pathogen that has emerged, in part, due to anthropogenic introduction (Daszak et al., 2007). Elasmobranchs (sharks, skates, and rays) are also greatly affected by anthropogenic stressors. Overexploitation and bycatch have been cited as factors having the largest impact on shark populations (Stevens et al., 2000). Many studies have documented the negative effects of capture stress on elasmobranch physiology (Hoffmayer and Parsons, 2001;

Manire et al., 2007; Mandelman and Skomal, 2008; Frick et al., 2010; Brooks et al., 2012; Hoffmayer et al., 2012; Marshall et al., 2012; Gallagher et al., 2014; Jerome et al., 2018), behavior (Skomal, 2007; Guida et al., 2016; Hyatt, 2016; Bouyoucos et al., 2017; Raoult et al., 2019), and survival (Moyes et al., 2006; Skomal, 2007; Braccini et al., 2012; Kneebone et al., 2013; French et al., 2015).

When sharks are hooked during fishing, the general adaptation syndrome is initiated. Upon capture, sharks react by thrashing and struggling (Hoffmayer and Parsons, 2001) and with great disruptions to their physiology (Skomal, 2007). Due to difficulties in isolating and measuring the unique primary stress hormone of elasmobranchs, 1 $\alpha$ -hydroxycorticosterone (1 $\alpha$ -OHB) produced from the interrenal tissue, little work has been conducted on corticosteroid changes during the primary response. The steroid hormone 1 $\alpha$ -OH-B is a corticosteroid that is very similar to corticosterone with the modification of a hydroxyl group on the 1st carbon in the alpha orientation (Anderson, 2012). The most common method of measuring stress in elasmobranchs is to analyze levels of plasma glucose, lactate, hematocrit, pH, and osmolality as an indicator of the secondary stress response, or resistance stage. These studies have suggested that the severity of the response to stressors is species-specific (Mandelman and Skomal, 2008) and can vary depending on the season/water temperature (Hoffmayer et al., 2012). Little research has been conducted on the tertiary, or prolonged, stress response of elasmobranchs due to the logistics associated with long-term study, with the exception of studies observing short-term survival after release (Skomal, 2007; Hoolihan et al., 2011; Danylchuk et al., 2014). However, negative impacts have been shown in studies with teleosts, such as suppression of important immune functions after prolonged exposure to a stressor (Tort and Mackenzie, 2002). This



indicates that further research is necessary, especially for species of elasmobranchs that are commonly caught as bycatch.

The field of conservation physiology was officially termed in 2006 and is described as the study of physiological responses to anthropogenic stressors that might contribute to population declines (Wikelski and Cooke, 2006). This field is interdisciplinary and seeks to unite physiologists and conservation managers to result in informed conservation planning.

Conservation physiology studies may include topics such as investigations into the effects of infectious disease (Blaustein et al., 2012), stress (Wingfield et al., 1997), and climate change (Farrell et al., 2008) on the physiology, endocrinology, and whole organism performance, all of which can provide insight into population stability (Cooke and O'Connor, 2010), however, a handful of challenges with conservation physiology have been identified. The aforementioned study highlights the importance of physiological biomarkers that directly relate to the fitness of the animal, studies conducted in the field rather in a laboratory, and the translation of physiological results into conservation management plans. This dissertation involves studies intended to investigate the physiological effects of anthropogenic stressors, while also aiming to link these effects to the overall condition and/or fitness of the animals.

Despite the plethora of studies on the effects of capture stress on elasmobranchs, most of these studies focus on the secondary stress response and do not investigate methods to reduce capture stress. An understanding of the primary effects of the stress response and learning new methods for mitigating capture stress is important for the conservation of elasmobranchs by increasing the chance of post-release survival. This dissertation involves experiments that are focused on further describing the primary stress response and determining methods to reduce the magnitude of stress in response to capture. Specifically, this study was built around the following

objectives: (1) Understand how capture stress affects the primary stress response via ACTH quantification, (2) understand the effects of air exposure commonly experienced during capture on the physiology of elasmobranchs, and (3) determine if iso-eugenol sedation is effective at reducing the stress response to capture.

## STUDY SITE

All studies were conducted around Horn Island off of the Mississippi coast in the Gulf of Mexico (GOM) travelling out of the Gulf Coast Research Labs in Ocean Springs, MS. Sampling typically focused around the western tip of the island (approximately 30°14'40.55"N, 88°46'11.52"W) due to a higher abundance of *R. terraenovae* and *C. limbatus* in this location (Figure1). Horn Island is a barrier island in the northern GOM that has a westward migration (Fritz et al., 2007) with substantial historical land loss on the eastern tip. Horn Island holds a broad diversity of plants, invertebrates, and vertebrates (Richmond, 1962) and is one of a chain of barrier islands in the northern GOM that provides protection from waves and storms to the continental coastline (Feagin et al., 2010).



**Figure 1.** Shark collection was focused on the western tip of Horn Island off of the Mississippi coast.

## FOCAL SPECIES

Two species of elasmobranch were selected as models for these studies, the Atlantic sharpnose shark (*Rhizoprionodon terraenovae*) and the blacktip shark (*Carcharhinus limbatus*), both members of the requiem shark family, or Carcharhinidae. These species were selected due to their abundance in the northern Gulf of Mexico and because they are commonly captured as bycatch by commercial and recreational fishers. The selection of *R. terraenovae* was also intended to provide comparison with previous studies investigating the stress response in this species (Hoffmayer and Parsons, 2001; Hoffmayer et al., 2012, 2015). Blacktip sharks were selected for investigation into the effects of air exposure because this species is frequently targeted by recreational fishers due to their highly active response to capture. White-spotted bamboo sharks (*Chiloscyllium plagiosum*) were selected as a benthic comparison to *R. terraenovae* and because they are easily kept in captivity. Golden shiners (*Notemigonus*

*crysoleucas*) were selected as a teleost comparison to both *R. terraenovae* and *C. plagiosum* and because they are readily available at bait shops.

CHAPTER 2:  
CIRCULATING ADRENOCORTICOTROPIC HORMONE LEVELS, LACTATE LEVELS,  
AND OSMOLALITY IN RELATION TO CAPTURE STRESS IN ATLANTIC SHARPNOSE  
SHARK, *Rhizoprionodon terraenovae*

ABSTRACT

Incidental capture of sharks during commercial and recreational fishing is of major conservation concern because of the potential effects it can have on physiological stress responses and survival. Endocrine aspects of the stress response are, however, poorly understood in elasmobranchs because of difficulties in measuring the primary glucocorticoid (1 $\alpha$ -hydroxycorticosterone). Here, we combined measures of plasma adrenocorticotrophic hormone (ACTH), the highly conserved pituitary hormone responsible for stimulating the release of adrenal/interrenal glucocorticoids, with measures of plasma lactate, osmolality, and behavior to gain a greater understanding of the capture stress response in Atlantic Sharpnose sharks, *Rhizoprionodon terraenovae*. Individuals were subject to a non-repeated blood sampling protocol in which blood samples were obtained following exposure to capture stress for < 3 min (designated baseline), and 15, 30, 45 and 60 minutes, after which behavior was categorized during release. Results revealed that ACTH was significantly higher at 15, 30, 45, and 60 minutes than at baseline. Lactate levels were highest at 45 and 60 minutes whereas osmolality did not differ significantly among the sampling periods. Lactate was the only variable that

significantly predicted the shark's behavior upon release with higher lactate levels correlating with sluggish behavior upon release. Measurements of stress indicators are important in understanding the effects of capture on shark populations, which has been implicated in population declines.

## INTRODUCTION

Fishes often show large behavioral and physiological responses to the stress of capture and handling when compared to other vertebrates (Skomal, 2007). Incidental bycatch as a result of commercial and recreational fishing is thought to pose a significant conservation risk to elasmobranchs, even when animals are rapidly released, because it can alter the stress physiology and behavior of these animals and potentially increase mortality (Hoffmayer and Parsons, 2001; Marshall et al., 2015; Morgan and Burgess, 2015). Studies utilizing satellite tags after release have demonstrated that even when sharks are released alive after capture, they exhibit variable and species-specific rates of mortality. For example, while great hammerheads (*Sphyrna mokarran*) had low (54%) survival rates four weeks after capture, the tiger shark (*Galeocerdo cuvier*) had a survival rate of 100% (Gallagher et al., 2014). One of the aspects of addressing bycatch-induced mortality is a consideration of the capture-induced stress response. While the link between acute capture stress and survival is not well established, documenting the stress response and behavior at release may inform us regarding post-capture survival. Toward this end, the description of the physiological changes that take place during the stress response may be critically important to shark conservation.

The general adaptation syndrome (GAS) (Hans Selye, 1950) describes a suite of non-specific responses to a stressor, such as capture. The GAS consists of three stages: (1) alarm, (2)

resistance, and (3) fatigue (Selye, 1965). When a stimulus is perceived as a stressor, the alarm stage is marked by the release of catecholamines from the central nervous system and corticotropin releasing hormone (CRH) from the hypothalamus. Corticotropin releasing hormone activates the anterior pituitary to stimulate release of adrenocorticotrophic hormone (ACTH) from the pituitary gland which stimulates glucocorticoid production from adrenal/interrenal tissue. Increased levels of circulating catecholamines and glucocorticoids released during the alarm stage mediate the 'fight or flight' response. The persistence of the stressor results in transition to the resistance stage during which elevated glucocorticoids mobilize energy stores that can be allocated towards escaping the threat. Chronic stress often suppresses growth, reproduction, and immune responses and can lead to the depletion of peripheral energy stores, fatigue and reduced survival (Bonga, 1997; Skomal and Mandelman, 2012). For example, a study on brown trout (*Salmo trutta*) demonstrated that experimentally elevated cortisol levels resulted in significantly increased rates of mortality due to disease along with reduced growth of the gonadal tissue in both sexes (Pickering, 1989). Similarly, a study on eastern fence lizards (*Sceloporus undulatus*) involving experimental elevation of glucocorticoids resulted in lower hatching success and survival of offspring (MacLeod et al., 2018).

Several studies have examined physiological responses of sharks to capture stress. For example, capture stress results in an increase in anaerobic metabolism and a cascade of other physiological changes (Hoffmayer and Parsons, 2001; Mandelman & Skomal, 2008; Frick et al., 2010). Elevations in lactate can alter the physiology of a shark in a way that negatively affects survival. Upon capture, the animal will accumulate an oxygen debt through a drastic increase in activity or by failing to meet the regular energy demands of cellular respiration. This failure may lead to the production of lactate, a decline in blood pH, and an alteration in the structure of

proteins (Dumetz et al., 2008). However, little is known about endocrine aspects of the stress response in sharks.

Historically, there was no commercially available labeled and unlabeled forms of the unique interrenal glucocorticoid produced in this group (i.e.,  $1\alpha$ -hydroxycorticosterone, or  $1\alpha$ OHB, derived from hydroxylation of corticosterone, Lambert, 2014) and a lack of antibodies against this hormone that are required to develop an assay for quantification (Anderson, 2012). However, a monoclonal antibody against  $1\alpha$ -OHB has been recently developed (Wheaton et al., 2018), along with an assay for measurement of this hormone but it is not commercially available. Several studies (Rasmussen and Crow, 1993; Karsten and Turner, 2003; Manire et al., 2007) have quantified corticosterone, but because it is not the primary glucocorticoid produced by elasmobranchs it is unclear whether measures of this hormone accurately reflect the endocrine stress response. Therefore, a new approach is necessary to obtain a better understanding of the direct effects of stress on the alarm stage of the GAS.

To examine the endocrine stress response during capture in elasmobranchs, we measured levels of ACTH, which, in contrast to glucocorticoids, is conserved across chondrichthyans and other vertebrates (Costa et al., 2004). We combined measures of ACTH with measures of plasma lactate and osmolality in Atlantic Sharpnose Sharks, *Rhizoprionodon terraenovae*. This species is relatively abundant in the northern Gulf of Mexico and frequently captured as bycatch. We used a standard capture stress protocol to understand the potential impact of bycatch on ACTH levels, lactate levels, and osmolality. We also asked how capture stress affects release behavior.



## METHODS

### *General Procedures*

Sampling was conducted from May-August in 2017 and 2018 in the Mississippi Sound of the Gulf of Mexico. *Rhizoprionodon terraenovae* were collected via baited hook-and-line between 0800 and 1800 hours with chum as an attractant. When a shark was hooked, it was immediately brought to the surface for identification. A subset of sharks was sampled immediately after capture to obtain an approximation of baseline values for all measured variables. Blood samples were collected within 2-3 minutes of capture and may not represent a true baseline value. Sharks not subjected to immediate blood sampling were released and allowed to swim while hooked on the unspooled line for randomly (with a random number generator, [www.random.org](http://www.random.org)) assigned time periods of: 15, 30, 45, or 60 minutes. These sharks were then brought on the boat and rapidly (within three minutes) sampled for up to 1 ml of blood via caudal venipuncture with a Becton, Dickson and Company (BD) 21G 1" Vacutainer Eclipse blood collection needle (catalog no. 368650) and BD brand EDTA treated vacutainer (catalog no. 367835). Each shark was sampled in the same manner across all treatments to account for the stress caused by blood collection. Blood loss from being hooked could not be accounted for and could have influenced concentrations of stress indicators. Standard length (measured from the tip of the rostrum to the caudal peduncle), fork length (from the tip of the rostrum to the fork in the caudal fin), and stretched total length (from the rostrum to the tip of the upper lobe of the caudal fin) were then measured in centimeters. Weight (kg) was also measured by gently placing the animals in a bucket suspended on a hanging scale. Water temperature (°C) at the site of collection was recorded with an optical dissolved oxygen probe, the Yellow Springs Instrument

(YSI) ProODO Optical Dissolved Oxygen Instrument (SKU no. 626281). The shark was then released and post-release behavior was recorded using a subjective ranking based on its behavior immediately after release, which depending on condition, ranged from a few seconds to a minute (Table 1; modified from Manire et al., 2001). Post-release behavior was observed and recorded by the same individual for each shark to avoid inter-observer bias. All blood samples were kept on ice until they were brought to the laboratory, after which they were centrifuged (3,400 rpm, 4 minutes) and plasma was retained and kept in a -80°C freezer until analyzed for ACTH, lactate, and osmolality. All procedures were approved by the University of Mississippi's Institutional Animal Care and Use Committee (protocol # 15-002).

#### *Quantification of ACTH, lactate and osmolality*

*ACTH.* Undiluted plasma from 25 *R. terraenovae* (run in duplicate) was thawed and analyzed for ACTH using a commercially available ELISA kit (Cusabio Biotech, Houston, TX, Product Code: CSB-E15926FH). All 25 samples were assayed with a single kit. Validation of the kit was done by comparing the slope of the standard curve constructed as per the recommendation of the manufacturer to the slope of a second standard curve spiked with 10 µl of pooled *R. terraenovae* plasma as is (n = 20) and a third curve constructed with 10 µl of deionized water that replaced shark plasma to account for the volume of plasma in the spiked standard curve. Using pooled plasma from 20 individuals, increased the probability of detecting cross-reactivity. Curves consisted of 5 points (representing different ACTH concentrations) ranging from 75 to 1200 pg/ml that were each constructed in duplicate. Use of this assay requires confirmation that the ELISA antibody only binds to ACTH, and does not cross-react with other

plasma constituents. The observation that there were no statistical differences in the slopes of the standard curves would validate the assay for use in this species.

*Lactate and osmolality.* Plasma lactate concentrations were measured with a commercially available lactate kit (Eton Bioscience Inc., San Diego, CA, SKU #1200011002 ). Osmolality of the plasma samples (run in duplicate) was measured via use of an Osmette freezing-point depression osmometer (Precision Instruments Inc., Natick, MA).

### *Statistical analyses*

Analysis of covariance (ANCOVA) was utilized to test for significant differences in slopes of the three standard curves. Welch's t-tests were utilized to determine if there were significant differences in levels of ACTH, lactate, or osmolality between sexes. ANCOVA and Tukey HSD tests were used to determine if ACTH, lactate, and osmolality differed over the five sampling times and to determine if covariates (sex, weight, temperature, and time of day of sample collection) explained a significant amount of variation. The time that the shark was kept on the line was coded as a factor to compare parameters among time periods. An ordinal logistic regression model was utilized to determine if release behavior (factored as an ordinal categorical variable) could be predicted by duration of the stressor (time on line), ACTH levels, osmolality, and lactate levels. All analyses utilized a 95% level of significance and were performed in R version 3.5.1. (R Core Team, 2018) using car (v2; Fox and Weisberg, 2011), doBy (v4.6-2; Højsgaard and Halekoh, 2018), rms (v5.1-3.1.; Harrell, 2019), ggplot2 (Wickham, 2016), and sciplot (v1.1-1.; Morales, 2017) packages.

**Table 1.** A description of release behavior rating (subjective score between 0 and 5) based on the behavior of the shark observed from a few seconds to a minute post-release. Table was modified from Manire et al. (2001).

Rating	Behavior Displayed
--------	--------------------

- 0 Sank to bottom without swimming, no movement during revival or post-release
- 1 Sank to bottom without swimming, but exhibited some movement during revival
- 2 Exhibited weak swimming movements
- 3 Slowly swam away at surface
- 4 Did not remain at surface while swimming away
- 5 Quickly swam down into the water

## RESULTS

Twenty-five sharks were captured by hook-and-line and utilized in this study (5 per treatment) including 18 males and seven females (Table 2). Sampling took place between May and August of 2017 and 2018. There was no significant difference between the concentrations of ACTH ( $t(2.5)=0.24$ ,  $p=0.83$ ), lactate ( $t(2.13)=-0.4$ ,  $p=0.72$ ), or osmolality ( $t(1.8)=-1.36$ ,  $p=0.32$ ) between sexes, therefore, males and females were pooled for all analyses. All sharks were hooked in the mouth and did not show signs of impairment or injury upon capture. Sharks typically responded to being allowed to swim freely on the line by swimming around with minimal exhibition of an additional burst escape response. Blood samples were returned to the lab for processing after a 2-10 hour delay. This delay was required because blood was collected at sea and was returned to the lab for same-day processing. The delay in blood sample processing may have contributed to variation around the mean of measured parameters but this would not affect the overall interpretation of the results.

