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POPULATION GENETICS OF COWNOSE RAYS, RHINOPTERA SPP. IN THE WESTERN ATLANTIC

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POPULATION GENETICS OF COWNOSE RAYS, *RHINOPTERA* SPP. IN THE WESTERN ATLANTIC

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ABSTRACT

Using molecular tools (e.g., the NADH subunit II mitochondrial gene), this study was the first to document the Brazilian cownose ray, *Rhinoptera brasiliensis*, within the northwestern Atlantic (NWA), and was the first study to examine population structure in *R. brasiliensis* within the NWA or northern Gulf of Mexico (GMX), revealing novel insights into the population biology of the animal and extending its range by nearly 1,500 km. This study also examined the sympatrically occurring American cownose ray, *R. bonasus*, and found population structure between the NWA and the GMX and the NWA and the southwestern Atlantic (SWA). High levels of population structuring were detected for *R. bonasus* between the NWA, the GMX, and the SWA, a finding which was not supported for *R. brasiliensis*. Low levels of genetic diversity for both species were found within the NWA and GMX, and high levels were found in the SWA, indicating a possible genetic difference between the three regions. The demographic history of both species was investigated using neutrality tests and indicated an evolutionarily recent population expansion.

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DEDICATION

In loving memory of my grandfather, Edwin, and my brother, Trey.

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LIST OF ABBREVIATIONS

AL	Alabama, USA
AMOVA	Analysis of Molecular Variance
ATL	western Atlantic
BIC	Bayesian Information Criterion
BLAST	Basic Local Alignment Search Tool
bp	base pair
BSA	bovine serum albumin
COI	Cytochrome C oxidase subunit I
Cyt B	Cytochrome B
DNA	deoxyribonucleic acid
DnaSP	DNA Sequence Polymorphism
dNTP	deoxyribonucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
eGMX	eastern Gulf of Mexico
FL	Florida, USA
GA	Georgia, USA
IUCN	International Union for Conservation of Nature
JC + G	Jukes-Cantor +Gamma
LA	Louisiana, USA
$MgCl_2$	magnesium chloride
MS	Mississippi, USA

mtDNA	mitochondrial DNA
ND2	Nicotinamide adenine dinucleotide hydrogen
NC	North Carolina, USA
NCBI	National Center for Biotechnology Information
nDNA	nuclear DNA
NWA	northwestern Atlantic
PCR	polymerase chain reaction
QA	quality assurance
QC	quality control
SSD	sum of squared deviations
SWA	Southwestern Atlantic
TBE	tris-borate-EDTA
TX	Texas, USA
USA	United States
UV	ultraviolet
VA	Virginia, USA
wGMX	Western Gulf of Mexico

CHAPTER I – LITERATURE REVIEW

1.1 Taxonomy and distribution

The genus *Rhinoptera* is made up of eight circumglobal species, with two species present in the Western Atlantic: *Rhinoptera bonasus,* Mitchill 1815, and *R. brasiliensis*, Muller 1836 (Fricke, Eshmeyer, & Van der Laan, 2020). All eight species in the genus *Rhinoptera* appear morphologically conserved, making field identification challenging. Tooth series counts, which are the numbers of teeth in the upper and lower jaws counted horizontally, have been used to differentiate *Rhinoptera* spp. (Figure 1) (Bigelow & Schroeder, 1953; James 1971; Last et al., 2016).

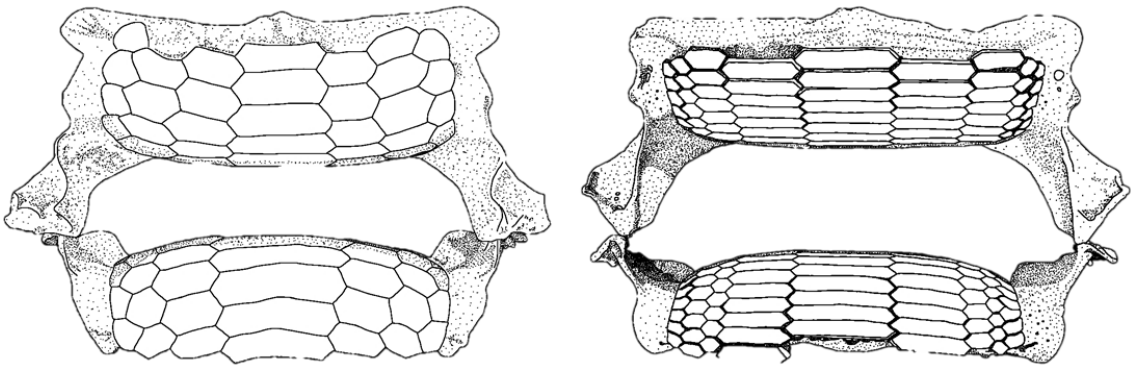

Figure 1.1 *Illustrations depicting tooth series counts*

Rhinoptera bonasus (left) and *R. brasiliensis* (right) (from Jones et al., 2017).

However, the use of tooth series counts alone to distinguish *Rhinoptera* spp. in the Western Atlantic may be unreliable due to overlaps in counts (*R. bonasus* = 5 – 15; *R. brasiliensis* = 7 – 13) (Jones et al., 2017). Additionally, anecdotal evidence suggests the feeding activities of *Rhinoptera* spp. may damage tooth plates, causing them to crack and

altering the appearance of tooth plate counts within the animal's lifetime, limiting the suitability of this diagnostic feature (November 2017, C. Jones, pers. comm.). A more reliable method of distinguishing *R. bonasus* from *R. brasiliensis* comes from cranial anatomy; specifically, the shape of the supra-cranial fontanelle (Jones et al., 2017). However, this method of species identification necessitates mortality, dissection, and expertise in *Rhinoptera* spp. morphology. Despite more reliable means of differentiating individuals to species, tooth plate counts are still relied upon in field identification where the two species' ranges overlap. In areas where only one species is thought to occur, range maps are relied upon to identify individuals to species.

The widely accepted range for the two species in the Western Atlantic (hereafter WAT) listed *R. bonasus* from Massachusetts, USA to Uruguay in 2019, and *R. brasiliensis* from the west coast of Florida to Uruguay in 2018 (Figure 2) (Carlson et al., 2019; Carlson et al., 2018). However, recent literature extends the range of *R. brasiliensis* into the northwestern Atlantic (hereafter NWA) along the eastern coast of Florida (Weber et al., 2020).

1.2 Habitat use

Rhinopterids are benthopelagic, most commonly found in warm, temperate, and tropical shallow marine and brackish waters along continental shelves (Last et al., 2016). *Rhinoptera bonasus* may have a greater tolerance for depth as they have been found in depths near 60 meters (Weigmann, 2016), while *R. brasiliensis* are found as deep as 20 meters, and along more sandy bottoms. Euryhaline, they reside primarily in estuaries and

river systems where salinity is as low as 5 ppt but can travel through salinities as high as 37 ppt during migration between seasonally utilized grounds (Neer, Rose, & Cortés 2007; Collins, Heupel, & Simpfendorfer, 2008). Throughout the year, *Rhinoptera* spp. are found within bays and estuaries, which are utilized for nurseries, and the lower reaches of coastal rivers, which may serve as home ranges while not in nurseries (Smith & Merriner, 1987; Blaylock, 1993; Collins et al., 2008).

Rhinopterids are highly mobile, traveling in organized groups, or fevers, numbering in the thousands during seasonal migration (Schwartz, 1965; Blaylock, 1989). Rhinopterids form shoals of 5 – 20 individuals, which congregate into larger fevers, consisting of 10 – 1,000 shoals or 50 – 20,000 individuals as they undertake large-scale migrations along coastlines. Fevers form based on disc width, and therefore age, containing members of both sexes as they migrate along continental shelves from wintering grounds to summer estuaries (Collins et al., 2007). It appears that in both hemispheres of the Atlantic, *Rhinoptera* spp. move away from the equator during spring, into the cooler portion of their range, as they move into estuaries to reproduce (Rangel, Rodrigues, & Moreira 2018). In the northern hemisphere, immigration into estuaries begins in early spring (April; November in southern hemisphere) where *Rhinoptera* spp. remain throughout summer to mate and pup (October; May in southern hemisphere). After leaving estuaries, *Rhinoptera* spp. are thought to retreat to their home range where they overwinter in riverine systems nearer to the equator (Collins et al., 2008). Wintering grounds may be utilized to escape temperatures falling near lethal levels (i.e., 12˚C) as migration is typically prompted by water temperatures falling below 22˚C (Collins et al.,

2007). It has been hypothesized that *R. bonasus* from the northern hemisphere migrates during winter months down to the southern portion of their range to Brazil, however, this may not be an accurate depiction of the migration patterns for all *R. bonasus*, as individuals of this species have been noted along the coast of Florida during winter months (Smith, 1987; Grusha, 2005; Omori & Fisher, 2017).

1.3 Diet

Rhinopterids in the Western Atlantic are benthic, generalist, opportunistic feeders, whose diet depends largely on the benthic prey available within their geographic range (Collins et al., 2007), and ontogeny variation, in which there may be resource partitioning between age groups (Grubbs, 2010; Ajemian & Powers, 2012). *Rhinoptera* spp. diet in the northwestern Atlantic (hereafter NWA) consist primarily of non-commercial clams (Bade et al., 2014), while those in the Gulf of Mexico (hereafter GMX) feed mostly on non-commercial bivalves (Ajemian & Powers, 2012), and those in the southwestern Atlantic (hereafter SWA) primarily feed on sea stars (Bornatowski et al., 2014). Previously thought to be highly durophagous, the foraging behavior of *R. bonasus* in seasonally-utilized estuaries in the NWA has implicated them in the decline of commercially important shellfish (Smith & Merriner, 1985; Blaylock 1992; Peterson et al., 2001; Myers et al., 2007). However, recent research suggests that *R. bonasus* do not have the bite force capability to consume commercially important shellfish, and therefore, feed on a number of non-shelled prey and small shellfish (Collins et al., 2007; Kohlman, 2017).

As mesopredators, Rhinopterids serve an important role in trophic dynamics, linking the lower and upper food levels by opportunistically feeding on the most abundant benthic species, while being preyed upon by apex predators such as bull sharks and cobia (Smith & Merriner, 1982; Blaylock, 1993; Arendt, Olney, & Lucy, 2001; Peterson et al., 2001; Myers et al., 2007). *Rhinoptera* spp. along the NWA and GMX have been associated with areas of low salinity (5 ppt) and near the inside edge of hypoxic bottom water (1 – 2 mg/L), which may suggest an enhanced foraging opportunity and lower osmoregulatory costs (Craig et al., 2010). One potential benefit of this hypoxia edge occurrence is the absence of competing benthic foragers, resulting in higher per capita food availability (Craig et al., 2010). Rhinopterids use their wings to excavate benthic prey, which, when combine with their association with hypoxic environments, may circulate nutrients in unproductive environments (Smith & Merriner, 1985).

1.4 Life history

While in estuaries, mating takes place between mature individuals from April – October in the northern hemisphere, and November – May in the southern hemisphere (Smith & Merriner, 1987; Blaylock, 1993; Rangel, Rodrigues, & Moreira, 2018). Females are considered mature when they are gravid or possess vitellogenin follicles, while males are considered mature when their claspers are calcified and open freely (Poulakis, 2013). Rhinopterids are aplacental viviparous, with only one noted functional ovary (Bigelow & Schroeder, 1953; Smith & Merriner, 1986; Neer & Thompson, 2005;

Poulakis, 2013). After fertilization, one embryo develops and is initially fed through a yolk sac, which typically dissolves by October, after which, nutrients are gained from uterine milk called histroph (Musick, 2010). Gestation lasts 11 – 12 months, and typically results in one offspring; although rare cases of two or more pups have been reported (Bigelow & Schroeder, 1953; Smith & Merriner, 1986; Neer & Thompson, 2005; Poulakis, 2013). Rhinopterids in the northern hemisphere typically pup in early summer (May- July; October – December in the southern hemisphere) (Blaylock, 1993).

Rhinopterids reach disc widths larger than 100 cm during adulthood, although maximum disc width varies between sexes (and likely between species), with females reaching approximately 5 – 10 cm greater maximum size than males; females are typically mature 62 – 92 cm, and males at approximately 64 – 85 cm (Smith & Merriner, 1987; McEachran & de Carvalho, 2002; Fisher, 2010; Last et al., 2016). Growth rates have been noted to vary between geographic region for *R. bonasus*, in the NWA, averaging 7 cm per year, opposed to 5 cm per year in the GMX (Smith & Merriner, 1987; Neer & Thompson, 2005; Fisher et al., 2013). Disc size has been used to infer sexual maturity, leading to maturity age variation among regions; maturing at 7 – 8 years old (75 – 85 cm) in the NWA, and 4 – 5 years old (64 – 70 cm) in the northern GMX (Smith & Merriner, 1986; Neer & Thompson, 2005). In comparison, in the SWA, slightly larger *Rhinoptera* spp. are mature at disc widths between 77 – 91 cm, although age data was not available (Vooren & Lamónaca, 2004). Growth rates and ages are based on vertebral counts, which may not be accurate for elasmobranch studies because of inter-specific differences in zinc deposits (Raoult et al., 2018). Additionally, a lack of genetic species

identification in age and growth studies may have affected the results by including individuals outside the study species. Current assumptions on the life history of *Rhinoptera brasiliensis* have largely been inferred from its congener, *R. bonasus*.

With an average life expectancy of 15 years, *Rhinoptera* spp. in the western Atlantic (hereafter WAT) average nine reproductive years (Neer & Thompson, 2005; Smith & Merriner, 1987). Throughout reproductive years, *Rhinoptera* spp. pup in estuaries (see 1.4) and are ready to mate in as little as a month after pupping, which is made possible by their ability to ovulate immediately after parturition, making annual reproduction possible and probable (Poulakis, 2013). However, even with annual reproduction, Rhinopterids have one of the lowest reproductive potentials of all elasmobranchs, averaging five offspring pupped per adult in their lifetime (Grubbs et al., 2016).

1.5 Conservation status and management implications

The utilization of coastal and estuarine habitats makes *Rhinoptera* spp. susceptible to fisheries by-catch, especially when combined with their schooling behavior near the water's surface, which allow a large number of *Rhinoptera* spp. to be caught easily in a single net (Vooren & Lamónaca, 2004). In Central and South America, the bycatch rates constitute a heavy and unregulated fishing pressure for both species (Vooren & Lamónaca, 2004; Carlson et al., 2018, 2019).