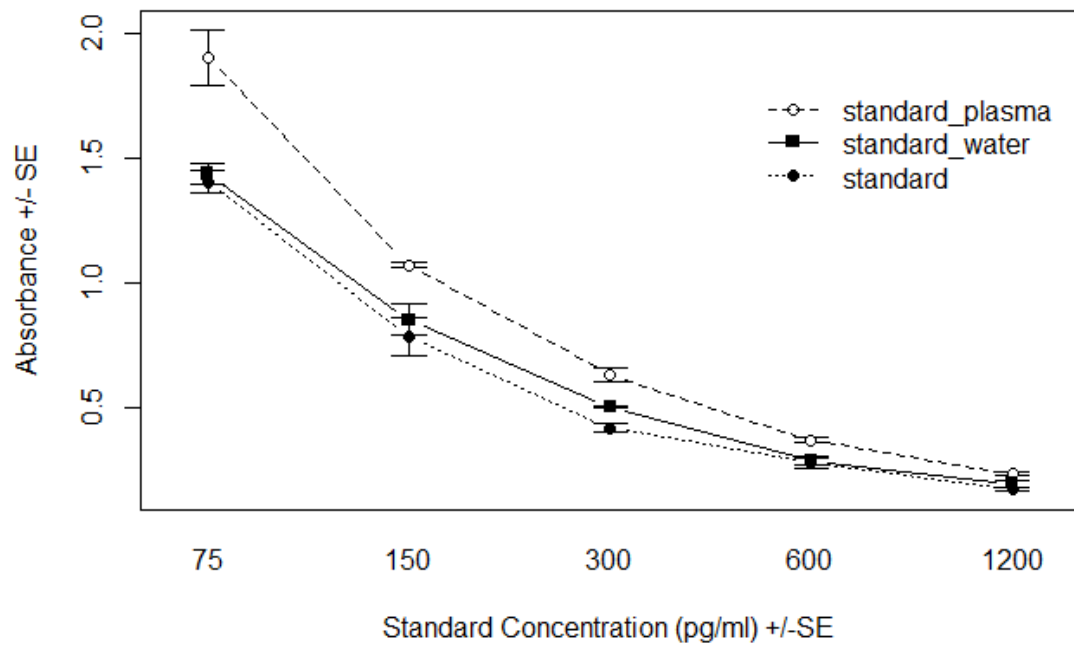
**Table 2.** Description of *R. terraenovae* collected for this study.

Time on Line	n	Sex Proportion (M/F)	Mean Total Length (cm) ± SE	Mean Weight (g) ± SE
0	5	5/0	81.2 ± 3.6	3137.5 ± 508.4

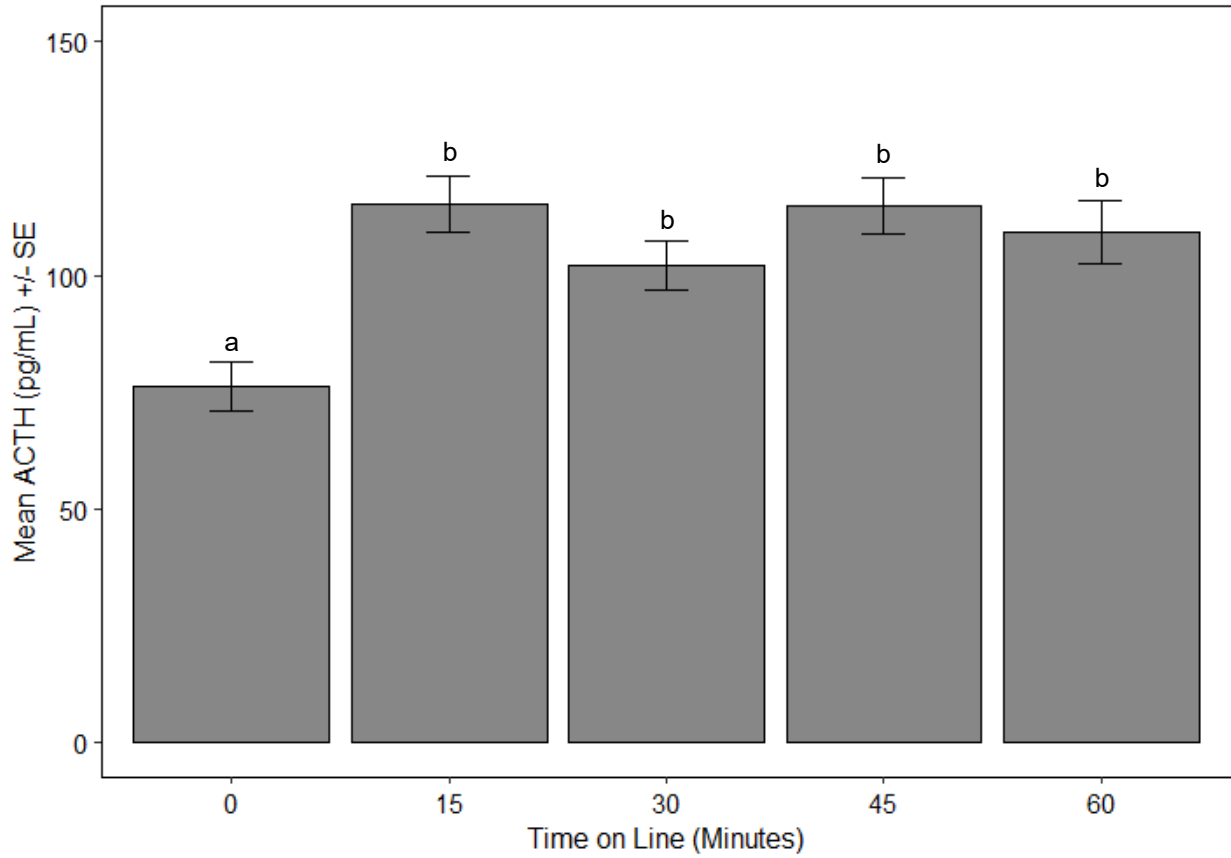
15	5	3/2	74.8 ± 4.9	1737.8 ± 407.7
30	5	3/2	72.7 ± 5.6	1602.5 ± 446.1
45	5	4/1	74.7 ± 4.5	1570.5 ± 385.05
60	5	3/2	74.5 ± 4.3	1686.2 ± 296.4
Total	N=25	18/7	75.7 ± 1.9	1910.1 ± 203.3

### *ACTH*

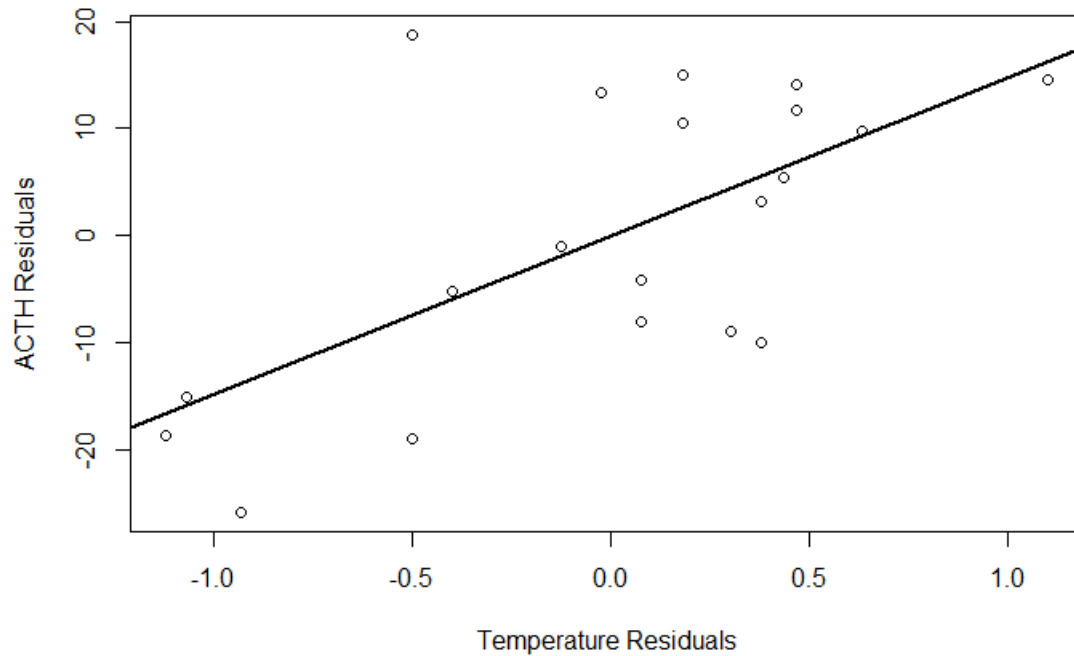
There was no evidence of heterogeneity of slopes among the standard and validation curves ( $F(2,36)=1.78$ ,  $p=0.18$ ; Figure 2). The standard mixed with plasma displayed higher concentrations of ACTH, as expected. While there was no significant difference between the standard alone and the standard mixed with water, it was expected that the diluted standard would exhibit lower concentrations of ACTH. This was not the case, possibly due to variation or pipetting error. The intra-assay coefficient of variation was calculated to be 5.27%, indicating negligible variation between duplicates. Adrenocorticotrophic hormone concentrations varied significantly over the sampling periods ( $F(4,15)=8.63$ ,  $p=0.0008$ ). Specifically, ACTH was higher after 15, 30, 45, and 60 minutes compared to baseline (0 minutes) ( $p=0.0004$ ,  $p=0.024$ ,  $p=0.002$ ,  $p=0.022$ , respectively) (Fig. 3). There was a significant relationship between ACTH and temperature in which higher temperatures were associated with higher ACTH levels ( $F(1,15)=12.06$ ,  $p=0.003$ ; Figure 4). Adrenocorticotrophic hormone concentrations were not significantly affected by sex ( $p=0.29$ ), weight ( $p=0.45$ ), or time of day of sample collection ( $p=0.17$ ).



**Figure 2.** Parallel relationship between three standard curves of ACTH: 1.) Standard only (represented by filled circles, 2.) Standard mixed with a pooled plasma sample from 20 *R. terraenovae* (represented by white circles) collected from the Mississippi sound of the Gulf of Mexico, and 3.) Standard mixed with deionized water (represented by boxes). Absorbance was measured at a wavelength of 450 nm.



**Figure 3.** The differences in ACTH concentration (pg/ml) of *R. terraenovae* captured from the Mississippi Sound in the Gulf of Mexico that were allowed to swim freely on the line for different sampling time intervals (n=5). Concentrations include +/- 1 standard error. Significant comparisons ( $p < 0.05$ ) indicated by different letters.

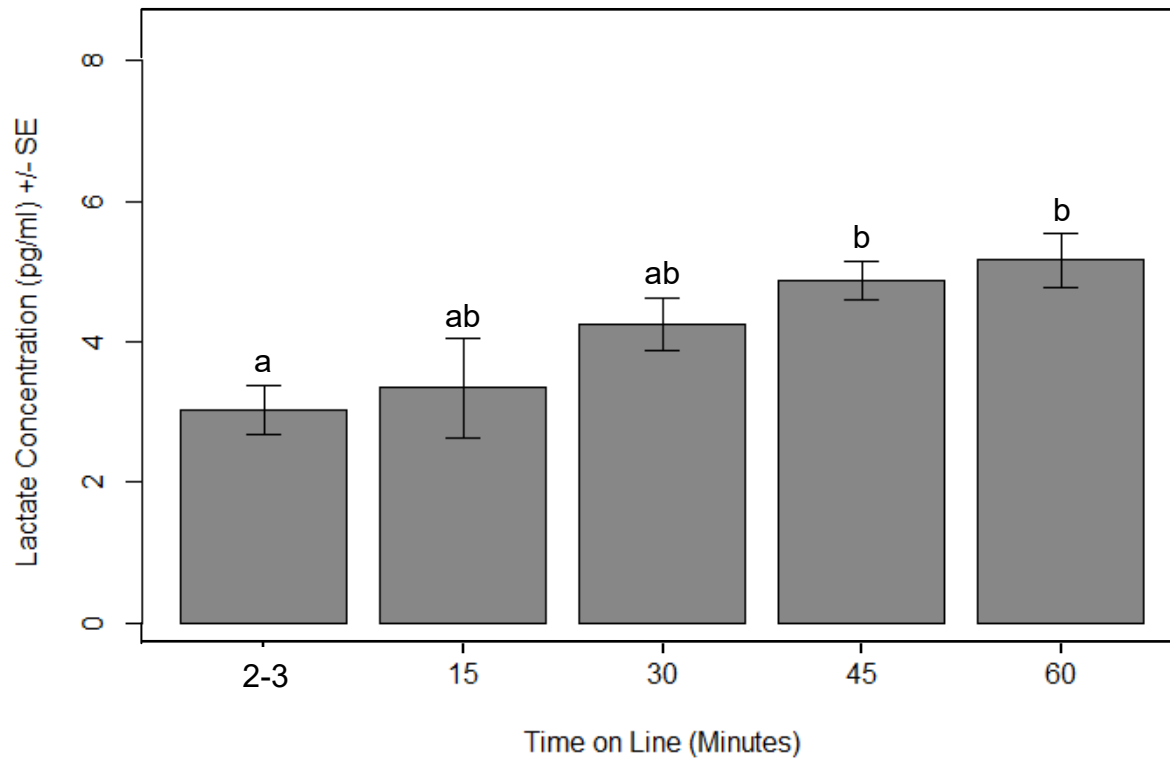


**Figure 4.** The relationships between ACTH (pg/ml) of *R. terraenovae* captured from the Mississippi Sound in the Gulf of Mexico and temperature (°C) at the site and time of collection ( $p < 0.05$ ;  $N = 21$ ). Axes represent regressed residuals of temperature and ACTH concentrations.

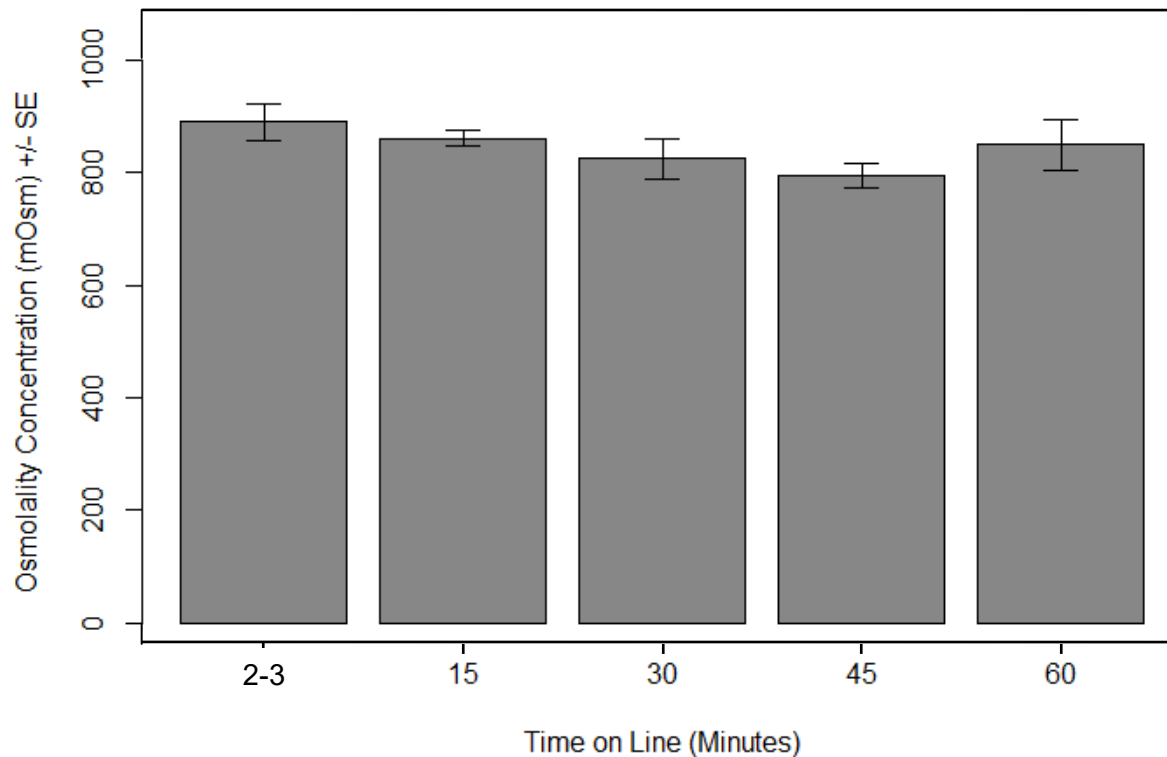
#### *Lactate and osmolality*

Plasma lactate concentrations differed significantly among treatments ( $F(4,20) = 4.60$ ,  $p = 0.008$ ). Lactate concentrations were significantly higher than baseline at both 45 and 60 minutes ( $p = 0.04$  and  $0.01$ , respectively) (Figure 5). Lactate concentrations were not significantly affected by sex ( $p = 0.43$ ), weight ( $p = 0.83$ ), temperature ( $p = 0.75$ ), or time of day of sample collection ( $p = 0.28$ ). Osmolality did not differ significantly among treatments ( $F(4,12) = 1.5832$ ,  $p = 0.24$ ) (Figure 6). However, osmolality was positively affected by the time of sample collection and temperature ( $F(1,12) = 7.0995$ ,  $p = 0.02$ ;  $F(1,12) = 31.0490$ ,  $p = 0.0001$  respectively). Osmolality was not affected by the other covariates, sex ( $p = 0.67$ ) and weight ( $p = 0.85$ ).





**Figure 5.** The relationship between lactate (pg/ml) of *R. terraenovae* captured from the Mississippi Sound in the Gulf of Mexico and time interval that the shark was held on the line ( $p < 0.05$ ;  $n = 5$ ). Significant comparisons ( $p < 0.05$ ) indicated by different letters and all other pairwise contrasts were nonsignificant ( $p \geq 0.05$ ).

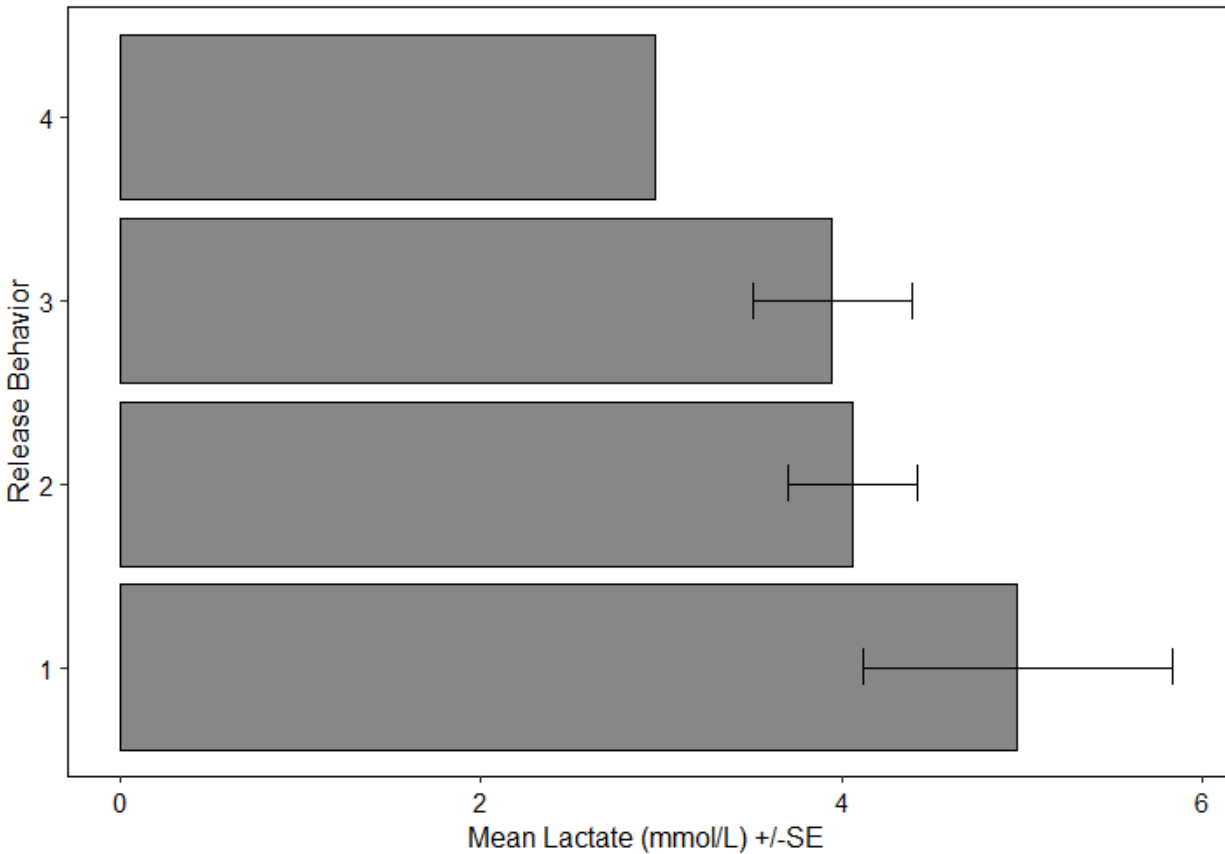


**Figure 6.** Changes in plasma osmolality of *R. terraenovae* captured from the Mississippi Sound in the Gulf of Mexico between time intervals that the sharks were held on the line (n=5). Bars include +/- 1 standard error. There was no significant difference in osmolality over time ( $p \geq 0.05$ ).

### *Release behavior*

No sharks had a release behavior score of 0, two sharks had a score of one, nine sharks had a score of two, nine sharks received a score of three, one shark received a score of four, and zero sharks had a score of 5. There was no significant relationship between the duration of the stressor and release behavior ( $\chi^2(4) = 4.25$ ,  $p = 0.37$ ). Levels of ACTH ( $\chi^2(1) = 1.74$ ,  $\beta = 0.04$ ,  $p = 0.19$ ) and plasma osmolality ( $\chi^2(1) = 1.03$ ,  $\beta = 0.01$ ,  $p = 0.31$ ) were also not significant predictors of release behavior. Lactate significantly predicted release behavior ( $\chi^2(1) = 7.73$ ,  $\beta = -2.1$ ,  $p =$

0.005). An inverse relationship was observed between these variables, such that individuals with higher lactate levels exhibited lower release behavior ratings (Figure 7).



**Figure 7.** The relationship between lactate concentration (mmol/L) of *R. terraenovae* captured from the Mississippi Sound in the Gulf of Mexico and release behavior ( $p < 0.05$ ;  $N=21$ ), which was coded on a scale of 0-5. Concentrations include +/- 1 standard error.

## DISCUSSION

### *ACTH*

During the alarm stage of the stress response, the recognition of the stressor stimulates the release of corticotropin-releasing hormone (CRH) from the hypothalamus, which binds to the anterior pituitary. This in turn results in the release of ACTH from the anterior pituitary. Adrenocorticotrophic hormone binds to the interrenal gland and stimulates the release of

glucocorticoids into the circulation, resulting in glucose increase in the blood. This cascade results in energy mobilization, aiding in homeostatic maintenance in response to the stressor.

Despite the importance of ACTH in this hormonal cascade, there is a surprising dearth of information on ACTH levels in the vertebrate response to stress. To our knowledge, this is the first study that has quantified ACTH in response to stress in elasmobranchs. For example, a literature search using Scopus revealed only six studies that quantified ACTH in response to stress; three in teleosts (Sumpter and Donaldson, 1986; Arends et al., 1999; Long et al., 2019) and three in mammals (Coe, 1978; Liberzon et al., 1997; Gadek-Michalska et al., 2019). Our results indicate that circulating levels of ACTH were higher (120 pg/ml) 15 minutes after capture in *R. terraenovae* and remained elevated for the subsequent sampling periods, which is expected to correspond to increased glucocorticoid production. Studies in birds indicate that blood samples collected within 2-3 minutes of capture represent baseline levels of glucocorticoids that can rapidly change following this time period (Wingfield et al., 1982; Romero and Wingfield, 2001; Romero and Reed, 2005). If glucocorticoids increase significantly from baseline in 3 minutes, then upstream increases in ACTH are likely to be detected more rapidly. This capture stress protocol is commonly used in studies of this nature, however, caution must be exercised when interpreting these as baseline values. We obtained blood samples as early as practically possible but these may not represent the lowest, un-stressed values. Nevertheless, the initial stress indicators were significantly lower than later time periods. In studies involving salmonids, ACTH was increased (50 pg/ml) from baseline after two minutes of handling and confinement stress and continued to increase for the duration of a 60 minute stressor (up to 140 pg/ml; Sumpter and Donaldson, 1986), similar to our findings. Similar results have also been found in rats where ACTH is significantly higher (150 pg/ml) five minutes after a stress event and

continued to rise for 30 minutes thereafter (up to 280 pg/ml; Liberzon et al., 1997). Arends (1999) did not observe a maximum ACTH level in the sea bream (*Sparus aurata*) until one hour after the stressor, whereas, in our study, we observed maximum ACTH levels after 15 minutes of exposure to the stressor. This could be due to temperature differences in our study (25-28° C) compared to the Arends (1999) study (18-22° C) or due to species differences.

Feedback inhibition can occur wherein elevated glucocorticoid levels inhibit further release of ACTH. Coe (1978) observed glucocorticoid feedback inhibition on ACTH release two hours after the stressor in squirrel monkeys (*Saimiri sciureus*). However, inhibition was not apparent in this study, indicated by the continued elevation of ACTH. If ACTH measurement had continued after the 60 minute period in this study, feedback inhibition may have been observed. Although there was no significant relationship between ACTH and the behavior of the sharks upon release, the persistent capture stress resulted in elevation of the stress response over the 60 minute sampling period.

### *Lactate*

No studies have investigated the relationship between indicators of the alarm stage of the GAS with indicators of the resistance stage. However, several studies have examined the resistance stage, describing elevations in lactate levels in fishes after a stressor. For instance, peak lactate levels (10 mmol/L) were observed in the gummy shark, *Mustelus antarcticus*, three hours after capture and lactate returned to baseline levels (0.5 mmol/L) 24 hours post-stress (Frick et al., 2012). Lactate levels also peaked (9 mmol/L) three hours after a capture stress event in juvenile sand tiger sharks, *Carcharhinus taurus*, and were significantly higher than baseline levels up to 12 hours post-stress (Kneebone et al., 2013). The elevated lactate values at 45 (5.6

mmol/L) and 60 (5.9 mmol/L) minutes in this study are supported by other studies as well. For example, *R. terraenovae* showed a significant increase in lactate (11 mmol/L) from baseline (1 mmol/L) after 30 minutes (Hoffmayer et al., 2015). Similarly, the sea bream, *Sparus auratus*, had higher lactate levels (1.5 mmol/L) 30 minutes after an air exposure event (Arends et al., 1999).

Our results revealed a relationship between lactate and release behavior, in which sharks with higher lactate levels scored lower on the release behavior scale. While this relationship may be strengthened by a higher sample size, similar results have been observed in other studies as well. For example, lactate levels were seen to be significantly higher in moribund sharks compared to sharks in good condition (Moyes et al., 2006; Marshall et al., 2012) and elevated lactate has been associated with impairment of reflexes (Jerome et al., 2018). The results of the present study suggest that elevated lactate affects the physiology of sharks by altering body chemistry, which is something that has been seen to affect post-release survival in other studies.

### *Osmolality*

Capture stress may lead to a disruption of the ability to osmoregulate in aquatic animals. Elasmobranchs possess unique osmoregulatory ability in that they are able to regulate internal ion concentrations via gill tissues but also through use of the rectal gland (MacLellan et al., 2015). This allows sharks to regulate their internal osmolality as a mechanism to compensate for fluxes in water between the shark and its environment. The increased energetic and respiratory demand during a stress response will result in increased ventilation, perfusion of the gills with blood, and the recruitment of additional gill lamellae. This may result in an increase in osmotic influx of water into the animal when in a hypotonic environment. The *R. terraenovae* sampled

from the Mississippi Sound in this study would have all been in a dilute environment, therefore, we would expect to see declines in osmolality when the animal was subjected to stress in a way to impact its ability to maintain homeostasis. Despite this, we found no evidence of a significant difference in osmolality between sampling periods which was likewise observed in the same species by Hoffmayer et al. (2015) and in *M. antarcticus* (Guida et al., 2016). The possibility of recovery at the later time periods could explain the lack of an expected significant decline in osmolality. Alternatively, significant differences in osmolality may have been observed for stressors applied for periods longer than 60 minutes. It is also possible that the salinity of the collection site had an influence on the osmolality of the shark that may have negated an effect of the stressor. However, there was a positive effect of temperature and time of day of sample collection on osmolality values, in which lower osmolality values were seen earlier in the day and at lower temperatures. This indicates that the sharks had a reduced ability to maintain a steady osmolality under these circumstances, potentially due to the presence of a stressor. Many studies have demonstrated that animals are subject to circadian rhythms of glucocorticoid levels, with levels peaking shortly before the active period of the day (Kuhn et al., 1986; Breuner et al., 1999; Dickmeis, 2008). It is possible that the circadian rhythm of glucocorticoids may explain the time of day effect on osmolality in this study. Similarly, the effect of temperature on osmolality could have also been due to a circadian rhythm of glucocorticoids since lower osmolality values were associated with lower temperatures experienced earlier in the day. While there have been studies on the effects of salinity on osmoregulation (Bryne et al., 1972; Cramp et al., 2015), the literature on the effects of other environmental parameters, such as temperature, are lacking. This highlights the need for studies that examine the effects of environmental parameters on osmoregulatory ability in elasmobranchs.

## CONCLUSIONS

Our validation of this commercial ELISA for quantification of ACTH in this species expands the toolkit available to researchers. These results provide the shark physiologist the means to easily assess the primary stress response in this group. Our results indicate an increase in ACTH within the first 15 minutes that remained elevated for the duration of the 60 minute stressor in Atlantic Sharpnose sharks. This provides insight into aspects of the endocrine stress response that was not previously available in elasmobranchs. Additionally, we show an increase in lactate levels over the course of the 60 min sampling period that corresponded to poor release behavior. This provides a potential link between a physiological indicator of stress and post-release survival. Future studies should examine how the stress response of sharks affects survival to better understand how capture stress associated with incidental bycatch impacts shark populations.

Conservation physiology is defined as “the study of physiological responses of organisms to human alteration of the environment that might cause or contribute to population declines” (Wikelski and Cooke, 2006). Our study of the fundamental stress indicators presented here provide the framework for potentially understanding the link between anthropogenic stress and species preservation. These results can inform researchers on the health of species. Our validation and quantification of ACTH represents a major step in understanding the alarm stage of the stress response in this species. For example, the ability to identify elevated ACTH levels provides a means for documenting chronic levels of stress that can be important in many conservation biology applications. Additionally, elevated lactate resulting from the stress response may reduce survivorship or ultimately alter fitness. Studies that combine quantification of physiological stress indicators and



estimates of post-release survival are necessary to understand the full impact of capture stress on elasmobranchs.

CHAPTER 3:  
THE EFFECTS OF AIR EXPOSURE ON THE STRESS RESPONSE OF BLACKTIP  
SHARKS, *Carcharhinus limbatus*

ABSTRACT

Air exposure is a common stressor that aquatic animals may experience during capture. Air exposure in fishes could result in collapse of the gills, the prevention of gas exchange and could result in the accumulation of lactate and other waste products in the blood. Although studies have investigated the effects of air exposure on both teleosts and elasmobranchs, few have utilized multiple durations of exposure. Therefore, we subjected blacktip sharks (*Carcharhinus limbatus*) to 0, 5, 10, or 15 minutes of air exposure and examined various blood parameters. We likewise tested the nictitating eyelid reflex (NER) after air exposure and categorized behavior during release. Results revealed that lactate was significantly higher after 10 and 15 minutes of air exposure compared to baseline levels. Release behavior was poorer after five, 10, and 15 minutes of air exposure compared to baseline and 15 minutes of air exposure resulted in complete impairment of the NMR. These results suggest that for blacktip sharks and perhaps other closely related species, fishers should limit exposure to air to no more than five minutes during capture in order to provide the highest chance of survival after release

## INTRODUCTION

Upon capture by commercial and recreational fishers, sharks are exposed to a variety of stressors, such as hook injury, fight time, handling, and exposure to air. Air exposure could occur during both commercial and recreational fishing while the remainder of the catch is being sorted, during photo opportunities, and while measurements are being obtained (Ferguson and Tuft, 1992). Fishers often view sharks as dangerous, so they are typically cautious during gear retrieval or even allow the shark to tire on the deck of the boat before handling. It is likely that exposure to air will result in effects upon the physiology of aquatic animals. The lamellae of the gills, the primary site of gas exchange in fishes, are suspended and separated due to water flowing through the gills. During air exposure the lamellae collapse onto each other which disrupts gas exchange so that new oxygen cannot diffuse into the blood and waste products cannot be released, resulting in an oxygen debt. This would force them to enter anaerobic respiration, resulting in a build-up of the byproduct lactate, which would acidify the blood and lead to significant physiological perturbation (Cicia et al., 2012). Because waste products would be unable to escape from the gills during air exposure, sharks would also experience a build-up of CO<sub>2</sub> in the blood, resulting in further acidification and a potential Bohr shift that decreases the affinity of hemoglobin for binding oxygen (Gingerich et al., 2007). Therefore, not only would the sharks be unable to acquire oxygen through gas exchange, they may also have lower efficiency of oxygen transport to oxygen starved tissues. This stress could result in severe physiological disturbance and increased mortality after capture.

A handful of studies have considered the effects of air exposure on teleosts that are frequently captured by fishers. Three minutes of air exposure resulted in a 50 fold increase in cortisol of gilthead sea bream (*Sparus aurata*) as well as increases in levels of plasma glucose and lactate (Arends et al., 1999). This indicates that air exposure elicits a stress response in fish that then leads to the secondary, metabolic stress response. Studies have also reported disturbances due to air exposure can lead to mortality in fishes. For example, increased mortality rates were noted after 30 minutes of air exposure and behavioral impairment (startle behavior and orientation) after 10 minutes of air exposure (Davis and Parker, 2004). Studies have also demonstrated that air exposure after a bout of exercise, typical of a capture event, exacerbates the stress response (Suski et al., 2007). Likewise, higher lactate and CO<sub>2</sub> were observed in the blood of rainbow trout (*Onchorynchus mykiss*) that had been aerially exposed after exercise (Ferguson and Tuft, 1992). Studies examining the physiological response of elasmobranchs to the stress of air exposure have reported elevations in plasma lactate along with changes in other physiological parameters (Frick et al., 2010; Cicia et al., 2012; Lambert et al. 2018). An increase in the temperature difference between water and air temperatures (thermal gradient) has also been indicated as having a compounding effect on the severity of the stress response (Cicia et al., 2012).

Measures of post-release survival can be difficult and costly when dealing with elusive marine animals. Despite this, there are a few studies that tracked movement and short term survival of elasmobranchs after capture (Moyes et al., 2006a; Afonso and Hazin, 2014; Gallagher et al., 2014; Hutchinson et al., 2015; Eddy et al., 2016, etc.). Other studies have quantified behavior during release and tested reflexes as a measure of condition of the shark (Manire et al., 2001; Gallagher et al., 2014; Jerome et al., 2018; Raoult et al., 2019). The nictitating eyelid

reflex (NER) is commonly used in fisheries (Musyl and Gilman, 2018) and involves observation of the movement of the nictitating eyelid over the eye. The addition of survival measurements, whether direct or indirect, to studies investigating the effect of capture stress can help to form a link between physiological measures of stress and fitness.

Blacktip sharks (*Carcharhinus limbatus*) are often targeted by recreational sport fishers due to their vigorous reaction when hooked. These sharks are common in Gulf of Mexico waters which makes them a good target for fisheries and for this study. In this study, we endeavored to answer the following questions: (1.) How does air exposure affect the stress response? (2.) Do environmental and individual variables explain a significant amount of variation in this effect? (3.) Will air exposure affect release behavior and can levels of stress indicators predict release behavior? (4.) Does air exposure affect the propensity to display the NER?

## METHODS

### *Aerial Exposure*

*Carcharhinus limbatus* were captured using hook and line from the Mississippi Sound in the Gulf of Mexico between May and October of 2016-2018. Sharks were brought onto the boat within an average of 3.5 minutes and remained on the deck during aerial exposure for 5, 10, or 15 minutes, after which sharks were rapidly (within 3 minutes) sampled for up to 1 ml of blood via caudal venipuncture with a 21G needle and BD brand EDTA treated vacutainer. A subset of sharks was sampled for blood immediately after capture (within 3 minutes) to serve as a control. The nictitating membrane response was examined in all sharks by ejecting seawater at the eye using a needle-less syringe and recording if the nictitating membrane closed over the eye. The total (from the rostrum to the tip of the upper lobe of the caudal fin), standard (measured from

the tip of the rostrum to the caudal peduncle), and fork (from the tip of the rostrum to the fork in the caudal fin) lengths were then measured from each shark after which it was revived by slowly moving it through the water to facilitate oxygen uptake at the gills before release. The immediate release behavior was categorized on a subjective scale based on its behavior during release (Table 1; modified from Manire et al., 2001). Environmental data was recorded when each shark was captured including dissolved oxygen (mg/L), water temperature (°C), and air temperature (°C). A thermal gradient, the difference between water and air temperatures, was also calculated to determine if it has an exacerbating effect on the stress response. All procedures were approved by the University of Mississippi's Institutional Animal Care and Use Committee (protocol # 15-002).

#### *Blood and Statistical Analyses*

Blood samples were kept on ice and returned to the lab for analysis. Whole blood samples were analyzed for lactate (mmol/L) with CG4+ cartridges and an I-Stat Vetscan 1. Glucose (mg/dL) was measured from whole blood using a handheld meter (ReliOn Prime Blood Glucose Monitoring System and ReliOn Prime Blood Glucose Test Strips) similar to other studies (Cooke et al., 2008; Awruch et al., 2011; Danylchuk et al., 2014; French et al., 2015; Bouyoucos et al., 2017). Hematocrit was measured by centrifuging whole blood in a capillary tube then measuring the proportion of red blood cells to whole blood.

Air exposure duration was coded as a categorical variable to be able to compare between the durations. Analyses of covariance (ANCOVAs) and Tukey HSD post-hoc tests were utilized to determine if response variables (lactate, glucose, and hematocrit) were significantly affected by air exposure duration or any measured environmental or individual variables (total length, sex, thermal gradient, and dissolved oxygen). An ordinal logistic regression model was utilized

to determine if the duration of air exposure or levels of stress indicators (lactate, glucose, and hematocrit) could significantly predict release behavior (factored as an ordinal categorical variable). A Monte-Carlo randomization test was utilized to determine if the duration of air exposure affected the propensity for sharks to display the NMR. All analyses were performed in R version 3.5.1 using *car*, *ggplot2*, and *sciplot* packages.

## RESULTS

Forty *C. limbatus* were captured by hook-and-line and utilized in this study (5 per treatment) including 25 females and 15 males (Table 3). All sharks utilized in this study were hooked in the mouth and did not show signs of impairment or injury upon capture.