Although the majority of fishery threats to *Rhinoptera* spp. in the NWA are indirect (bycatch), direct fishing efforts began in 2007 in Chesapeake Bay, Virginia, USA

after research implicated *R. bonasus* in the decline of commercially important shellfish. The goal of this targeted fishing effort was to alleviate predation on oysters through the removal of *R. bonasus* (Fisher, 2010; Fisher et al., 2013; Grubbs et al., 2016; Carney et al., 2017). Recent research, however, found no commercially important shellfish present in the gut contents of *R. bonasus* from Chesapeake Bay, suggesting the impact on commercially important shellfish abundances was overestimated (Bade, 2014; Grubbs et al., 2016). Furthermore, another study found that *Rhinoptera* spp. do not have the bite force ability to consume marketable prey items (Kohlman, 2018). These findings aided in the exoneration of Rhinopterids in the Chesapeake Bay, resulting in a moratorium on the hunting competitions until a management plan could be drafted for this species.

As a result of the heavy and unregulated fishing pressure of inshore Central and South America combined with their life history characteristics and declining population trends, *Rhinoptera bonasus* was assessed by the IUCN in 2019 as Vulnerable (Carlson et al., 2019) and *R. brasiliensis*, having the same pressures and presumed life history, was also assessed by the IUCN in 2018 as Vulnerable.

1.6 Previous genetic studies

Previous population genetic research using microsatellites, RAG-1 and a 850-base pair (bp) portion of the NADH-2 gene found that *R. bonasus* in Chesapeake Bay were genetically distinct from those in the Gulf of Mexico (McDowell et al., 2014). Further, McDowell assessed the Chesapeake Bay, VA, USA and the Tampa Bay, FL, USA to be separate stocks (McDowell et al., 2014). Additionally, another study using a 540-bp

portion of Cyt B and 585-bp portion of the COI gene reported that although the same haplotypes were found in both Chesapeake Bay and the Tampa Bay, the frequencies of those haplotypes differed significantly, suggesting two genetically distinct populations (Carney et al., 2017). A third study has confirmed the presence of *R. brasiliensis* with in the southern GMX in Veracruz, Mexico, however, the study did not include tissues samples from *Rhinoptera spp.* from the northern GMX for comparison, nor did it provide population level analysis (Palacios-Barreto et al., 2017).

1.7 Project aims

The aim of this project was to better understand the occurrence and population genetics of *Rhinoptera* spp. in the western Atlantic. This research used opportunistic sampling from *Rhinoptera* spp. throughout both species' ranges to serve as the most thorough sampling and analysis of cownose rays in the western Atlantic to date. With these samples, this project:

1) refined the ranges and occurrences of *R. bonasus* and *R. brasiliensis* in the western Atlantic

2) assessed population structure and genetic diversity in *R. bonasus* and *R. brasiliensis* in the western Atlantic

3) examined the demographic history of *R. bonasus* and *R. brasiliensis* in the western Atlantic

Genetically identifying individuals to species was necessary to determine the spatial scale for population level analysis. By determining the spatial scale of each

species occurrences (their ranges), populations could be parsed out and genetic diversities of each population could be calculated. Understanding a species' true range sets the larger spatial scale for management and conservation plans. Population structure informs how the species has grouped, refining the spatial scale for management, and allowing for inferences into life history. Genetic diversities of each species and population give insight into the overall viability of each group and can focus conservation efforts to specific populations or regions. Such information will facilitate management plans throughout both species' ranges, spanning multiple states and countries. The resultant information should be used to improve management and conservation of *Rhinoptera* spp. by providing appropriate spatial scales and insights into viability for management within the Western Atlantic.

CHAPTER II – GENETIC EVIDENCE SUPPORTS A RANGE EXTENSION FOR THE BRAZILIAN COWNOSE RAY *RHINOPTERA BRASILIENSIS* IN THE WESTERN NORTH ATLANTIC

Weber, H. K.[1], Jones, C.M.[2], Ajemian, M. J.[3], McCallister, M.P.[3], Winner, B. L.[4], Poulakis, G. R.[5], Bethea, D. M.[6], Hollensead, L. D.[7], Zapf, D.[8], Swenson, J. D.[9], Hendon, J. M.[10], Daly-Engel, T. S.[11], Phillips, N. M.[1]

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2.1 Abstract

We report 24 new records of the Brazilian cownose ray Rhinoptera brasiliensis outside its accepted geographic range. Sequencing of a 442-base pair portion of the mitochondrial NADH dehydrogenase subunit 2 gene for 282 Rhinoptera samples revealed eight records off the east coast of the USA and 16 from the eastern Gulf of Mexico. Both sexes of all life stages were documented in all seasons over multiple years in the Indian River and Lake Worth lagoons, Florida, indicating that their range extends further in the western North Atlantic than previously described.

2.2 Introduction

The cownose rays (Rhinopteridae) are comprised of eight morphologically similar species in the genus *Rhinoptera* (Last et al., 2016). Rhinopterids are circumglobally distributed, with two recognized species in the western Atlantic: the Endangered Brazilian cownose ray *Rhinoptera brasiliensis* Müller 1836 (Vooren & Lamónaca, 2004) and the Near Threatened American cownose ray *Rhinoptera bonasus* (Mitchill 1815) (Barker, 2006). Both species are benthopelagic and indigenous to tropical and temperate shallow waters along continental shelves (Last et al., 2016). Historically, tooth series counts were used to distinguish between these two species, despite ambiguity arising from overlap in series counts (Bigelow & Schroeder, 1953). Recent research documented changes in tooth morphology with age, making this characteristic unreliable (Jones et al., 2017). More reliable methods of distinguishing *R. brasiliensis* from *R. bonasus*, such as cranial anatomy and number of spiral valve lamellae, necessitate either dissection or

advanced analytical techniques, such as computed tomography or magnetic resonance imaging (Jones et al., 2017).

In lieu of clear, external diagnostic characteristics to distinguish *Rhinoptera* spp., species identifications in the western North Atlantic have largely relied on assumed geographic ranges. This approach, however, grossly underestimated the range of *R. brasiliensis* in the western Atlantic. The range of *R. bonasus* extends from New England in the United States to northern Argentina (Last et al., 2016). Historically, *R. brasiliensis* was considered endemic to Brazil, restricted to the waters of southern Brazil (Barker, 2006; Bigelow & Schroeder, 1953). Recent literature extended the range of *R. brasiliensis* to include Central America (McEachran & Carvalho, 2002), the southern Gulf of Mexico (Palacios-Barreto et al., 2017), and the northern Gulf of Mexico (Jones et al., 2017), using a combination of morphological and molecular methods in species identifications. This range extension of *R. brasiliensis* substantially increased overlap in the ranges of *R. brasiliensis* and *R. bonasus*, and given the absence of clear field characteristics, the range of *R. brasiliensis* is still poorly understood. Given this uncertainty, existing datasets of *Rhinoptera* spp. in the western Atlantic may reflect a species complex rather than single species, which could lead to the use of spurious data from biological and ecological studies in their management. Here, we used genetic methods to identify *Rhinoptera* spp. to evaluate the extent of occurrence of *R. brasiliensis* beyond its currently described range in the western North Atlantic, specifically in the eastern Gulf of Mexico (hereafter eGOM) and along the east coast of the United States (hereafter Atlantic).