**Table 3.** Description of *C. limbatus* collected for this study.

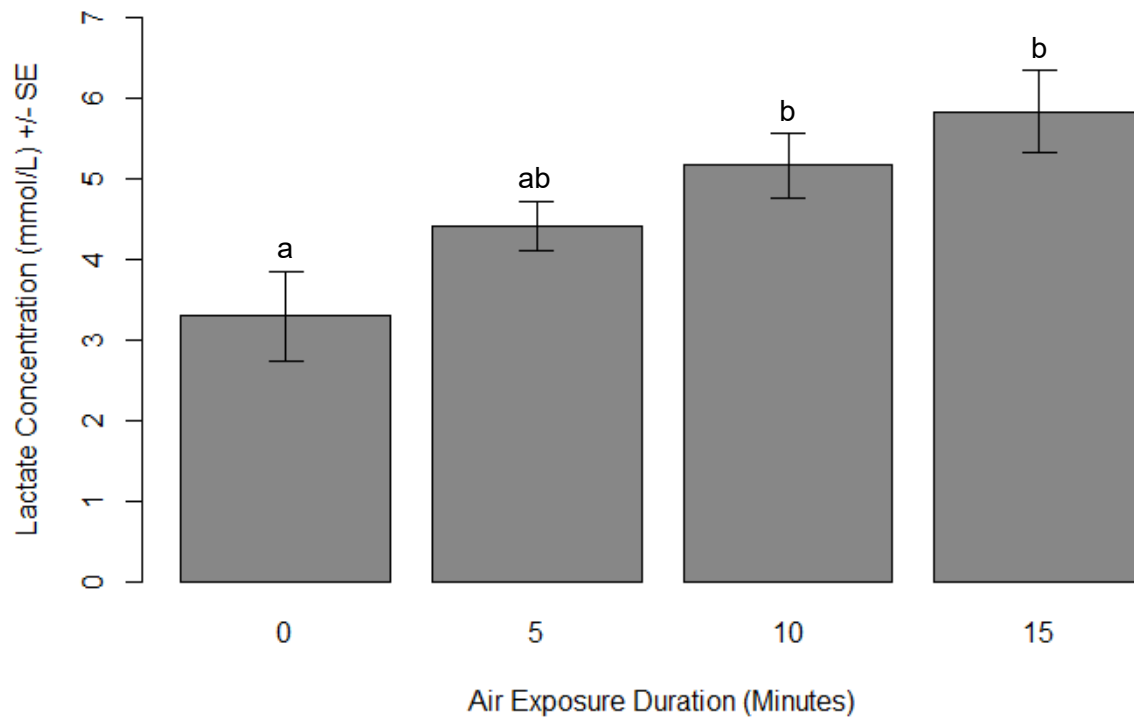
Air Exposure Duration	n	Sex Proportion (M/F)	Mean Total Length (cm) $\pm$ SE
0	10	5/5	91.3 $\pm$ 11.2
5	10	4/6	87.6 $\pm$ 7.5
10	10	4/6	83.9 $\pm$ 9.3
15	10	2/8	78.6 $\pm$ 9.1
Total	N=40	15/25	84.9 $\pm$ 4.4

### *Effects on Stress Indicators*

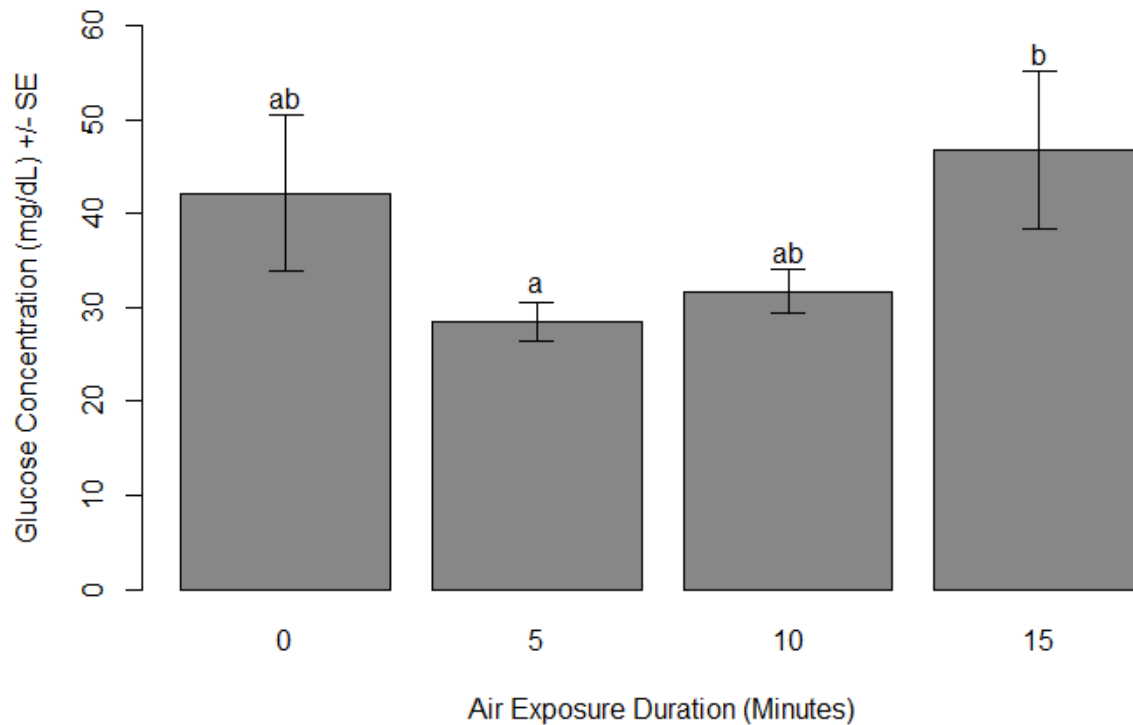
The duration of air exposure significantly affected lactate concentrations ( $F(3,35)=5.83$ ,  $p=0.0024$ ) with longer air exposure leading to higher lactate levels. Lactate was significantly higher than baseline levels after 10 and 15 minutes of air exposure ( $p=0.028$  and  $0.0018$ , respectively; Figure 8). Duration of air exposure significantly affected blood glucose concentrations ( $F(3,22)=3.34$ ,  $p=0.038$ ) with longer air exposure leading to higher glucose

levels. Glucose was significantly higher after 15 minutes of air exposure than after 5 minutes of air exposure ( $p=0.046$ , Figure 9). None of the environmental or observational variables significantly affected glucose ( $p>0.07$ ). Neither air exposure nor environmental variables had an effect on hematocrit levels ( $F(3,23)=1.69$ ,  $p=0.18$ ).





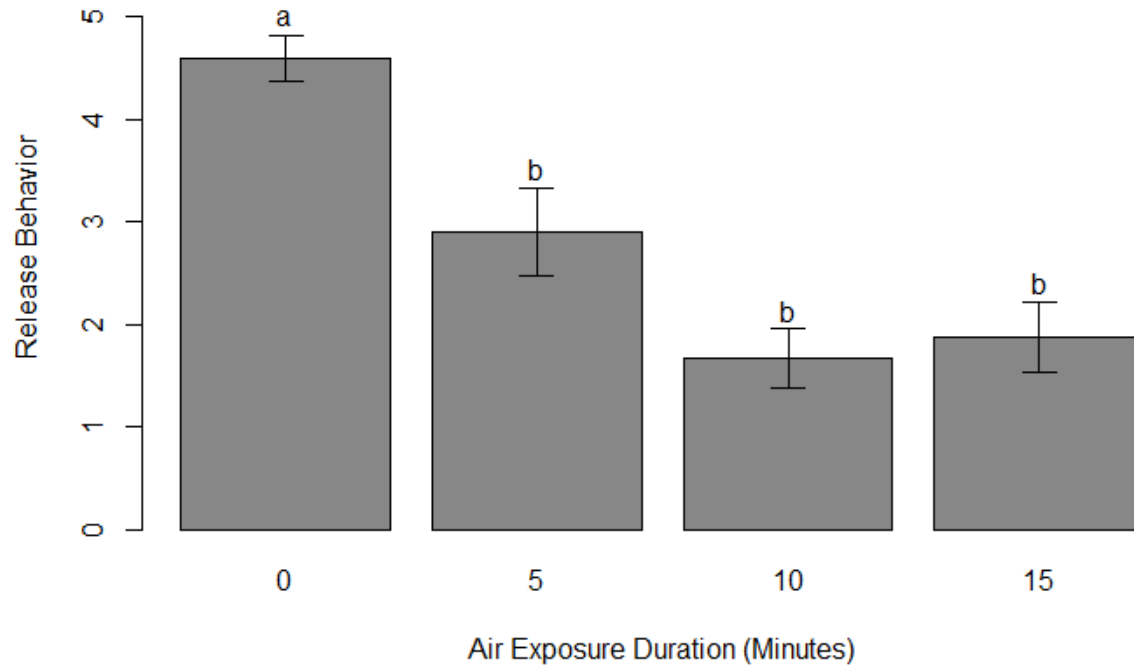
**Figure 8.** The differences in plasma lactate concentration (mmol/L) of *C. limbatus* captured from the Mississippi Sound in the Gulf of Mexico between air exposure duration (minutes). Concentrations include +/- 1 standard error. Significant comparisons ( $p < 0.05$ ) indicated by different letters.



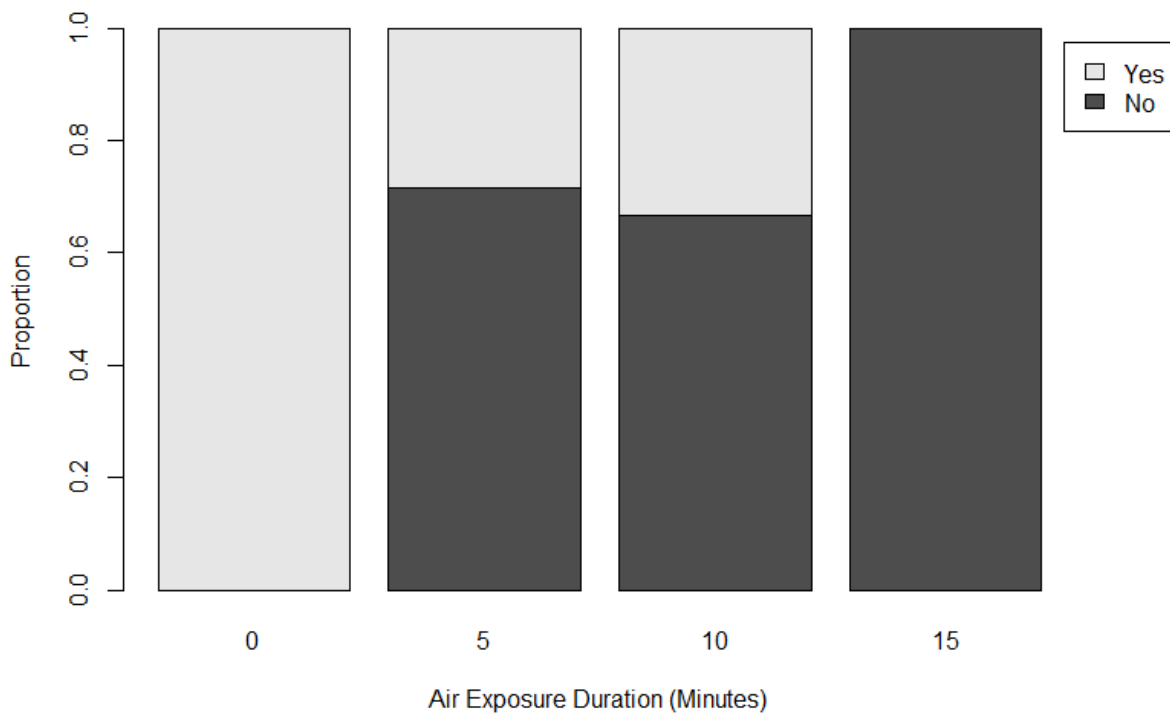
**Figure 9.** The differences in plasma glucose concentration (mg/dL) of *C. limbatus* captured from the Mississippi Sound in the Gulf of Mexico between air exposure durations (minutes). Concentrations include +/- 1 standard error. Significant comparisons ( $p < 0.05$ ) indicated different letters.

#### *Effects on Release Behavior and NER*

Air exposure duration significantly affected release behavior ( $\chi^2(3) = 21.32$ ,  $p = 1e-04$ ), with release behavior scores indicating more sluggish sharks after 5, 10, and 15 minutes of air exposure compared to baseline scores (all  $p < 0.0038$ , Figure 10). Other stress indicators (lactate, glucose, and hematocrit) were not significant indicators of release condition ( $p > 0.08$ ). Air exposure significantly affected the propensity of sharks to display the nictitating membrane reflex ( $\chi^2 = 9.95$ ,  $p = 0.016$ ) in which higher levels of air exposure resulted in decreased displays of the NER (Figure 11).



**Figure 10.** The differences in release behavior (ranked on a scale of 0-5) of captured *C. limbatus* captured from the Mississippi Sound in the Gulf of Mexico between air exposure durations (minutes). Significant comparisons ( $p < 0.05$ ) indicated by different letters.



**Figure 11.** The proportion of *C. limbatus* captured from the Mississippi Sound in the Gulf of Mexico exhibiting (yes) the NER versus those not exhibiting (no) the NER across different durations (minutes) of air exposure. The NER was tested by squirting water at the eye and recording whether the nictitating eyelid covered the eye.

## DISCUSSION

### *Effects on Stress Indicators*

Our study demonstrated significant effects of air exposure on the physiology of captured *C. limbatus*. Our results indicate an increase in plasma lactate concentrations with increasing duration of air exposure. Specifically, after only 10 minutes of air exposure, lactate levels were significantly higher than baseline levels with 15 minutes of air exposure resulting in an even larger difference. This effect is well-supported by existing literature. For example, an increase in lactate levels due to air exposure has been documented in teleosts such as the gilthead sea bream (*Sparus aurata*; Arends et al., 1999) and bonefish (*Albula vulpes*; Suski et al., 2007) as well as elasmobranchs such as the Atlantic stingray (*Hypanus Sabina*; Lambert et al., 2018), sparsely

spotted stingaree (*Urolophus paucimaculatus*; Heard et al., 2014), and the little skate (*Leucoraja erinacea*; Cicia et al., 2012). The majority of these studies examined air exposure over much longer time periods. The exposure durations used here (5, 10 and 15 minutes) provided the opportunity to examine fine scale stress response development in *C. limbatus*. Additionally, we believe that these selected intervals are reasonable approximations of the amount of time that fishers hold sharks out of water. Lactate levels observed here after only 10 minutes of air exposure (~5 mmol/L) were equivalent to those observed after one hour of gill net capture stress (~4.7 mmol/L; Manire et al., 2001) in *C. limbatus*. This highlights the severity of air exposure as a stressor compared to capture stress alone, as well as the severe physiological effects that exposure may have. Elevated lactate as a result of the stress response can cause alterations in protein structure and protein-protein interactions (Dumetz et al., 2008) along with a drop in pH of the blood. Similar to the results presented here, other studies on capture stress have reported high lactate levels correlated with poor release condition ( Moyes et al., 2006; Marshall et al., 2012).

Not only does air exposure result in a shift to anaerobic respiration, but it also stimulates the hypothalamic-pituitary-interrenal (HPI) axis. The perception of a stressor will result in the stimulation of the hypothalamus and anterior pituitary, causing a release in glucocorticoids from the interrenal tissue. Glucocorticoids will stimulate the mobilization of glucose as an energy source to the body. Previous studies on the effects of air exposure on plasma glucose levels have reported mixed results ranging from no effect (Suski et al., 2007; Cicia et al., 2012), to an increase in glucose (Arends et al., 1999; Lambert et al., 2018; Heard et al., 2014; Cicia et al., 2012), and a decrease in glucose (Frick et al., 2010). Our results show glucose levels decreasing, although not significantly, after 5 minutes of air exposure compared to baseline levels, followed

by an incremental increase in glucose after 10 and 15 minutes of air exposure. This is possibly due to the increased levels of activity that *C. limbatus* engage in during the first few minutes of capture and air exposure. Without exception, sharks thrashed on the deck in an attempt to escape the stressor. Physiologically, this could imply that the increased activity during the first five minutes of air exposure resulted in a depletion of blood glucose. This increased activity abated after longer periods of air exposure which may explain why glucose levels were observed to increase again at the 10 and 15 minute time periods. Within the first three minutes of a stressor, glucocorticoids will be released, which will allow for breakdown of glycogen and production of more glucose, which could also account for the elevated glucose levels after 10 and 15 minutes of air exposure. This pattern has been observed in *H. sabina* in which glucose levels decrease within the first 15 minutes of air exposure but then increase at the 15 and 30 minute time periods (Lambert et al., 2018). However, it is also possible that there may have been a large amount of variation in initial glucose measurements due to the possibility of some sharks having eaten more recently than others before capture or due to the low sample size.

During a stress response, catecholamines (epinephrine and norepinephrine) are released from the chromaffin tissue, which result in increased blood flow throughout the body. Blood flow will also increase at the gills, allowing for more gill lamellae to be perfused with blood, a process known as lamellar recruitment (Bennett and Rankin, 1987). This results in an increase in gill permeability to water between the body of the shark and the environment which can lead to hemodilution or hemoconcentration, depending upon salinity. Despite this possibility, the literature surrounding the effects of air exposure on hematocrit typically show no effect (Lambert et al., 2018; Cicia et al., 2012). An increase in hematocrit with the addition of air exposure to an exercise stressor was observed when compared to the effects of exercise alone in *A. vulpes*

(Suski et al., 2007). In the present study, hematocrit was not significantly affected by air exposure duration, which fits with the previously mentioned literature on elasmobranchs (Lambert et al., 2018; Cicia et al., 2012).

### *Effects on Release Behavior*

The scoring of release behavior has the potential to provide insight into the condition and, possibly, chances of survival of the animal after release when direct measures of survival are not feasible (Campbell et al., 2009; Brownscombe et al., 2017). Previous studies investigating the effect of air exposure on behavior after release are fairly consistent. For example, *A. vulpes* that were exposed to air took significantly longer to regain equilibrium than those not exposed to air. They also noted that most predation events of the released *A. vulpes* occurred within 20 minutes of release, possibly relating to more sluggish behavior after the stressor (Suski et al., 2007). Behavioral impairment following 10 minutes of air exposure was also observed in sablefish, *Anoplopoma fimbria*, along with an effect of size class in which smaller fish exhibited more impairment and mortality in response to air exposure than large fish (Davis and Parker, 2004). A study on the draughtboard, *Cephaloscyllium laticeps*, and piked spurdog, *Squalus megalops*, sharks noted lower post-release activity levels following 15 minutes of air exposure (Raoult et al., 2019). In the present study, release behavior was categorized as significantly more sluggish after 5, 10, and 15 minutes of air exposure when compared to sharks that were not air exposed. This indicates that even 5 minutes of air exposure could impair the animal's ability to escape a threat immediately after release. Although not observed in the current study, others have demonstrated an interaction between air exposure duration and temperature in which animals exposed to air during high temperatures exhibited greater behavioral impairment (Gingerich et

al., 2007) and higher mortality rates (Gingerich et al., 2007; Cicia et al., 2012). The only mortality experienced in this study was during the highest air exposure treatment (15 minutes).

Despite our results of air exposure on release behavior and the common use of immediate release behavior as an indicator of survival, caution must be taken when drawing conclusions because long-term survivorship could not be verified. In a study that observed behavior up to five minutes post-release, it was noted that behavior of elasmobranchs did not differ significantly between air exposed and control sharks during the first 30 seconds after release, whereas activity was significantly lower in air exposed sharks five minutes after release (Raoult et al., 2019). This indicates that immediate release behavior (within 30 seconds after release) may not be representative of long-term release behavior or survivorship.