2.3 Genetics methods

A total of 282 archived or opportunistically collected *Rhinoptera* spp. tissue samples collected between 2002 and 2018 were analyzed from 12 locations in the eGOM and Atlantic, spanning from Santa Rosa Sound, Florida, to Chesapeake Bay, Virginia (Figure 1). *Rhinoptera* spp. were caught throughout the year, primarily by gillnet, and fin clips were collected and stored in 95% ethanol. Total genomic DNA was extracted from 15 mg of tissue using a Qiagen DNeasy DNA extraction kit (Hilden, Germany), according to the manufacturer's protocol, with the exception that tissue samples were digested overnight. A 442-base pair region of the mitochondrial (mtDNA) NADH dehydrogenase subunit 2 (ND2) gene was targeted using a forward primer (RhinND2F1: 5`-GAACCCYTTAATCCTCTYCATC-3`) designed by McDowell and Fisher (unpubl. data) and a reverse primer (RayND2R: 5`-GGATTGATAGTACGCCTATGG-3`) designed for this study. Polymerase chain reaction (PCR) mixtures contained 25 – 50 ng template DNA, 10 mM Taq buffer (Invitrogen, Carlsbad, California, USA), 1.5 mM $MgCl_2$, 0.3 μM of each primer, 0.1 mM deoxynucleotide triphosphates (dNTP mix, Promega, Madison, Wisconsin, USA), 1 U of Taq polymerase (Invitrogen) and PCR-grade water for a final reaction volume of 25 μL. PCR cycling conditions consisted of an initial denaturation at 95°C for 5 min, then 35 cycles of 30 s denaturation at 95°C, 30 s annealing at 58°C, 30 s extension at 72°C, followed by a 5 min final extension at 72°C. Amplicons were cleaned using 3 μL of ExoSAP-IT (ThermoFisher, Waltham, Massachusetts, USA) and sequenced in the forward and reverse directions on an Applied

Biosystems 3730XL DNA Analyser using a BigDye Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA) following the manufacturer's protocol except that all sequences were run using half reactions.

Consensus sequences were generated by aligning the forward and reverse sequences for each individual in CodonCode v 9.0.1 (CodonCode Corporation, Dedham, Massachusetts, USA). Resultant haplotypes were compared to mtDNA ND2 haplotypes generated, using the methods described here, for 10 specimens each of *R. brasiliensis* and *R. bonasus* collected from Mississippi and Alabama waters (United States), which were verified using a suite of 21 external morphological measurements and genetic analysis of the cytochrome c oxidase subunit I (CO1) gene (see Jones et al., 2017). The Jukes–Cantor substitution model (Jukes & Cantor, 1969) was determined to be the nucleotide substitution model of best fit for the data in jModelTest 2 v 2.1.10 using Bayesian Information Criterion (BIC) values (Darriba et al., 2012). Phylogenetic relationships between these haplotypes were inferred using a maximum likelihood approach with 10,000 bootstrap replicates and the Jukes–Cantor substitution model in MEGA v X (Kumar et al., 2018), with the bat ray *Myliobatis californica* Gill 1865 (GenBank accession no. KM364985) serving as an outgroup. Relationships among haplotypes were estimated using the maximum parsimony method of Polzin and Daneshmand (2003) and visualized as a median-joining haplotype network using Network v 10.1.10 (Bandelt et al., 1999).

2.4 Results

The 282 tissue samples revealed 14 haplotypes that formed two distinct genetic clades. Three haplotypes representing 24 individuals clustered together with the single haplotype (RBRA1) sequenced for the 10 verified *R. brasiliensis* (GenBank accession nos. MT410205– MT410207) and 11 haplotypes representing 258 individuals clustered with the single haplotype sequenced for the 10 verified R. bonasus (RBON1) (GenBank accession nos. MT410194–MT410204) (Figure 2.1). The sampled *R. brasiliensis* and *R. bonasus* haplotypes were differentiated by 5.43–7.24% sequence divergence, based on the number of base pair differences (Figure 2.2). In contrast, the maximum sequence differentiation between sampled haplotypes within each species was much less: 0.68% and 1.13% for *R. brasiliensis* and *R. bonasus*, respectively (Figure 2.2). The minimum sequence differentiations between *M. californica* and *R. brasiliensis* and *R. bonasus* were 13.80% and 12.90%, respectively.

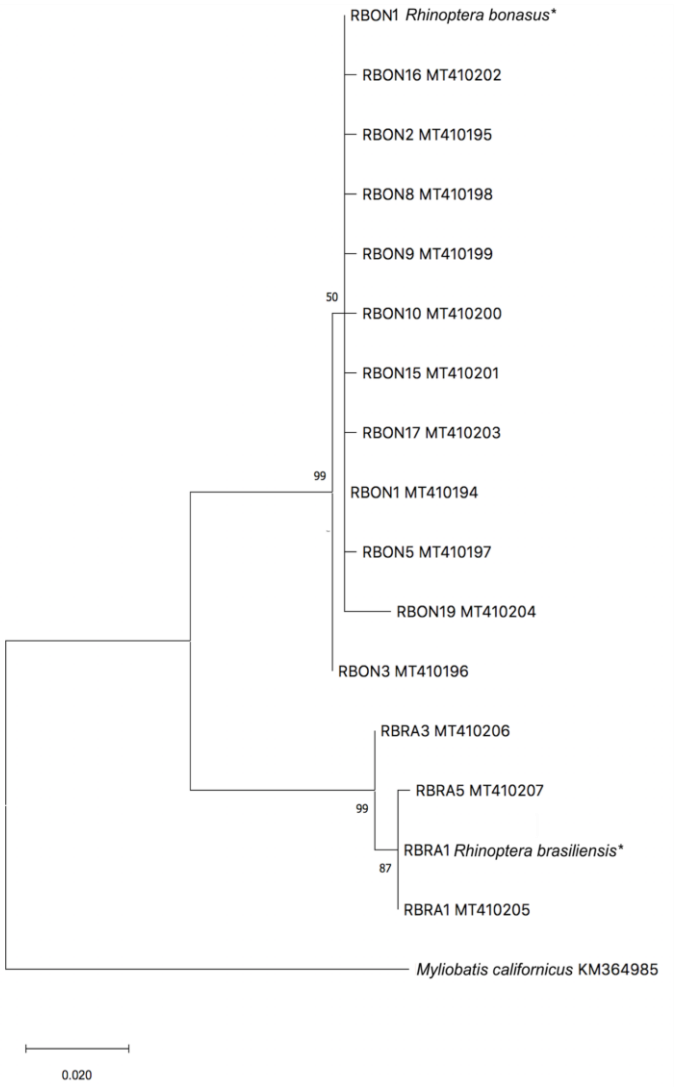

Figure 2.1 *Phylogeny for* Rhinoptera *spp. in the western North Atlantic*

Maximum likelihood phylogeny derived from a 442-base pair portion of the mitochondrial DNA NADH dehydrogenase subunit 2 gene in Rhinoptera spp. from the western North Atlantic, with GenBank accession numbers. Haplotypes generated from morphologically and genetically verified specimens of the Brazilian cownose ray R. brasiliensis Müller 1836 and the American cownose ray R. bonasus (Mitchill 1815) (see Jones et al., 2017) are indicated by * and the bat ray Myliobatis californica Gill 1865 served as the outgroup. Bootstrap values are indicated at nodes and branch lengths were measured as the number of substitutions per site.