#### *Effects on the Nictitating Eyelid Reflex*

Reflex impairment can greatly affect an animal's fitness by decreasing the likelihood of a timely escape from a threat (Campbell et al., 2010). Capture stress has a high possibility of resulting in reflex impairment, due to the animal utilizing their energy stores in an attempt to escape the stressor. Because of this, measures of reflex impairment are common in capture stress studies. For instance, reflexes were significantly impaired in red snapper (*Lutjanus campechanus*) exposed to capture from deeper waters when compared to shallow waters (Campbell et al., 2010). The nictitating membrane reflex (NMR) is commonly utilized by fishers and scientists as an index of the condition of a captured animal (Poisson et al., 2014; Dapp et al., 2017; Musyl and Gilman, 2018) and it has been suggested that the NMR should be measured across fisheries studies for comparison (Musyl and Gilman, 2018). A variety of reflexes can be measured, along with the NMR, as a measure of condition. Despite the widespread utilization of



the NMR as a measure of condition, impairment of the jaw reflex has been implicated as a more informative reflex as it was associated with multiple physiological stress indicators and, therefore, may better reflect physiological disturbance (Jerome et al., 2018). No other study investigating the effects of air exposure measured the NMR as a response variable so direct comparison with the current study was not possible. Our results demonstrate that the duration of air exposure had a significant effect on the NMR, with all sharks displaying the NMR immediately after capture and no displays of the NMR after 15 minutes of air exposure, indicating reflex impairment in air exposed sharks. These results, along with the negative effect of air exposure on release behavior, and the equivalence of stress indicators between 10 minutes of air exposure and an hour of capture stress indicate that air exposure is a more severe stressor to which aquatic animals may be subjected during capture.

## CONCLUSION

Our results demonstrated elevated lactate after 10 and 15 minutes of air exposure compared to baseline levels, indicating a negative physiological disturbance after only 10 minutes of air exposure. Air exposure also resulted in poorer release behavior after five minutes along with complete impairment of the NMR after 15 minutes. These results highlight the severe negative impact that even short durations of air exposure can have on captured sharks. We suggest that fishers limit air exposure of these animals to no more than five minutes in order to provide the highest chance of post-release survival. Due to species-specific variation in the response to capture stress, future studies should investigate the effects of acute air exposure durations on various species and attempt to relate these physiological and behavioral stress indicators to direct measures of post-release survival.

CHAPTER 4:  
EFFECTS OF ISO-EUGENOL ON THE STRESS AND RESPIRATION RATES OF  
ATLANTIC SHARPNOSE SHARKS (*Rhizoprionodon terraenovae*), WHITE-SPOTTED  
BAMBOO SHARKS (*Chiloscyllium plagiosum*), AND GOLDEN SHINERS (*Notemigonus  
crysoleucas*)

ABSTRACT

Sedatives are utilized by researchers, fisheries managers, veterinarians, and aquarium personnel in order to reduce stress and activity of fishes, which is useful during surgery, transport, and handling. A variety of sedatives are currently utilized such as MS-222, quinaldine, metomidate, benzocaine, etc., but these sedative are not all FDA approved for use in fishes due to negative side-effects. Clove oil, with an active component of eugenol, has shown potential as a natural fish sedative; however, few studies have investigated its effects on elasmobranchs. This study is an investigation into the effects of iso-eugenol on physiological stress indicators, including lactate, glucose, hematocrit, and respiration, of two elasmobranchs, Atlantic sharpnose sharks (*Rhizoprionodon terraenovae*) and white-spotted bamboo sharks (*Chiloscyllium plagiosum*), and a teleost, golden shiners (*Notemigonus crysoleucas*). Our results demonstrated that immersion in a 10 mg/L solution of iso-eugenol significantly reduced lactate, glucose, and hematocrit levels in *R. terraenovae*, while also resulting in more sluggish release behavior. The effects on respiration rates were variable, with 5 mg/L iso-eugenol resulting in higher rates in *R. terraenovae*, lower rates in *C. plagiosum*, and no significant difference in *N. crysoleucas*.

These results highlight the extreme variation in effects of sedatives between species and indicate that preliminary trials must be conducted on any species of interest before utilization of iso-eugenol as a sedative. While iso-eugenol had a negative effect on release behavior, it also demonstrated potential in reducing the magnitude of stress in response to capture.

## INTRODUCTION

Sedation and anesthesia have proven to be powerful tools to reduce stress in fishes during handling and transport by lowering cortisol levels (Iversen et al., 2003, 2009) and by reducing plasma lactate in response to low oxygen (Small, 2004). Anesthetics can also minimize the effects of stress after a stress response has already been initiated (Wagner et al., 2003). Sedation can reduce metabolic rate and, therefore, decrease oxygen demand, reduce activity, and allow for easier handling for scientists or fishers (Cooke et al., 2004). In contrast, other studies have shown negative effects of sedation, such as increased cortisol levels (Weber et al., 2011) and a risk of ventilatory failure when high doses are administered (Sladky et al., 2001).

In fishes, studies have shown an increase in heart rate at low concentrations of anesthesia and a decrease at higher concentrations (Sneddon, 2012). Iso-eugenol is a derivative of clove oil, a commonly used sedative for fishes. Fishes exposed to clove oil had a calmer induction into anesthesia than fishes exposed to other sedatives (Munday and Wilson, 1997). Similarly, rainbow trout (*Oncorhynchus mykiss*) were found to have a quicker induction into anesthesia with low concentrations of clove oil compared to other sedatives (Keene et al., 1998). However, Sladky et al. (2001) found that red pacu (*Piaractus brachypomus*) exposed to tricaine-methanesulfate had a much larger margin of safety than those exposed to clove oil, meaning that fishes exposed to clove oil were more likely to have ventilatory failure. Few studies are available

regarding the effects of sedation on elasmobranchs. Aquarium fishes and elasmobranchs anesthetized with quinaldine did not resist gentle handling over a short time period (e.g. 5 mins) (Dempster, 1968). A minor increase in plasma lactate was seen in sharks after sedation with Aqui-S, a clove oil-derived sedative (Frick et al., 2009). These results emphasize the species-specific responses to anesthetics and the general lack of studies dealing with elasmobranchs.

Only a handful of studies have been conducted to investigate the effects of sedatives on physiological indicators of the secondary stress response. Studies on the sedative effects of stress reduction in fishes have focused on quantification of cortisol, the primary glucocorticoid in teleosts. The majority of studies have reported increases in cortisol in response to sedative exposure (Thomas and Robertson, 1991; Iversen et al., 2003, 2009; Davis and Griffin, 2004; Weber et al., 2011; Yousefi et al., 2018) with the exception of metomidate, which has been seen to block the cortisol stress response (Thomas and Robertson, 1991; Davis and Griffin, 2004). Due to difficulties quantifying the glucocorticoid stress response of elasmobranchs, stress studies typically focus on physiological indicators of the secondary stress response. Despite this, there is only one (to the author's knowledge) study that has examined the effects of a sedative on the secondary stress response of an elasmobranch (Frick et al., 2009). This study noted significantly higher lactate levels in Australian swellsharks (*Cephaloscyllium laticeps*) exposed to sedation with Aqui-S (a commercial form of iso-eugenol) than control sharks. Similar results have been found in teleosts in which sedation with either clove oil, eugenol, or iso-eugenol resulted in increased lactate levels (Iversen et al., 2003; Weber et al., 2011; Yousefi et al., 2018). These results indicate that, with the exception of metomidate, sedatives may be perceived as noxious stimuli by fishes, resulting in a glucocorticoid and secondary stress response.

The objective of this study was to identify a possible method that could be applied to reduce the stress response of captured sharks. Specifically, we aimed to determine if a sedative, iso-eugenol, has an effect on the development of the stress response and respiration rates of wild-caught sharpnose sharks, *Rhizoprionodon terraenovae*, captive white-spotted bamboo sharks, *Chiloscyllium plagiosum*, and golden shiners, *Notemigonus crysoleucas*. This allowed for comparison between sharks with different life histories and for comparison between elasmobranchs and teleosts. For *R. terraenovae*, we aimed to answer the following questions: (1.) Does sedation affect levels of stress indicators (such as lactate, glucose, and hematocrit) over time and do covariates (water temperature, salinity, dissolved oxygen, and weight) explain a significant amount of variation? (2.) Does sedation affect the respiration rate of sharks over the 30 minute trial and do environmental variables explain a significant amount of variation in this effect? (3.) Can release behavior be predicted by the type of sedation or by levels of stress indicators at the time of release? For both *C. plagiosum* and *N. crysoleucas* we aimed to determine if sedation significantly affected the respiration rates of the subjects while also controlling for covariates of temperature (°C) and weight (g).

## METHODS

### *Atlantic Sharpnose*

#### Collection and Experimental Procedure

*Rhizoprionodon terraenovae* were collected by hook-and-line around Horn Island off the coast of Mississippi in the Gulf of Mexico between May and October of 2016, 2017, and 2018. Upon capture and identification as *R. terraenovae*, the individual was sampled (within 3 minutes) for up to 1 ml of blood as a baseline blood sample via caudal venipuncture with a 21G

needle and BD brand EDTA treated vacutainer. The shark was then placed into a respiration tank (a 204.2 L sealed tank with a dissolved oxygen probe inserted in the lid; Figure 12) on board for 30 minutes. Each shark was randomly assigned a treatment, either iso-eugenol (5 or 10 mg/L; mixed with ethanol), ethanol concentrations equal to that which was mixed with iso-eugenol (5 or 10 mg/L), or control (seawater only) which was mixed with seawater from the sampling site. During the 30 minute trial, blood was serially sampled and dissolved oxygen recorded every 10 minutes. This required us to open the chamber and remove the shark during the experiment which could have introduced oxygen into the chamber. To try and account for this effect, all dissolved oxygen measurements were taken immediately before opening the chamber and removing the shark. After the 30 minute trial, the standard (measured from the tip of the rostrum to the caudal peduncle), fork (from the tip of the rostrum to the fork in the caudal fin), and stretched total length (from the rostrum to the tip of the upper lobe of the caudal fin) (cm) and weight (g) was measured for each shark before release. The immediate release behavior was categorized on a subjective scale based on its behavior during release (Table 1; modified from Manire et al., 2001). Environmental data was recorded when each shark was captured including dissolved oxygen (mg/L), water temperature (°C), and salinity (ppt).



**Figure 12.** The respiration tank constructed and utilized for sedation experiments with wild *R. terraenovae*.

## Blood Analysis

Blood samples were kept on ice and returned to the lab for analysis. Whole blood samples were analyzed for glucose (mmol/L) with a glucose meter (Reli-On Prime Glucose Monitoring System and associated strips) similar to other studies (Cooke et al., 2008; Awruch et al., 2011; Danylchuk et al., 2014; French et al., 2015; Bouyoucos et al., 2017). Hematocrit (%) was measured as the proportion of red blood cells to whole blood in centrifuged samples. Plasma was separated from whole blood by centrifugation (3,400 rpm) and was kept in a -80°C freezer until analyzed for lactate (mmol/L) with a lactate kit (Eton Bioscience Inc., San Diego, CA, SKU #1200011002).

## Calculations and Statistical Analyses

A linear mixed-effects model was performed in R using the lmer function in the lmerTest package to determine if treatment, time, and covariates (weight, water temperature, salinity, and environmental dissolved oxygen) had an effect on lactate, glucose, and hematocrit. An individual ID was assigned to each shark and that was introduced as a random effect to account for repeated measures from the same individual.

Total relative respiration rates ( $\text{mgO}_2/\text{g}/\text{hr}$ ) were calculated by measuring the decline in dissolved oxygen of the tank over the 30 minute trial per gram mass of the shark. Respiration rates ( $\text{mgO}_2/\text{g}/\text{hr}$ ) were also calculated by measuring the decline in dissolved oxygen in the tank between only the last two time periods (20 and 30 minutes) to try and remove a potential effect of stress of introduction to the chamber. ANCOVAs were conducted using the aov function in R followed by Tukey HSD tests to determine if treatment or covariates (water temperature and environmental DO) had an effect on either respiration rate.

A logistic regression model was conducted to determine if treatment or levels of stress indicators (lactate, glucose, hematocrit, and respiration rate) at time of release had a significant effect on release behavior (coded as an ordinal categorical variable).

### *White-Spotted Bamboo Sharks*

#### Husbandry

Five juvenile female *C. plagiosum* with a mean weight of 272 grams ( $\pm 102.7$  SE) were kept in a 350 gallon saltwater aquarium. Water quality parameters (nitrites, nitrates, ammonia, pH, temperature, dissolved oxygen, and salinity) were checked and the aquarium was cleaned and vacuumed daily. The sharks were fed a mixed diet of shrimp, tilapia, and squid by hand every three days.

#### Respiration Chamber

The respiration chamber consisted of an acrylic tube with a threaded PVC cleanout adapter and screw-in plug fitted onto each end and secured with aquarium-safe silicone. The plug on one end was permanently secured with aquarium-safe silicone, allowing only one end to place the shark into and out of the chamber. A hole was drilled into the secured cleanout adapter into which a vacutainer lid was secured to allow for injection of the treatment into the chamber. A hole was drilled into the acrylic tube to securely fit a dissolved oxygen probe. A small submersible pump was connected to the chamber via clear vinyl tubing attached to adapters securely fixed into the acrylic chamber. This allowed for low water flow through the chamber and over the dissolved oxygen probe (Figure 13). The respiration chamber was submerged in a tank filled with saltwater to facilitate filling of the chamber tubing without air bubbles.





**Figure 13.** The respiration chamber constructed and utilized for sedation experiments with *C. plagiosum*.

#### Experimental Procedure

A shark was captured from the main aquarium and quickly (within 30 seconds) placed into a submerged respiration chamber that was then sealed. A treatment was randomly assigned to each trial and injected into the respiration chamber. Possible treatments included iso-eugenol (5 mg/L) mixed with ethanol for dissolving, ethanol concentration equal to that which was mixed with iso-eugenol (5 mg/L), or control (saltwater only). The shark remained in the chamber for 30 minutes, during which dissolved oxygen was measured and the number of gill ventilations was counted (if possible) every minute. After 30 minutes, the shark was carefully removed from the chamber and weighed on a digital scale. Blood sampling to evaluate stress indicators was not conducted for this study due to the small size of the animals. Sharks were allowed to recover for 30 minutes in a holding tank before being placed back into the main aquarium.

#### Calculations and Statistical Analyses

The relative respiration rate (mgO<sub>2</sub>/g/hr) was calculated from each 30 minute trial using the decline in total oxygen in the respiration chamber, the mass of the shark, and the time period. An ANCOVA was performed using the lmer function in the lmerTest package in R to determine if treatment or covariates, water temperature (°C) and weight (g), significantly affected the respiration rates of *C. plagiosum*. The number of gill ventilations per minute was averaged for each shark and an ANCOVA was utilized to determine if treatment or the same covariates significantly affected the average number of ventilations. Because each shark was utilized for experiments more than once, an identification number was assigned to the sharks and was introduced into the model as a random effect to account for repeated measures. The emmean function from the emmeans package in R was utilized for post-hoc analyses. The bargraph.CI function in the sciplot package was utilized for creation of figures.

#### *Golden Shiners*

#### Specimen Collection and Experimental Treatment

*Notemigonus crysoleucas* were purchased locally and transported to the lab where they were held under aeration until experimental trials. Respiration chambers consisted of 1 L plastic Ziploc containers with a hole drilled in the lid to fit a dissolved oxygen probe. Treatments consisted of iso-eugenol (5 mg/L) in an ethanol solution, ethanol concentration equal to that which was mixed with iso-eugenol (5 mg/L), or a control (reverse osmosis water only). Treatments were randomly assigned to each subject and either ethanol, iso-eugenol, or no treatment was added to the water in each chamber prior to sealing the fish in the chamber. Dissolved oxygen (mg/L) and temperature (°C) was measured every 10 minutes for an hour. After the last measurement, the subject was removed from the chamber and weighed (g).

Subjects were euthanized after experimentation via submergence in concentrated iso-eugenol under a fume hood.

#### Calculations and Statistical Analyses

Relative respiration rates for *N. crysoleucas* were calculated over each one hour trial using the same method described for *C. plagiosum* along with respiration rates calculated from the difference in dissolved oxygen between the 50 and 60 minute time periods. Similarly, an ANCOVA was performed using the lmer function in the lmerTest package in R to determine if treatment or covariates, water temperature (°C) and weight (g), significantly affected either respiration rate of the shiners. The bargraph.CI function in the sciplot package was utilized for construction of figures. All statistical analyses were conducted with R version 3.4.2 (“Short Summer”).

## RESULTS

### *Atlantic Sharpnose*

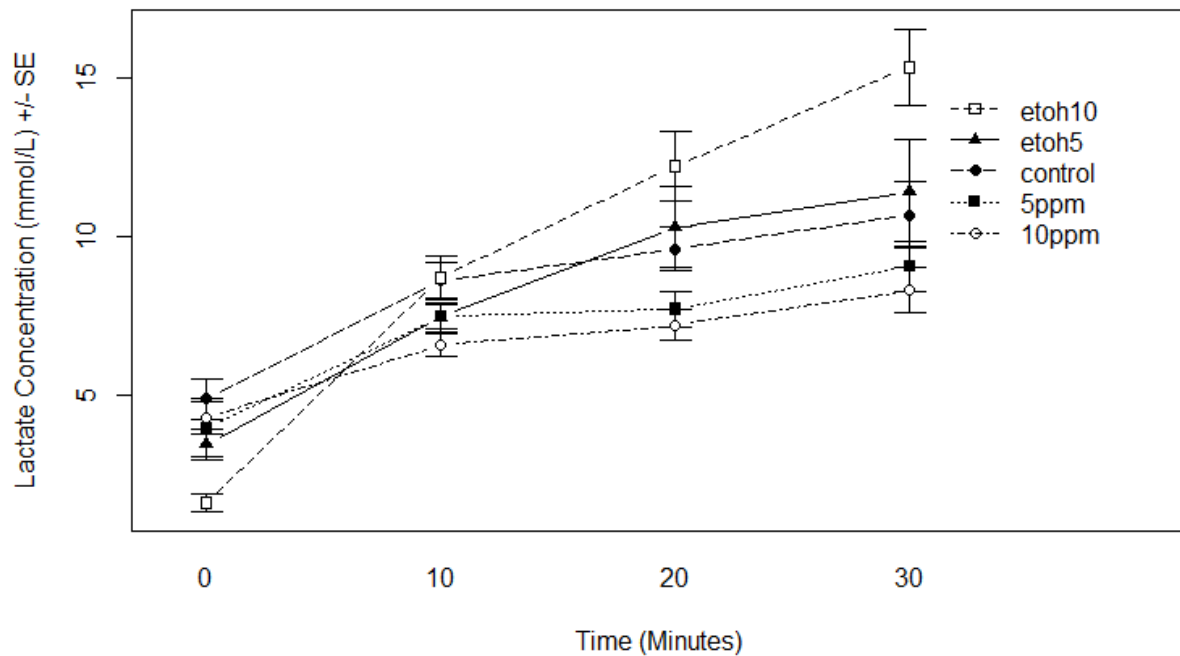
Forty-nine male *R. terraenovae* were captured by hook-and-line and utilized in this study (Table 4). Ten sharks were utilized for the treatments of 10 mg/L of iso-eugenol, 5 and 10 mg/L of ethanol, and control while nine sharks were utilized for the 5 mg/L dose of iso-eugenol.

**Table 4.** Description of *R. terraenovae* collected for this study.

Treatment	n	Sex Proportion (M/F)	Mean Total Length (cm) ± SE	Mean Weight (g) ± SE
Control	10	10/0	80.8 ± 3.4	2273.6 ± 318.4
5 mg/L Ethanol	10	10/0	77.8 ± 2.2	1916.5 ± 229.3
10 mg/L Ethanol	10	10/0	79.0 ± 3.2	2154.9 ± 307.4

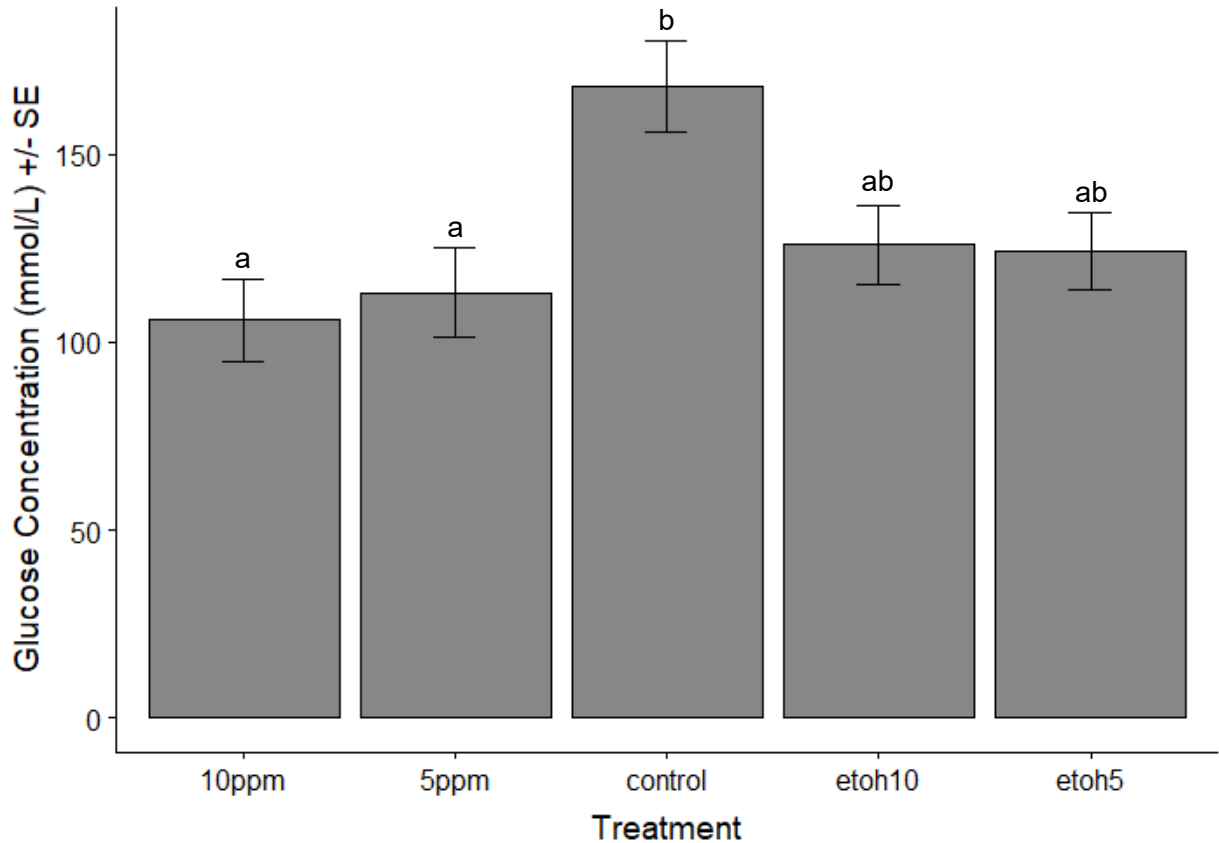
5 mg/L Iso-eugenol	9	9/0	76.8 ± 3.5	1734.0 ± 210.7
10 mg/L Iso-eugenol	10	10/0	76.8 ± 4.2	1974.3 ± 313.8
Total	N=49	49/0	78.3 ± 1.5	2013.4 ± 123.5

Lactate concentration (mmol/L) was significantly affected by a treatment by time interaction ( $F(12,130)=5.28, p=3.335e-07$ ; Figure 14) in which lactate increased over the course of the 30 minute trial in all treatments. Lactate levels in sharks exposed to 10 mg/L of ethanol were significantly higher than those exposed to 10 mg/L of clove oil ( $p=0.0023$ ). There was no significant difference between sharks exposed to 5 mg/L of ethanol and 5 mg/L of clove oil ( $p=0.6$ ) or between control and any other treatment ( $p>0.11$ ). Lactate in sharks exposed to 5 mg/L clove oil was significantly lower than sharks exposed to 10 mg/L of ethanol ( $p=0.019$ ). No covariate (weight, water temperature, salinity or environmental DO) had a significant effect on lactate levels ( $p>0.15$ ).



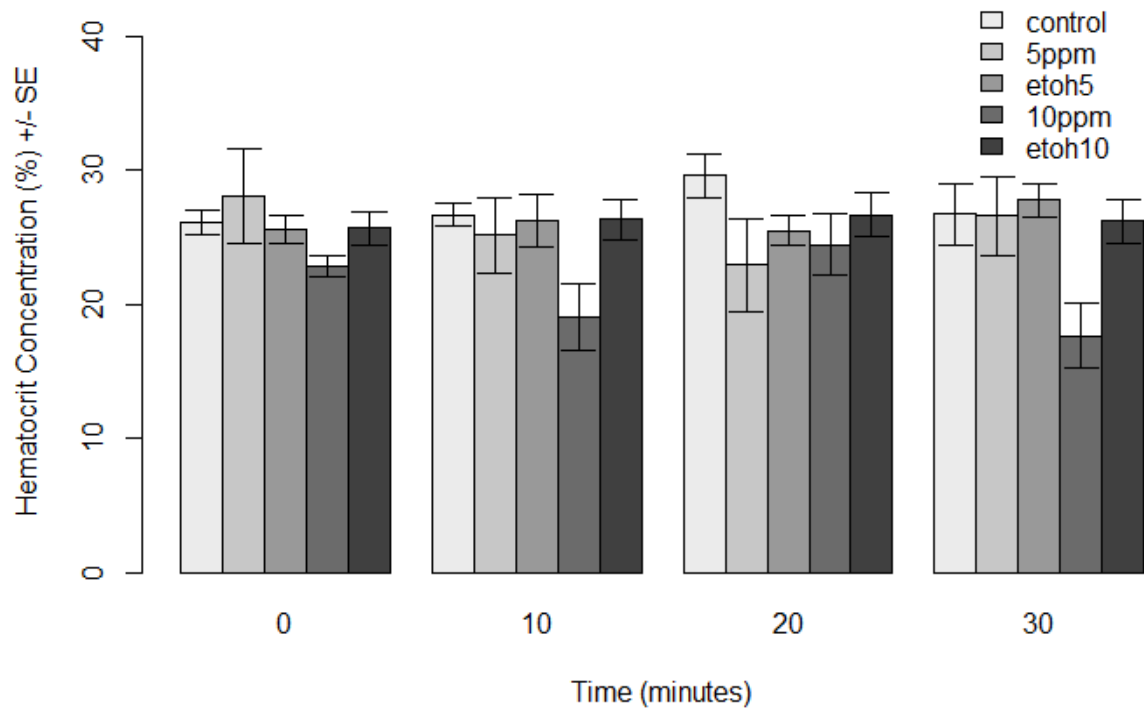
**Figure 14.** Difference in plasma lactate (mmol/L) change over time (minutes) of *R. terraenovae* captured from the Mississippi Sound in the Gulf of Mexico between treatments. Concentrations include mean +/- 1 standard error.

Treatment had a significant effect on glucose levels ( $F(4,38)=3.35$ ,  $p=0.019$ ) in which glucose was significantly higher in control sharks than those exposed to 5 or 10 mg/L of clove oil ( $p=0.03$  and  $p=0.01$ , respectively; Figure 15). Glucose levels were significantly different between sampling times ( $F(3,128)=18.24$ ,  $p=6.402e-10$ ) in which glucose levels were higher than baseline after 10, 20, and 30 minutes of treatment ( $p=0.0012$ ,  $<0.0001$ , and  $<0.0001$ , respectively). Glucose levels were also significantly higher after 30 minutes of treatment when compared with the 10 minute treatment ( $p=0.03$ ). Covariates (weight, water temperature, salinity or environmental DO) all had a non-significant effect on glucose ( $p>0.11$ ).



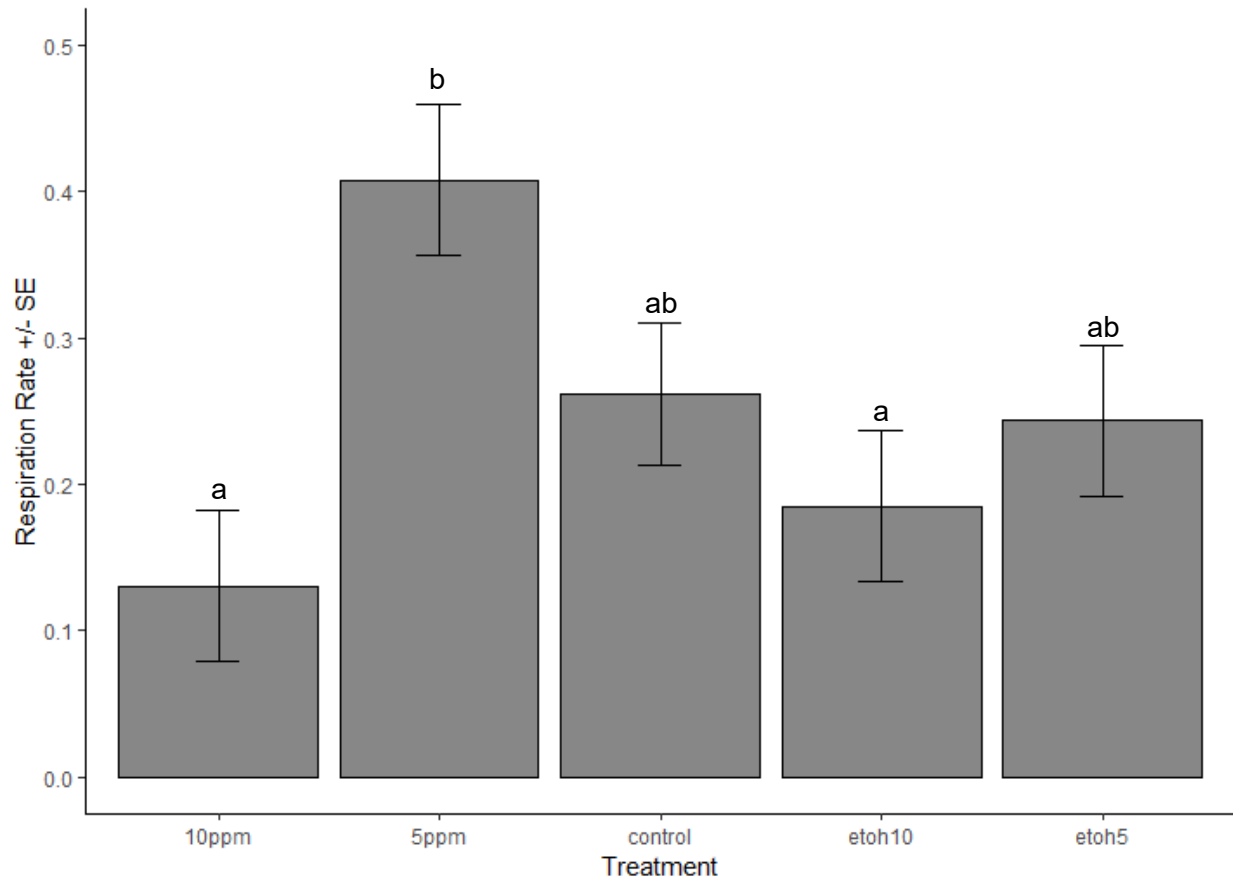
**Figure 15.** Difference in glucose concentrations (mmol/L) of *R. terraenovae* exposed to different treatments averaged over the 30 minute experimental trial. Sharks were captured from the Mississippi Sound in the Gulf of Mexico. Concentrations include mean +/- 1 standard error. Significant comparisons indicated by different letters.

Hematocrit levels were significantly influenced by a treatment by time interaction ( $F(12,99)=1.94, p=0.038$ ; Figure 16). Sharks exposed to 10 mg/L of clove oil had significantly lower hematocrit than sharks exposed to 5 and 10 mg/L of ethanol ( $p=0.04$  and  $0.048$ , respectively) and control sharks ( $p=0.013$ ). Hematocrit did not differ between sampling times alone ( $p>0.58$ ). No covariate (weight, water temperature, salinity or environmental DO) had a significant effect on hematocrit levels ( $p>0.26$ ).



**Figure 16.** Comparison of hematocrit (%) between treatments and at the different sampling periods (minutes) in *R. terraenovae* captured from the Mississippi Sound in the Gulf of Mexico. Concentrations include +/- 1 standard error.

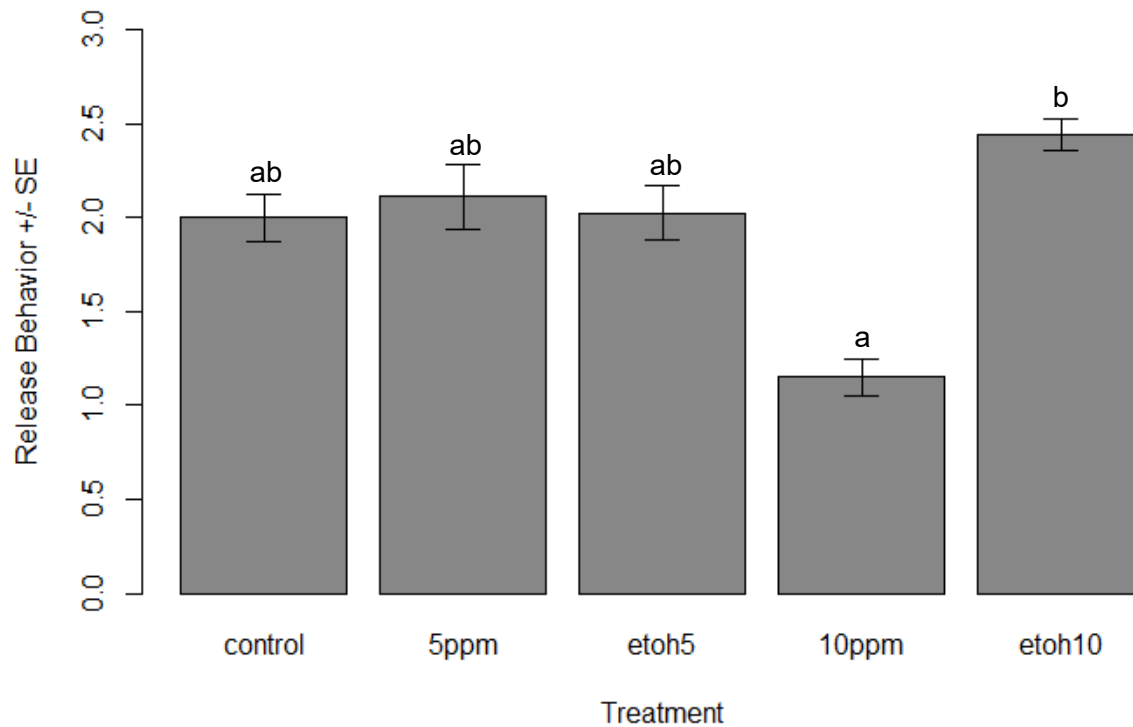
Respiration rates were significantly different between treatments ( $F(4,35)=3.91$ ,  $p=0.01$ ). Respiration rates were significantly higher in sharks exposed to 5 mg/L of clove oil than those exposed to 10 mg/L of clove oil ( $p=0.007$ ; Figure 17). Respiration rates calculated between the 20 and 30 minute time periods were not significantly affected by treatment ( $F(4, 41)=1.39$ ,  $p=0.25$ ). Covariates (water temperature and environmental DO) did not significantly affect either respiration rates ( $p>0.11$ ).



**Figure 17.** Difference in total respiration rates (mgO<sub>2</sub>/g/hr) of *R. terraenovae* exposed to different treatments. Sharks were captured from the Mississippi Sound in the Gulf of Mexico. Concentrations include +/- 1 standard error. Significant comparisons are indicated by different letters.

Release behavior was significantly different between treatments ( $\chi^2(4) = 12.36, p = 0.015$ ) in which sharks exposed to 10 mg/L of clove oil had significantly lower release behavior ratings than sharks exposed to 10 mg/L of ethanol ( $p = 0.0087$ ; Figure 18). No other variable was a significant predictor of release behavior ( $p > 0.20$ ).





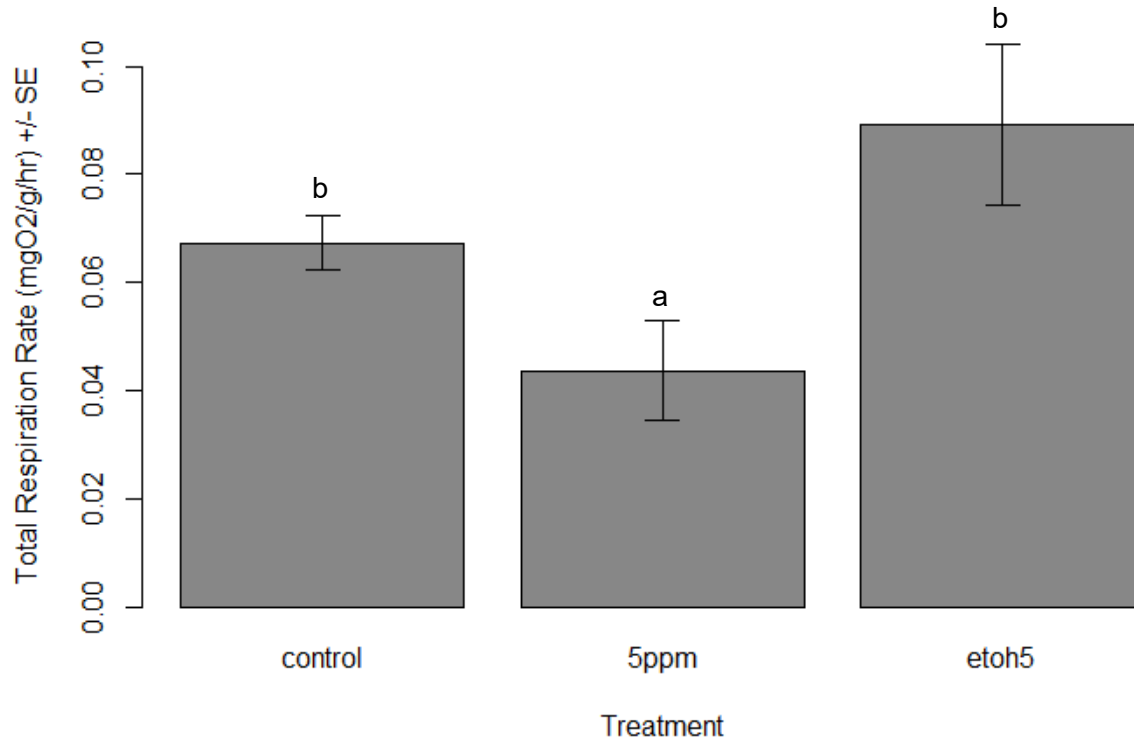
**Figure 18.** Difference in release behavior (a subjective ranking based on the behavior of the shark upon release, +/- 1 standard error) of *R. terraenovae* exposed to different treatments. Sharks were captured from the Mississippi Sound in the Gulf of Mexico. Significant comparisons are indicated by different letters.