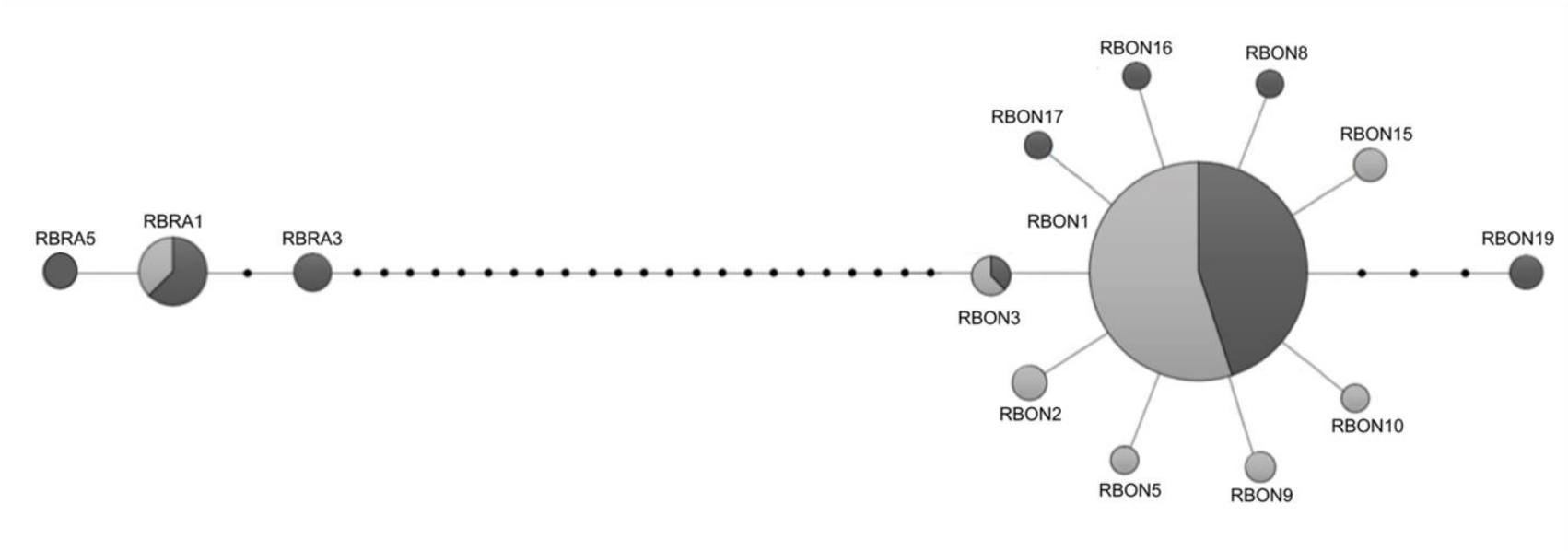

Figure 2.2 *Relationships among* Rhinoptera *spp. haplotypes in the western Atlantic*.

Haplotype network used a 442-base pair portion of the mitochondrial DNA NADH dehydrogenase subunit 2 gene in the Brazilian cownose ray Rhinoptera brasiliensis Müller 1836 (RBRA) and the American cownose ray R. bonasus (Mitchill 1815) (RBON) from the eastern Gulf of Mexico (light gray) and the along the east coast of the USA (dark gray). Each circle represents a unique haplotype and the relative size of the circle reflects its frequency. Small black circles indicate unsampled, intermediate haplotypes.

The 24 genetically identified *R. brasiliensis* were collected from eight locations in the western North Atlantic; eight were from two estuaries in the Atlantic and 16 were from six estuaries in the eGOM (Figure 2.3). In the Atlantic, five *R. brasiliensis* were caught at the mouth of the St. Sebastian River in the Indian River Lagoon, Florida (hereafter IRL) and three were caught in Lake Worth Lagoon, Florida. In the eGOM, six were caught in Apalachicola Bay, five in Santa Rosa Sound, two in Charlotte Harbor and one in each of St. Andrew Bay, St. Joseph Bay and Cedar Key. Individuals included *R. brasiliensis* (GenBank accession nos. MT410205– MT410207) and 11 haplotypes representing 258 individuals clustered with the single haplotype sequenced for the 10 verified *R. bonasus* (RBON1) (GenBank accession nos. MT410194–MT410204)

(Figure S1). The sampled *R. brasiliensis* and *R. bonasus* haplotypes were differentiated by 5.43–7.24% sequence divergence, based on the number of base pair differences (Figure S2). In contrast, the maximum sequence differentiation between sampled haplotypes within each species was much less: 0.68% and 1.13% for *R. brasiliensis* and *R. bonasus*, respectively (Figure 2.2). The minimum sequence differentiations between *M. californica* and *R. brasiliensis* and *R. bonasus* were 13.80% and 12.90%, respectively.

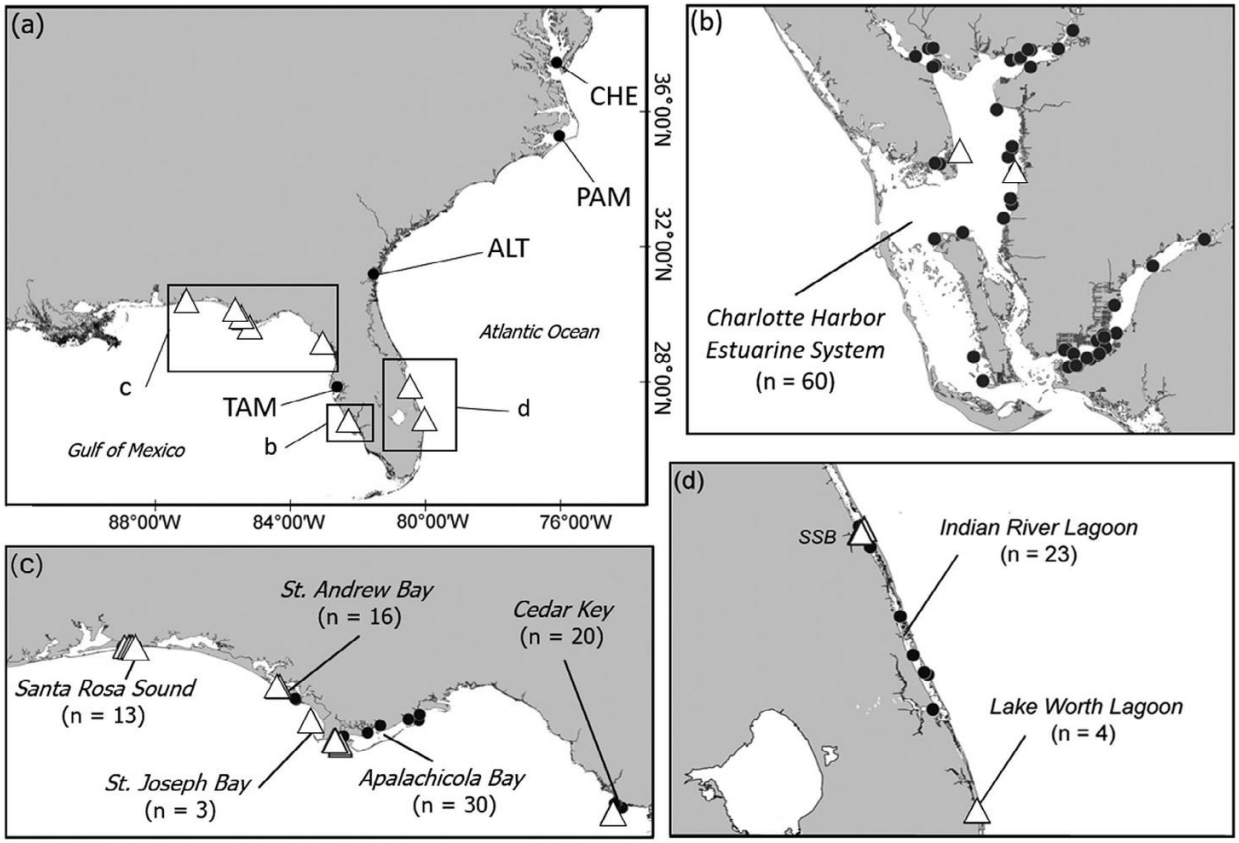

Figure 2.3 Rhinoptera *spp. sampling sites in the western North Atlantic*

Triangles represent general locations where the Brazilian cownose ray *R. brasiliensis* Muller 1836 and the American cownose ray *R. bonasus* (Mitchill 1815) co-occurred and circles represent general locations where only *R. bonasus* occurred. TAM, Tampa Bay, n =22; ATL, Altamaha River, n = 2; PAM, Pamlico Sound and its tributaries, n = 66; CHE, Chesapeake Bay, n = 23. For locations of co-occurrence, inset maps b – d show sites where each species was caught, with overall sample size for each location indicated. *R. brasiliensis*, triangles; *R. bonasus*, circles; SSB, St. Sebastian river.

The 24 genetically identified *R. brasiliensis* were collected from eight locations in the western North Atlantic; eight were from two estuaries in the Atlantic and 16 were from six estuaries in the eGOM (Figure 2.3). In the Atlantic, five *R. brasiliensis* were caught at the mouth of the St. Sebastian River in the Indian River Lagoon, Florida (hereafter IRL) and three were caught in Lake Worth Lagoon, Florida. In the eGOM, six were caught in Apalachicola Bay, five in Santa Rosa Sound, two in Charlotte Harbor and one in each of St. Andrew Bay, St. Joseph Bay and Cedar Key. Individuals included males and females, juveniles, and adults, and were caught across all seasons in multiple years between 2003 and 2018 (Table 2.1). Evidence of reproductive habitat use in the Atlantic was identified by the presence of two particular *R. brasiliensis* near the mouth of the St. Sebastian River, a gravid female caught in summer (August) and a juvenile male caught in winter (January). None were identified from estuaries in the Atlantic north of the St. Sebastian River in the IRL, including the Altamaha River, Georgia (n = 2), Pamlico Sound and its tributaries, North Carolina (n = 66), and Chesapeake Bay, Virginia (n = 23) (Figure 2.3).

Table 2.1 *Biological details for the Brazilian cownose ray*

Rhinoptera brasiliensis Muller 1836 genetically identified via a 442-base pair fragment of the mitochondrial NADH dehydrogenase subunit 2 gene from sites off Florida in the eastern Gulf of Mexico (eGOM) and along the east coast of the USA (Atlantic).

Region	Location	Date collected	Season[a]	Sex	Life stage[b]	Disc width (cm)	Haplotype
eGOM	Santa Rosa Sound	2013–2017	NA	NA	NA	NA	RBRA1
		2013–2017	NA	NA	NA	NA	RBRA1
		2013–2017	NA	NA	NA	NA	RBRA1
		2013–2017	NA	NA	NA	NA	RBRA1
		2013–2017	NA	NA	NA	NA	RBRA1
	St. Andrew Bay	25 October 2013	Fall	F	Juvenile	55.0	RBRA1
	St. Joseph Bay	14 August 2012	Summer	M	Adult	90.0	RBRA1
	Apalachicola Bay	20 June 2013	Summer	F	Adult	88.5	RBRA1
		20 June 2013	Summer	F	Adult	71.5	RBRA1
		20 June 2013	Summer	F	Juvenile	49.0	RBRA1
		14 June 2012	Summer	F	Juvenile	48.0	RBRA1
		14 June 2012	Summer	M	Juvenile	40.0	RBRA1
		24 October 2013	Fall	M	Juvenile	49.0	RBRA1
	Cedar Key	6 December 2017	Winter	F	Adult	77.5	RBRA3

	Charlotte Harbor	17 October 2003	Fall	M	Juvenile	62.5	RBRA1
		15 December 2008	Winter	M	Juvenile	66.7	RBRA1
Atlantic	Lake Worth Lagoon	14 February 2018	Winter	M	Adult	74.0	RBRA1
		14 February 2018	Winter	F	Adult	78.4	RBRA1
		14 February 2018	Winter	F	Adult	80.2	RBRA1
	Indian River Lagoon	12 January 2018	Winter	M	Juvenile	61.3	RBRA1
		12 April 2017	Spring	M	Adult	87.0	RBRA5
		12 April 2017	Spring	M	Adult	87.5	RBRA1
		12 April 2017	Spring	M	Adult	85.1	RBRA1
		7 August 2017	Summer	Fc	Adult	93.2	RBRA1

Specific dates and biological details were not available (NA).

[a]Spring, March – May; summer, June – August; fall, September – November; winter, December – February.

[b]Life stage of *R. brasiliensis* was inferred from disc width at 100% maturity for both sexes (> 71.2 cm disc width) of *R. bonasus* (Mitchill 1815) from Charlotte Harbor, Florida by Poulakis (2013).

[c]Gravid.

2.5 Discussion

The discovery of 24 *R. brasiliensis* based on mtDNA ND2 sequence data across sites in Florida waters further confirms its occurrence in the eGOM (see Carney et al., 2017; Jones et al., 2017; McDowell & Fisher, unpubl. data) and documents that the range extends further in the western North Atlantic than previously described, at least as far north as the St. Sebastian River in the IRL. Since mtDNA is maternally inherited, this finding assumes that these individuals are not hybrid crosses between female *R. brasiliensis* and male *R. bonasus,* which would require analysis of additional nuclear data.

Hybridization has not yet been documented in cownose rays, but has been shown in freshwater rays (Potamotrygonidae, Crus et al., 2015) and fiddler rays (Rhinobatidae, Donnellan et al., 2015). While it is possible to detect patterns of introgression from mtDNA markers alone, robust evidence necessitates powerful datasets that typically use much larger DNA fragments (e.g., Rosenzweig et al., 2016).

These new records are part of a growing body of literature documenting the presence of *R. brasiliensis* in the western North Atlantic. Prior species accounts that used genetic identifications of *R. brasiliensis* in the Atlantic include four records with no life history data from Georgia in fall (October) 2007 (Quattro, unpubl. data), one of unknown size from North Carolina with an unknown capture date in 2008 (Naylor et al., 2012), and New Jersey in summer (August) 2017 (Stoeckle et al., 2020). There is one additional historic record from North Carolina, but since this identification was based on tooth series counts alone (Bigelow & Schroeder, 1953), it is not considered reliable. Despite

these records, the Atlantic was not considered part of the accepted range of *R. brasiliensis* in subsequent accounts (e.g., Last et al., 2016), possibly because they were discounted as 'vagrants'. The term vagrant was recently defined for elasmobranchs by Grant et al. (2019) as 'individuals found outside of the species' distribution, in a habitat not biologically utilized by the species. Our work has shown the presence of both male and female *R. brasiliensis* of all life stages, documented across all seasons over multiple years as far north as the St. Sebastian River in the IRL in the Atlantic. This indicates that *R. brasiliensis* is unlikely to be a vagrant, at least in the Atlantic off Florida, and that this region is part of its range, although the northernmost extent of its range remains uncertain. Whether *R. brasiliensis* have long occurred in these waters but were obscured by the presence of morphologically similar *R. bonasus*, or this reflects a more recent range expansion, remains unknown.