### *White-spotted Bamboo Sharks*

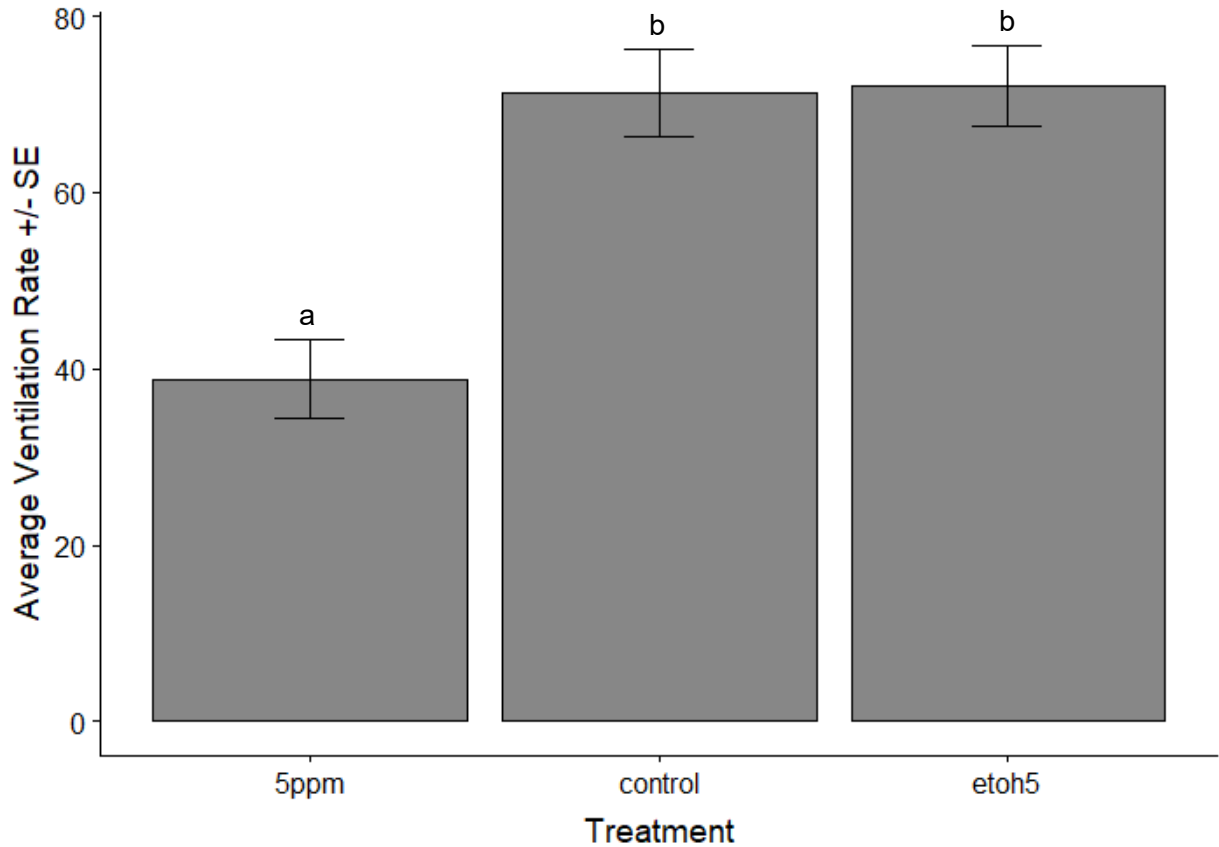
Treatment had a significant effect on respiration rates ( $F(2,8.7)=15.15$ ,  $p=0.0015$ ) in which respiration rates of sharks exposed to clove oil were lower than those exposed to control and ethanol (Figure 19). Neither water temperature nor weight had a significant effect on respiration rates ( $p>0.27$ ).

Treatment had a significant effect on the mean number of gill ventilations ( $F(2,3.04)=141.84$ ,  $p=0.00099$ ). Sharks exposed to 5 mg/L of iso-eugenol exhibited significantly

lower respiration rates than sharks exposed to 5 mg/L of ethanol ( $p=0.0013$ ) or saltwater only ( $p=0.0022$ ) (Figure 20).



**Figure 19.** Difference in total respiration rates ( $\text{mgO}_2/\text{g/hr}$ ) of captive *C. plagiosum* exposed to different treatments. Respiration rates include +/- 1 standard error. Significant comparisons indicated by different letters.



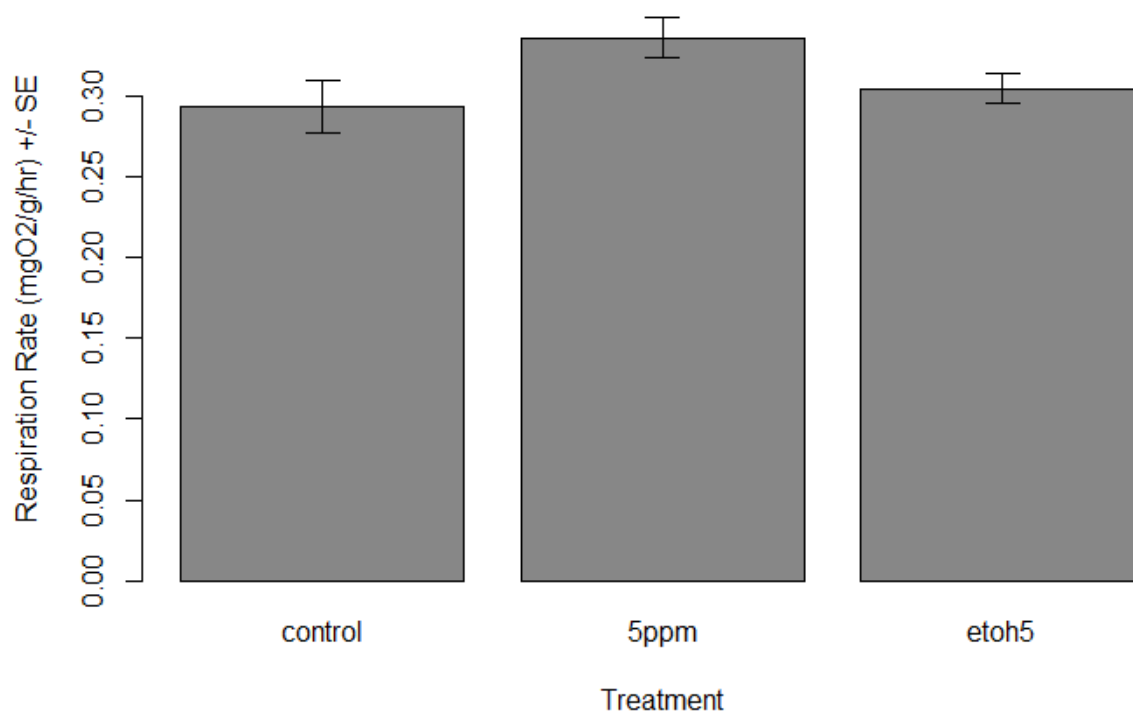
**Figure 20.** Difference in average ventilation rates of captive *C. plagiosum* exposed to different treatments. Ventilation rates include +/- 1 standard error. Significant comparisons indicated by different letters.

### *Golden Shiners*

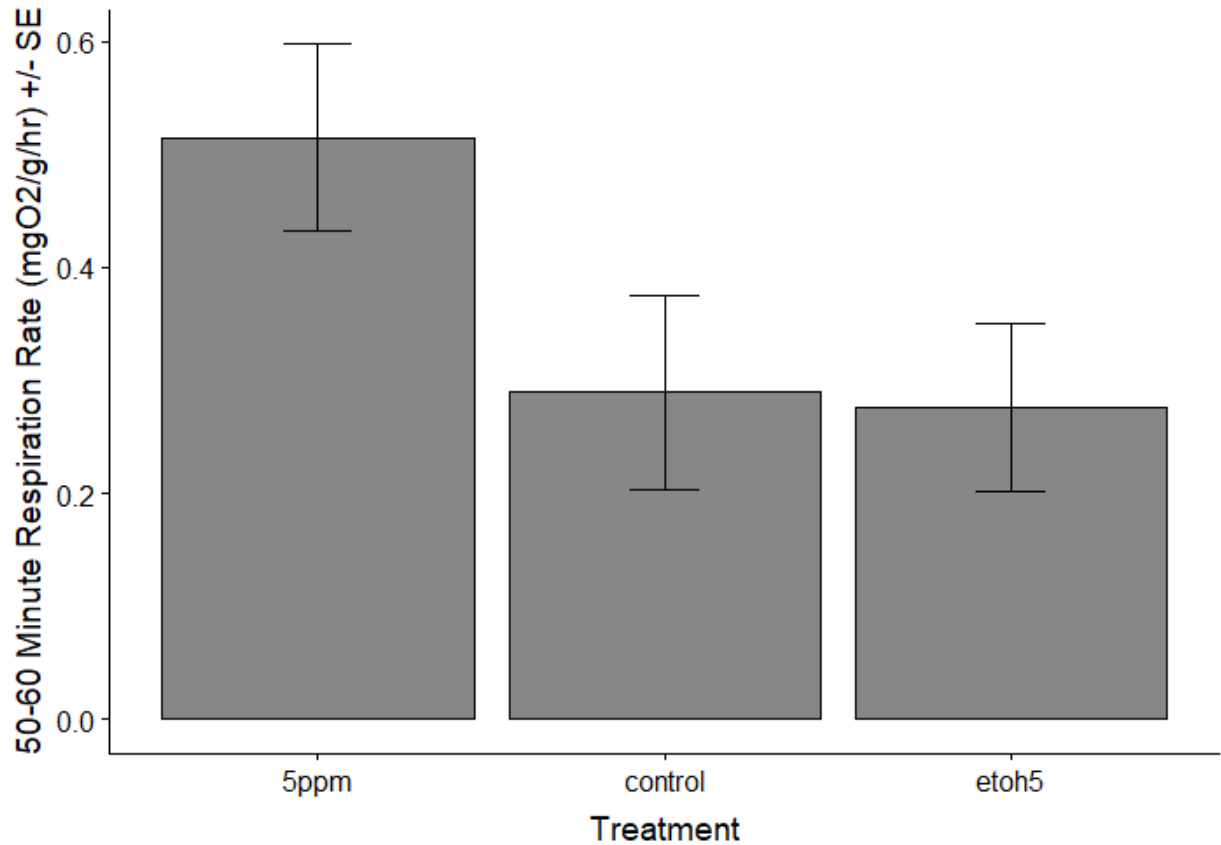
Of the fifty-nine *N. crysoleucas* utilized in this study, 20 were exposed to 5 mg/L of ethanol, 19 to 5 mg/L of iso-eugenol, and 20 controls (Table 5). Treatment did not have a significant effect on total respiration rates ( $F(2,56)=2.9$ ,  $p=0.063$ ; Figure 21). Treatment did not have a significant effect on the respiration rates of *N. crysoleucas* ( $F(2,48)=0.59$ ,  $p=0.56$ ; Figure 22) calculated between the 50 and 60 minute time periods. Neither temperature nor weight had a significant effect on either respiration rate ( $p>0.66$ ).

**Table 5.** Description of *N. crysoleucas* collected for this study.

Treatment	n	Mean Weight (g) ± SE
Control	20	2.0 ± 0.09
5 mg/L Ethanol	20	1.9 ± 0.1
5 mg/L Iso-eugenol	19	1.8 ± 0.09
Total	N=59	1.9 ± 0.06



**Figure 21.** Non-significant difference in total respiration rates (mgO<sub>2</sub>/g/hr) of *N. crysoleucas* exposed to different treatments. Respiration rates include +/- 1 standard error.



**Figure 22.** Non-significant difference in respiration rates calculated between the 50 and 60 minute time periods (mgO<sub>2</sub>/g/hr) of *N. crysoleucas* exposed to different treatments. Respiration rates include +/- 1 standard error.

## DISCUSSION

### *Effects on Physiological Stress Indicators*

Our results demonstrate lower lactate and glucose concentrations in *R. terraenovae* exposed to 10 mg/L of clove oil, which may be indicative of a stress reducing effect. This is in contrast to results commonly seen in the literature. However, it is possible that rather than clove oil having a stress-reducing effect, ethanol may have a stress-inducing effect. Ethanol was not observed to have a significant effect on behavior or ventilation rate in flowerhorn (*Amphilophus labiatus* x *Amphilophus trimaculatus*) (Tarkhani et al., 2017). This lack of an effect of ethanol

supports the idea that clove oil may have a stress-reducing effect in *R. terraenovae*. The difference in our results compared to previous studies could also be a result of species-specific variation in the effects of clove oil. A study examining the effect of clove oil sedation on six species of teleost (brown trout, *Salmo trutta*; Atlantic salmon, *Salmo salar*; rainbow trout, *Oncorhynchus mykiss*; whitefish, *Coregonus lavaretus*; perch, *Perca fluviatilis*; and roach, *Rutilus rutilus*) observed large variation in sedative effects between species and even individuals of the same species, forcing the authors to conclude that finding an optimal dose for a species may be difficult (Hoskonen and Pirhonen, 2004).

The primary stress response commonly seen in other studies would, theoretically, lead to changes in glucose concentrations as a function of both catecholamines and cortisol, both of which mobilize glucose throughout the body (Thomas and Robertson, 1991). It is possible that sedatives could prevent the breakdown of excess energy stores of the liver, therefore, not resulting in an increase in blood glucose. The literature on the physiological effects of sedatives has demonstrated mixed results when it comes to changes in glucose levels. Many studies have observed increases in glucose following elevations in glucocorticoids (Thomas and Robertson, 1991; Sladky et al., 2001; Davis and Griffin, 2004; Weber et al., 2011). These studies, similar to the results seen with cortisol during sedation, implicate a minor stress response due to exposure to sedatives as the cause for these results. However, some studies have observed no effect of sedative exposure on glucose levels (Iversen et al., 2003; Davis and Griffin, 2004). Additionally, the only study examining the physiological response of an elasmobranch to sedation observed no change in glucose in sharks exposed to clove oil (Frick et al., 2009). The above results are all in contrast to the results seen in the present study in which iso-eugenol at both 5 and 10 mg/L resulted in significantly lower glucose levels than control sharks, indicating a potential reduction

in either the sympathetic or HPI-axis based stress response (both of which can be responsible for elevated glucose). Despite this, we also noted significantly poorer release behavior in sharks exposed to the high dose of clove oil compared to ethanol. This could be indicative of negative physiological effects of clove oil on parameters not measured in this study or it could be due to the longer recovery times that are typical of clove oil sedation (Stamper and Neiffer, 2009).

During a stress event, a process known as lamellar recruitment may occur as a result of increased blood flow in the gill lamellae due to catecholamine release. This process can increase the permeability of the gills to water fluxes between the shark and its environment, leading to hemodilution, fluxes of water across vascular compartments, and potentially severe disruptions to osmotic balance. However the potential of sedatives to reduce the stress response could also reduce the likelihood of these negative effects. Some studies investigating the physiological effects of sedatives in fishes have not observed a significant change in hematocrit upon sedation (Cooper and Morris, 1998; Weber et al., 2011), while one study observed an increase in hematocrit in response to sedation (Sladky et al., 2001). Our results demonstrate a reduction in hematocrit of sharks exposed to clove oil at 10 mg/L than that observed in sharks exposed to the equivalent amount of ethanol contained in the 10 mg/L clove oil mixture. This could represent hemodilution that may be indicative of a stress response, contrary to the results from other stress indicators, such as glucose and lactate.

### *Effects on Respiration*

The stress response is likely to have direct or indirect effects on respiration and ventilation rates. During stress, an oxygen debt may be generated due to a shift to anaerobic respiration and an increase in activity, which will also result in a build-up of carbon dioxide and

acidification of the blood. Hyperventilation is a common coping mechanism for blood acidification because it allows for the release of built up carbon dioxide and the introduction of more oxygen into the body (Cooper and Morris, 1998). There is evidence of elevated carbon dioxide levels stimulating hyperventilation in fishes along with a possible role of catecholamines in directly stimulating hyperventilation (Wood and Munger, 1994). It is likely that some combination of these factors stimulates increased respiration and ventilation rates as a way of offsetting acidosis. Because of this, measures of respiration and ventilation are commonly utilized as a reliable indicator of physiological impairments due to stress and have also been utilized to determine the physiological effects of sedatives.

The majority of sedatives commonly used in fishes (clove oil, metomidate, 2-phenoxyethanol, and quinaldine) have a suppressing effect on the respiratory system. Lower rates of either respiration or ventilation have been reported as a result of sedative exposure in fishes (Sneddon, 2012). However, some studies on fishes have noted increased ventilation rates upon immediate exposure to sedatives such as MS-222 and benzocaine (Sneddon, 2012). Sedation with a form of eugenol (eugenol, iso-eugenol, or clove oil) typically results in suppressed respiratory function (Sladky et al., 2001; Javahery et al., 2012; Sneddon, 2012) and can even result in complete respiratory failure, requiring resuscitation (Sladky et al., 2001). It is thought that this is due to either inhibition of the respiratory center of the medulla oblongata or due to neurotoxic or hepatotoxic properties (Javahery et al., 2012). Studies with mammals have revealed neurotoxic, hepatotoxic, and irritant properties of clove oil (Sladky et al., 2001). Our results revealed that lower doses of clove oil (5 mg/L) resulted in respiration rates that were significantly higher in this treatment than 10 mg/L of both clove oil and ethanol in *R. terraenovae*. It is possible that the low dose of clove oil is not enough to fully sedate the animal



so that exposure to a novel substance results in a minor stress response. In fact, sedating doses of clove oil in other studies have been higher than those used here, such as 10-15, 20, 24, 30, 50, and 80 mg/L (Iversen et al., 2003; Cooke et al., 2004; Pattanasiri et al., 2008; Frick et al., 2009; Weber et al., 2011; Tarkhani et al., 2017), indicating that 5 mg/L was likely too low of a dose to result in full sedation. Our results also showed significantly lower ventilation rates in *C. plagiosum* exposed to iso-eugenol, similar to reports in the literature (Sneddon, 2012).