Juvenile *R. brasiliensis* occurred near the mouth of the St. Sebastian River during winter, in St. Andrew and Apalachicola bays during summer and fall, and in Charlotte Harbor during fall and winter. The presence of juveniles in these estuaries along both coasts of Florida, as well as the presence of a gravid *R. brasiliensis* in the IRL during the time associated with their parturition (Rangel et al., 2017), suggests these bays may serve as nurseries. Juvenile *R. bonasus* are abundant in estuaries in the northern Gulf of Mexico and have been shown to tolerate low temperatures (e.g., < 12°C, reported down to 8.3 – 9.1°C), potentially residing in estuaries over winter, and possibly until they reach maturity (Ajemian, 2011; Ajemian & Powers, 2016; Bigelow & Schroeder, 1953).

Additional research is needed to better understand how and to what extent *R. brasiliensis* uses and relies on these estuarine habitats in the Atlantic and eGOM.

The opportunistic nature of the sampling regime in this study likely limited our understanding of the occurrence and frequency of *R. brasiliensis* throughout the study area. For example, for some locations four samples were collected at a single site in a single day and represented the same life stage, while elsewhere 52 samples were selected from archived samples to include all life stages, seasons, and sexes over 9 years. Despite these differences, the presence of *R. brasiliensis* at locations outside of its accepted range, detected over multiple years, implies this species has a wider range than previously thought.

As documented here, the range of *R. brasiliensis* extends at least to the St. Sebastian River in the IRL in the Atlantic. More comprehensive, year-round surveys paired with genetic species identifications are still needed to clarify the northern extent of the range of *R. brasiliensis* in the Atlantic and seasonal patterns of occurrence in these waters. This range extension further increases range overlap between *R. brasiliensis* and *R. bonasus*, emphasizing the need to verify species identities using genetic methods prior to undertaking biological or ecological studies of either species. Furthermore, existing biological and relative abundance datasets require careful consideration as they may reflect a Rhinoptera species complex rather than single species. Future studies should use a suite of approaches, including tagging and molecular methods, to better understand and disentangle the biology and ecology of these morphologically similar, sympatric species.

CHAPTER III – POPULATION GENETICS AND GENETIC DIVERSITY OF *RHINOPTERA* SPP. IN THE WESTERN ATLANTIC

3.1 Introduction

Management of a species typically requires information on a species' geographic range (e.g., Chapter 2) and the delineation of populations to inform appropriate spatial scales of management (Allendorf et al., 1987; Laikre et al., 2005). True continuous ranges of marine species are rare, even for coastal migratory species as evident in the finetooth shark, *Carcharhinus isodon* (Carlson et al., 2003), and the lemon shark, *Negaprion brevirostris*, (Feldheim et al., 2001), both of which have population structure across a continuous range. Migratory species may be geographically wide-reaching, but individuals may only utilize a portion of their range rather than its entirety (Avise, 2000; Hellberg et al., 2002; Guillot et al., 2009). Continuous distribution is often broken up by barriers to dispersal, both hard and soft, creating genetically distinct populations across a species' range (Palumbi, 1994; Feldheim et al., 2001; Irwin, 2002; Carlson et al., 2003; Chapman et al., 2015; Hirschfeld et al., 2021). Hard barriers are barriers that physically limit dispersal; in the marine environment these can include the formation of lang bridges that split marine populations (e.g., the Isthmus of Panama) (Knowlton et al., 1993; Palumbi, 1994; O'Dea et al., 2016). Soft barriers are physically permeable (see Bowen et al., 2016; Teske et al., 2011), but dispersal across them is limited due to body size, environmental tolerance, and vagility of a species (see Cowman & Bellwood, 2013; Phillips et al., 2021).

Migratory marine species with broad geographic ranges and high dispersal potential may be genetically structure in the absence of phylogeographic barriers due to behavior (e.g., philopatry) (Mayr, 1963; Palumbi, 1996; Heuter et al., 2005; Speed et al., 2010; Chapman et al., 2015). Site fidelity and sex-mediated philopatry can cause demographic isolation resulting in population structure, and has been documented in taxa spanning elasmobranchs, marine mammals, and sea turtles (Waser & Jones, 1983; Phillips et al., 2017; Baltazar-Soares et al., 2020). Many elasmobranchs, including cownose rays, have high vagility, but effective dispersal and population structure patterns reflect a complex interaction of biology, behavior, and environment (Grubbs, 2010). Highly migratory pelagic species are typically structured over larger spatial scales than coastal or benthic species (Pardini et al., 2001; Keeney et al., 2005; Dudgeon et al., 2009; Karl et al., 2012; Phillips et al. 2021).

Unlike other elasmobranch groups, batoids typically exhibit bi-parental philopatry (Li et al., 2013, 2015; Flowers et al., 2016). While many batoids exhibit philopatry, the majority are benthic and have limited movement. Cownose rays pose an interesting group to study because like other batoids, they exhibit bi-parental philopatry, however they behave more like a coastal pelagic shark.

Cownose rays (*Rhinoptera* spp.) within the western Atlantic have a broad, continuous distribution (see Chapter 1), which may be partitioned by soft barriers to dispersal. Both male and female *R. bonasus* exhibit biparental philopatry in the northwestern Atlantic (Fisher et al., 2013), but less is known about the movements and reproductive biology of *R. bonasus* elsewhere, or of *R. brasiliensis* throughout its range.

The only study examining the population structure of *Rhinoptera* spp. in the western Atlantic to date found that *R. bonasus* in Tampa Bay, FL within the Gulf of Mexico and Chesapeake Bay, VA in the northwestern Atlantic, were considered separate stocks, separated by a known phylogeographic barrier, the Gulf Stream (Carney et al., 2017).

It is the hypothesis of this study that well documented phylogeographic barriers to dispersal in coastal elasmobranchs in the western Atlantic (e.g., the Gulf Stream separating the Gulf of Mexico from the northwestern Atlantic, the Mississippi River outflow separating the west and east northern Gulf of Mexico, and the Amazon River separating northern and southern Brazil via a large plume of freshwater (Cronin & Cowsett, 1996; Dudgeon et al., 2012)) will limit dispersal in *Rhinoptera* spp..

The current study is the first to examine population structure of *R. bonasus* throughout the entirety of its geographic range, and the first to examine the population structure of *R. brasiliensis*. This study aims to identify genetically differentiated populations of each of *R. bonasus* and *R. brasiliensis* to inform on their management as both species are listed as Vulnerable by the IUCN (Carlson et al., 2018; 2019). Knowledge on the population structure of *Rhinoptera* spp. in the western Atlantic is needed for proper management and conservation of the species, which is necessary due to the fishing pressures they face ~~which the biology of the species cannot sustain~~ (see Chapter 1). Separate stocks (i.e., subpopulations) may have novel genetic, physiological, or behavioral adaptations that result in differences in life history (e.g., reproductive success, growth rates, and resistance to disease), which may result in increased survivability against anthropogenic or environmental stressors (Stepien, 1995).