The extreme interspecific variation in the physiological effects of sedatives reported by Hoskonen and Pirhonen (2004) were also observed in the present study. Significantly lower respiration rates were seen in *C. plagiosum* exposed to 5 mg/L of clove oil compared to ethanol and control sharks, indicating a potential stress-reducing effect of 5 mg/L clove oil in these sharks, whereas this was not the case in *R. terraenovae*. Our results also demonstrate that respiration rates of *N. chrysoleucas* were not affected by exposure to 5 mg/L of clove oil. The contrasting results between these species highlight the species-specific variation in effects of sedatives. It is possible that this variation is due to differences in life history and lifestyle of the sharks, in which *R. terraenovae* are ram ventilators and *C. plagiosum* can utilize a buccal pump while resting on the ocean floor. This typically leads to differences in activity levels with ram ventilators being more active. Even though *N. chrysoleucas* also utilize a buccal pump, they are likely more active than *C. plagiosum* since *N. chrysoleucas* are not a benthic species. However, higher activity levels should lead to higher metabolic rates which are thought to increase the effect of and recovery from sedation (Javahery et al., 2012). Despite this *C. plagiosum*, the least active species seemed to experience a sedative effect of clove oil at a low dose than was not experienced by *R. terraenovae* or *N. chrysoleucas*. It is also possible that this species-specific difference could be related to differences in size of the fishes and may require the use of larger

doses in larger animals. Our results on sharks would support this, in that larger *R. terraenovae* had reductions in stress indicators with only the high dose of iso-eugenol, whereas the smaller *C. plagiosum* had potential detrimental effects on ventilation with the low dose of iso-eugenol.

## CONCLUSIONS: CLOVE OIL AS A SEDATIVE

Many researchers and fisheries biologists are intrigued by the possibility of clove oil as a sedative due to quick induction to sedation, availability, low cost, and efficacy at a range of temperatures. However, effects such as elevated cortisol (Thomas and Robertson, 1991; Iversen et al., 2003; Davis and Griffin, 2004; Yousefi et al., 2018), lactate (indicative of a stress response) (Iversen et al., 2003; Frick et al., 2009; Weber et al., 2011; Yousefi et al., 2018), and respiratory failure (Sladky et al., 2001; Javahery et al., 2012) indicate that further study is necessary to determine if it is a safer alternative to the commonly utilized sedatives in fishes. Our results demonstrated that 10 mg/L of clove oil resulted in lower lactate, glucose, and hematocrit levels, indicating a possible reduction in the stress response of *R. terraenovae*. However, release behavior was significantly lower in sharks exposed to 10 mg/L of clove oil in comparison with the equivalent amount of ethanol. This effect may have been observed due to the longer recovery times that are typically necessary for animals exposed to clove oil compared to other sedatives (Stamper and Neiffer, 2009). A low dose of clove oil appears to be effective at reducing respiration and ventilation rates of *C. plagiosum*, but the possibility of ventilatory failure persists. It is possible that a higher dose of clove oil could effectively reduce respiration rates of *N. chrysoleucas*, therefore, further research is necessary. The variation between species in this study indicates that the effect of clove oil cannot be generalized across species and trials are necessary

to determine a safe dosage for each species of interest. Although more research is necessary, the potential for ventilatory failure and the poor release condition of sharks exposed to high concentrations of clove oil indicate that it may not be a safe alternative for *R. terraenovae*.

## CHAPTER 5:

### RECOMMENDATIONS TO MANAGERS, FISHERS, AND SCIENTISTS

The purpose of this project was to perform descriptive and experimental research with the goal of having results that could be directly applicable to fishers, fisheries managers, and scientists. The type of applied research helps to bridge the gap between information that exists in scientific literature and the information that managers utilize when developing management plans. The publication of these types of research in journals with an applied management focus can also help to bridge the gap between scientists and managers.

First, this work can provide recommendations to other elasmobranch stress physiologists. Our work validating and quantifying ACTH with a commercially available ELISA kit opens up this opportunity to other researchers as well. We have developed a reliable method of validating the kit for use in a particular species through ensuring cross-reactivity does not occur with other constituents in plasma. This procedure can easily be adopted by other researchers aiming to validate this ELISA in a different species. Our use of a commercial kit also makes it much easier and less time-intensive to understand more about the alarm stage of the stress response. While researchers are making progress towards measurement of  $1\alpha$ -OHB (Wheaton et al., 2018), this process still involves synthesis of the hormone and development of an antibody against it. Until this hormone is made readily available, quantification of ACTH will still provide quick and easy insight into the alarm stage. We recommend that researchers focusing on the resistance stage,

through measurement of lactate, glucose, hematocrit, osmolality, etc., add quantification of ACTH to the suite of physiological indicators being investigated. Doing this will broaden the view of the stress response that researchers are able to obtain through measurement of indicators of the alarm and resistance stages. The difference in timing of the alarm and resistance stage makes it important to understand more about the effects of both. For example, the alarm stage involves the immediate effects within minutes of the perception of the stressor (Sumpter and Donaldson, 1986; Liberson et al., 1997), which may be important to researchers interested in the acute response to stress. However, the resistance stage can occur and last anywhere from 15 minutes to hours after perception of the stressor (Arends et al., 1999; Frick et al., 2012; Kneebone et al., 2013; Hoffmayer et al., 2015), therefore, this can provide a view of the delayed or more long-term effects of the stressor. Including measurement of both of these stages will help to strengthen studies on the stress physiology of elasmobranchs.

Next, we can make recommendations directly to fishers and managers through applied experimental research. While there are many studies that investigate the physiological effects of air exposure on fishes (Arends et al., 1999; Davis and Parker, 2004; Suski et al., 2007; Frick et al., 2010; Cicia et al., 2012; Lambert et al., 2018), none of these have utilized a fine-scale of air exposure durations to help determine how long fishers can keep fish out of the water. Our use of 0, 5, 10, and 15 minutes of air exposure allow us to achieve that goal. With this, we can confidently recommend to fishers and managers that captured *C. limbatus* be released back into the water within five minutes. According to our results, doing this will result in lactate levels that are not significantly higher than those measured within minutes of capture. Limiting air exposure to five minutes will also avoid negative effects on release behavior and reflex impairment. While

we were not able to link these effects directly to post-release survival, we can assume that limiting these effects will allow the animals the best chance of survival after capture.

Lastly, our investigation into iso-eugenol as a means of reducing the magnitude of the stress response provides a base of information on which future studies may build. Iso-eugenol demonstrated potential stress reduction through decreases in secondary stress indicators, such as lactate, glucose, and hematocrit. However, this research also demonstrated the need for extended recovery times from the sedative before release. Our study noted dramatic species-specific variation in the effects of iso-eugenol with potentially stress reducing effects in *R. terraenovae*, possibly detrimental respiratory effects in *C. plagiosum*, and no obvious effect in *N. crysoleucas*. Because of this, we can only recommend the use of iso-eugenol for stress reduction after having run preliminary trials on the species in question and with a range of doses to determine what is appropriate on an individual basis.

We hope that the results of these studies can be utilized by researchers, managers, and fishers to improve our understanding of the elasmobranch stress response and to aid in reduction of stress as a means to increase chances of post-release survival. While there have been many studies to quantify the physiological response to stress, very few have investigated methods to reduce stress of captured sharks. The continued decline in populations of some elasmobranchs due to overfishing and the stress associated with bycatch makes it critical to take the next step and find reliable methods of stress reduction in these animals.

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## APPENDIX

**Table A. Summary of statistical results**

Response Variable: ACTH		Chapter 2 ANCOVA			
Predictor Variables:	Sum of Squares	DF	F	<i>p</i>	
Fixed effects					
Time on line	4790.5	4	8.63	<b>0.0008</b>	
Sex	210.11	1	1.24	0.29	
Weight	105.06	1	0.62	0.45	
Temperature	1675.0	1	12.06	<b>0.003</b>	
Time of day of collection	382.65	1	2.26	0.17	
Response Variable: Lactate		Chapter 2 ANCOVA			
Predictor Variables:	Sum of Squares	DF	F	<i>p</i>	
Fixed effects					
Time on line	32.49	4	4.60	<b>0.008</b>	
Sex	0.53	1	0.67	0.43	
Weight	0.037	1	0.047	0.83	
Temperature	0.087	1	0.11	0.75	
Time of day of collection	1.05	1	1.33	0.28	
Response Variable: Osmolality		Chapter 2 ANCOVA			
Predictor Variables:	Sum of Squares	DF	F	<i>p</i>	
Fixed effects					
Time on line	7880	4	1.58	0.24	
Sex	277.0	1	0.18	0.67	
Weight	55.2	1	0.04	0.85	
Temperature	38634	1	31.05	<b>0.0001</b>	
Time of day of collection	8834	1	7.10	<b>0.02</b>	
Response Variable: Release Behavior		Chapter 2 Ordinal Logistic Regression			
Predictor Variables:	X <sup>2</sup>	<i>df</i>	<i>p</i> (>  <i>t</i>  )		
Fixed effects					
Time on line	4.25	4	0.37		
Lactate	7.73	1	<b>0.005</b>		
ACTH	1.74	1	0.19		
Osmolality	1.03	1	0.31		
Response Variable: Lactate		Chapter 3 ANCOVA			
Predictor Variables:	Sum of Squares	DF	F	<i>p</i>	
Fixed effects					
Air exposure duration	24.3	3	4.35	<b>0.01</b>	
Total length	2.10	1	1.05	0.32	
Sex	6.25	1	3.11	0.09	
Thermal Gradient	1.58	1	0.79	0.38	
Dissolved oxygen	1.72	1	0.86	0.36	

Response Variable: Glucose					
Chapter 3 ANCOVA					
Predictor Variables:	Sum of Squares	DF	F	p	
Fixed effects					
Air exposure duration	0.16	3	3.34	<b>0.038</b>	
Total length	0.04	1	3.64	0.07	
Sex	0.000001	1	0.0001	0.99	
Thermal Gradient	0.0007	1	0.06	0.81	
Dissolved oxygen	0.015	1	1.33	0.26	
Response Variable: Hematocrit					
Chapter 3 ANCOVA					
Predictor Variables:	Sum of Squares	DF	F	p	
Fixed effects					
Air exposure duration	2.64	3	1.02	0.4	
Total length	0.13	1	0.20	0.66	
Sex	1.21	1	1.82	0.20	
Thermal Gradient	0.03	1	0.05	0.83	
Dissolved oxygen	2.47	1	3.70	0.07	
Response Variable: Release Behavior					
Chapter 3 Ordinal Logistic Regression					
Predictor Variables:	X <sup>2</sup>	df	p (> t )		
Fixed effects					
Air exposure duration	21.32	3	1e-04		
Lactate	3.07	1	0.08		
Glucose	-1.551	1	0.51		
Hematocrit	4.587	1	0.19		
Response Variable: Lactate					
Chapter 4 Linear Mixed Effects Model					
Predictor Variables:	Sum of Squares	Mean Square	Df (Num/Den)	F	p
Fixed effects					
Treatment	49.02	12.26	4/128.85	2.16	0.077
Time	1317.32	1317.32	1/140.45	231.94	<b>&lt;2.2e-16</b>
Mass	0.05	0.05	1/16.4	0.011	0.92
Water temperature	4.06	4.06	1/15.9	0.88	0.36
Salinity	13.32	13.32	1/19.5	2.35	0.14
Dissolved oxygen	8.07	8.07	1/22.7	1.42	0.25
Treatment*time interaction	322.38	80.59	4/140.4	14.2	<b>9.45e-10</b>
Response Variable: Glucose					
Chapter 4 Linear Mixed Effects Model					
Predictor Variables:	Sum of Squares	Mean Square	Df (Num/Den)	F	p
Fixed effects					
Treatment	5104.7	1276.2	4/51.79	3.06	0.025
Time	19891.8	19891.8	1/126.13	47.65	<b>2.2e-10</b>

Mass	1097.9	1097.9	1/16.1	2.78	0.11
Water temperature	848.4	848.4	1/16.0	2.15	0.16
Salinity	484.2	484.2	1/16.0	1.23	0.28
Dissolved oxygen	5421.5	5421.5	1/16.1	1.37	0.19
Treatment*time interaction	1292.6	323.2	4/69.14	0.82	0.52

Response Variable: Hematocrit

**Chapter 4 Linear Mixed Effects Model**

Predictor Variables:	Sum of Squares	Mean Square	Df (Num/Den)	F	p
Fixed effects					
Treatment	33.6	8.40	4/22.5	0.47	0.76
Time	15.04	15.04	1/55.2	0.84	0.36
Mass	0.061	0.061	1/13.7	0.003	0.95
Water temperature	22.87	22.87	1/12.9	1.28	0.28
Salinity	2.21	2.21	1/13.0	0.12	0.73
Dissolved oxygen	0.37	0.37	1/14.01	0.02	0.89
Treatment*time interaction	47.02	47.02	4/55.63	0.66	0.62

Response Variable: Respiration Rate

**Chapter 4 ANCOVA**

**Sharpnose**

Predictor Variables:	Sum of Squares	DF	F	p
Fixed effects				
Treatment	0.10	4	3.91	<b>0.0099</b>
Temperature	0.03	1	0.05	0.27
Dissolved oxygen	0.00017	1	0.03	0.87

Response Variable: Release Behavior

**Chapter 4 Ordinal Logistic Regression**

**Regression**

Predictor Variables:	X <sup>2</sup>	df	p (> t)
Fixed effects			
Treatment	12.36	4	<b>0.015</b>
Lactate	0.03	1	0.86
Glucose	0.08	1	0.78
Hematocrit	1.62	1	0.20
Respiration rate	0.94	1	0.33

Response Variable: Respiration Rate

**Chapter 4 ANCOVA Bamboo Sharks**

Predictor Variables:	Sum of Squares	Df (Num/Den)	F	p
Fixed effects				
Treatment	0.006	2/8.7	15.15	<b>0.0015</b>
Temperature	0.0003	1/6.6	1.45	0.27
Weight	0.00017	1/2.07	0.82	0.46

Response Variable: Ventilation Rate

**Chapter 4 ANCOVA Bamboo Sharks**

Predictor Variables:	Sum of Squares	Df (Num/Den)	F	p
Fixed effects				
Treatment	2327.02	2/3.04	141.84	<b>0.001</b>

Temperature	102.16	1/3.33	1.23	0.33
Weight	2.15	1/2.89	0.26	0.65
Response Variable: Respiration Rate		<b>Chapter 4 ANCOVA Shiners</b>		
Predictor Variables:	Sum of Squares	<i>Df</i>	F	p
Fixed effects				
Treatment	0.019	2	2.90	0.063
Temperature	0.0007	1	0.20	0.66
Weight	0.00004	1	0.01	0.92

Bold = Significant



## VITA

### Lauren Fuller

#### ***Education:***

- 2013-July,19 Graduate Education, Biology, 4.0 GPA, University of Mississippi at Oxford  
Dr. Glenn R. Parsons, advisor. Graduation: July, 2019.  
Dissertation title: Description and Alleviation of the Stress Response  
in Sharks.
- 2009-2013 B.S. Organismal Biology, 3.48 GPA, University of Arkansas at Monticello

#### ***Research Experience:***

- 2013-Present Research on the effects of air exposure on blacktip sharks with results and  
recommendations directly applicable to fishers and managers. University of  
Mississippi Biology Department.
- 2013-Present Investigation into a sedative, iso-eugenol, to determine if it is effective at  
reducing the stress response of Atlantic sharpnose sharks, white-spotted bamboo  
sharks, and golden shiners. University of Mississippi Biology Department.
- 2015-2018 Research on the jumping characteristics of invasive Asian carp with intentions of  
improving barrier design. University of Mississippi Biology Department.
- June, 2017 Assistant on a research trip investigating the efficacy of the greenstick tuna  
fishing technique as an alternative to long-line tuna fishing to reduce bycatch  
under Dan Foster. National Oceanographic and Atmospheric Administration.
- 2015-2016 Research Assistant on a shark bycatch reduction project involving development  
and production of fishing techniques to encourage shark bite-off. University of  
Mississippi Biology Department.
- 2012-2013 Research investigating the effects of ATV traffic in the Saline River, AR on  
freshwater mussel abundances. The University of Arkansas at Monticello.

#### ***Teaching Assistantships/Experience:***

- Physiology Lab, 7 semesters, University of Mississippi
- Biometry Lab, 1 semester, University of Mississippi
- Biology 1 and 2 Labs, 2 semesters each, University of Mississippi
- Biology of Sharks and Their Relatives Lecture, 2 lectures
- Herpetology Lab, 1 semester, The University of Arkansas at Monticello
- Comparative Vertebrate Anatomy Lab, 1 semester, The University of Arkansas at Monticello

#### ***Outreach/Volunteering:***

- Volunteer at the Memphis Zoo; Memphis, TN. January, 2018-present.
  - Docent Training, August, 2018-present. Involves training from current docents and Zookeepers and engaging and educating guests about the animals in exhibits.
  - Volunteer at Stingray Bay, Summer 2018. Duties involved teaching guests how to safely pet and feed stingrays, engaging and educating guests on the biology of sharks and rays.

- Zoo Brew, May, 2018. Duties involved pouring beer for guests and explanation of the differences between the various beers available.
- Volunteer for Explorers Program, February, 2018. Duties involved helping Education staff with educational activities for elementary school students.
- Impromptu lecture for visiting Junior High students; July 2017; The University of Mississippi. Demonstrated anatomy of shark jaws, discussed shark biology, question and answer session.
- Volunteer Educator at Bramlett Elementary School's Shark Week for First Graders; Oxford, MS; May 2017. Title: All About Sharks. Topics: Basic shark biology and question and answer session.
- Volunteer Educator at the University of Mississippi Field Station's Science Day; October 2017; Oxford, MS. Duties involved demonstration and identification of representative biota.
- Volunteer Educator at the Outdoor Wildlife Day for the Monticello Elementary School; April 2011; Monticello, AR. Duties involved preparing a lecture and poster display on marine ecology and conservation, design and construction of a trophic pyramid display for hands-on teaching.
- Volunteer clean-up of the Saline River, AR with the University of Arkansas at Monticello Biology Club; Spring 2011. Tasks included picking up and proper disposal of trash.

**Notable Oral Presentations:**

- Fuller, L.N. Stress in Sharks: Quantification of Adrenocorticotropic Hormone and its Application to Fisheries Conservation. 2019. Oral presentation at the Mississippi Chapter of the American Fisheries Society meeting; Jackson, MS. Awarded 2<sup>nd</sup> place presentation.
- Fuller, L.N. Stress Reduction in Sharks. 2018. Oral presentation at the University of Mississippi's Three Minute Thesis Competition; University, MS. Awarded 3<sup>rd</sup> place.
- Fuller, L.N., Parsons, G.R. A Note on Associations Observed between Sharks and Teleosts in the Gulf of Mexico. 2017. Oral presentation at the Mississippi Chapter of the American Fisheries Society annual meeting; Biloxi, MS.
- Fuller, L.N., Parsons, G.R. Sedation as a means to reduce capture stress of sharks. 2016. Oral presentation at the Joint Meeting of Ichthyologists and Herpetologists meeting; New Orleans, LA.

**Publications:**

- Buchholz, R., Banusiewicz, J.D., Burgess, S., Crocker-Buta, S., Eveland, L., & Fuller, L. 2019. Behavioural research priorities for the study of animal response to climate change. *Animal Behaviour*. 150:127-137.
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***Software Proficiencies:***

- Microsoft Office suite, Blackboard learning software, statistical softwares (R, Primer, JMP), PowerLab Physiological Software