3.2 Methods

3.2.1 Sample collection

A total of 528 tissue samples of *Rhinoptera* spp. were collected primarily via gillnet across 23 sampling locations in the western Atlantic between 2003 – 2018 and were subsampled for this study. Individuals were identified to species by comparison of known genetic sequences of *R. bonasus* and *R. brasiliensis* (see Chapter 2). Of these, 440 were from *R. bonasus* collected across all sampling locations; in the southwestern Atlantic (SWA) (N=13), northern Gulf of Mexico (GMX) (N=317), and northwestern Atlantic (NWA) (N=110) (Table 3.1). A total of 88 samples of *R. brasiliensis* were collected across 16 sampling locations in the SWA (N=9), GMX (N=71), and NWA (N=8) (Figure 3.1).

Rhinoptera spp. tissue samples were mostly fin clips collected from adults (43.3%), although juveniles (16.8%) and young-of-the-year (4.5%) were also sampled (see Figure 3.2). However, over a third of the samples did not have associated age data (35.4%). Samples from males were more common (43.5%) than females (35.2%), although sex data were missing for 21.3% of the samples (see Figure 3.3). Tissue samples of each species had representatives from each season from at least one location, although samples were not collected at every location during each season due to the opportunistic nature of sample collection (see Figure 3.3). In some instances, samples were collected from localities during a single fishing excursion (ex: Lake Worth Lagoon, FL, USA, February 2018), while in other cases, samples were collected in each season over the course of several years (ex: Charlotte Harbor, FL, USA, 2003 – 2018).

Table 3.1 *Collection sites and sample sizes of Rhinoptera bonasus and R. brasiliensis.*

Region	Water Body	Abbreviation	*R. bonasus*	*R. brasiliensis*	Total
SWA	Mucuripe Cove, Fortaleza, Brazil	MUC	2	9	11
	Paramaribo, Suriname	PAR	11	0	11
GMX	Laguna Madre, Texas	LAG	18	0	18
	Baffin Bay, Texas	BAF	12	1	13
	Aransas Bay, Texas	ARA	8	1	9
	Matagorda Bay, Texas	MAT	27	2	29
	Gulf of Mexico, Texas	GTX	1	4	5
	Gulf of Mexico, Louisiana	GLA	4	3	7
	Mississippi Sound, Mississippi	MIS	57	33	90
	Pelican Bay, Alabama	PEL	17	11	28
	Santa Rosa Sound, Alabama	SRS	8	5	13
	St. Andrew Bay, Florida	SAB	15	1	16
	St. Joseph Bay, Florida	SJB	2	1	3
	Apalachicola Bay, Florida	APA	24	6	30

	Cedar Key, Florida	CED	17	1	18
	Tampa Bay, Florida	TAM	22	0	22
	Charlotte Harbor, Florida	CHA	58	2	60
	Caloosahatchee River, Florida	CAL	27	0	27
NWA	Lake Worth Lagoon, Florida	LWL	1	3	4
	Indian River Lagoon, Florida	IRL	18	5	23
	Altamaha River, Georgia	ALT	2	0	2
	Pamlico Sound, North Carolina	PAM	66	0	66
	Chesapeake Bay, Virginia	CHE	23	0	23
		Totals:	440	88	528

Samples collected throughout the southwestern Atlantic (SWA), northern Gulf of Mexico (GMX), and northwestern Atlantic (NWA). Abbreviations are based off of the first three letters of the water body, or the most commonly accepted abbreviation for the location (i.e., IRL for Indian River Lagoon).

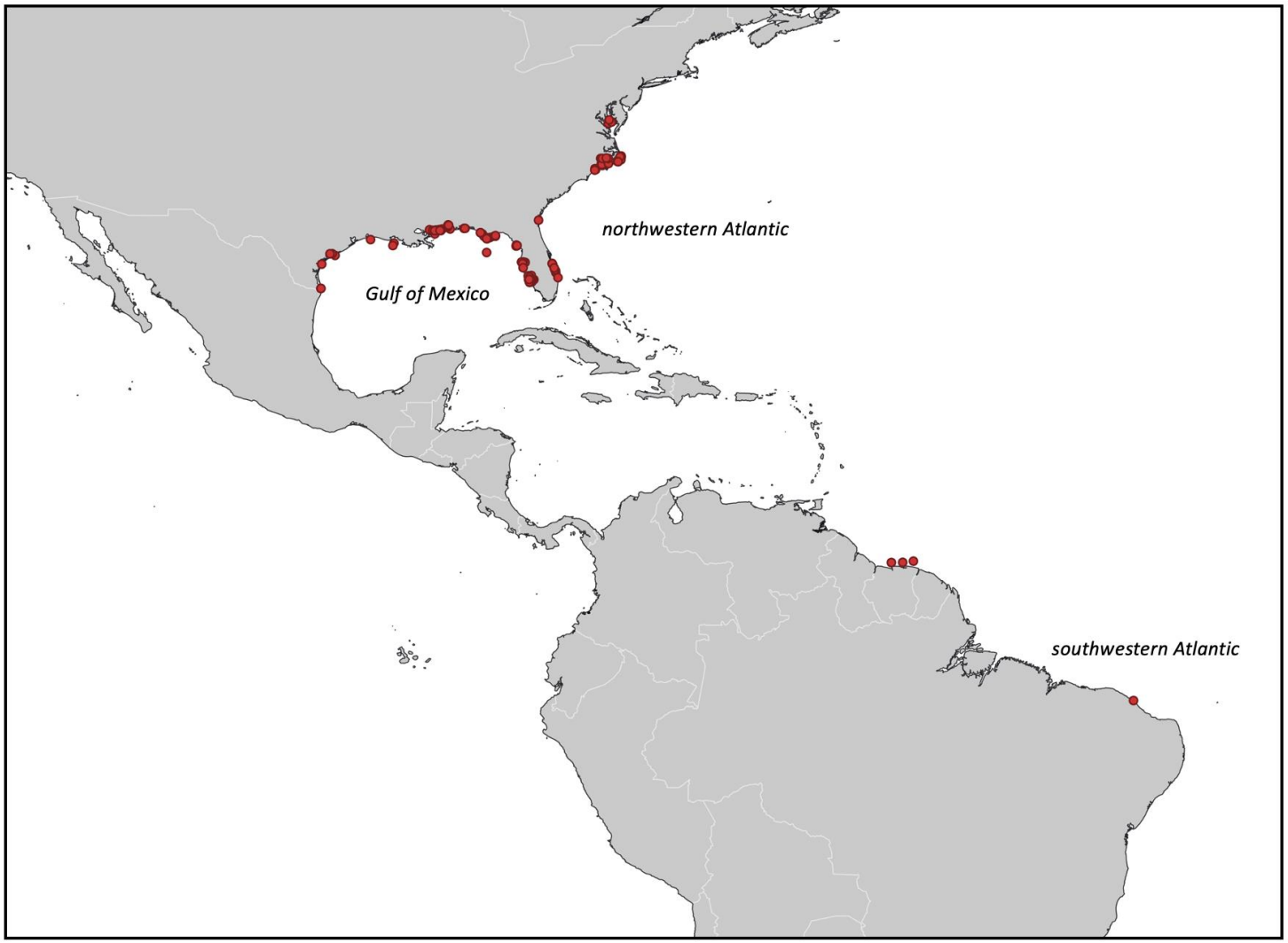

Figure 3.1 *Samples of* Rhinoptera bonasus *collected throughout the western Atlantic.*

Figure 3.2 *Samples of* Rhinoptera brasiliensis *collected throughout the western Atlantic.*

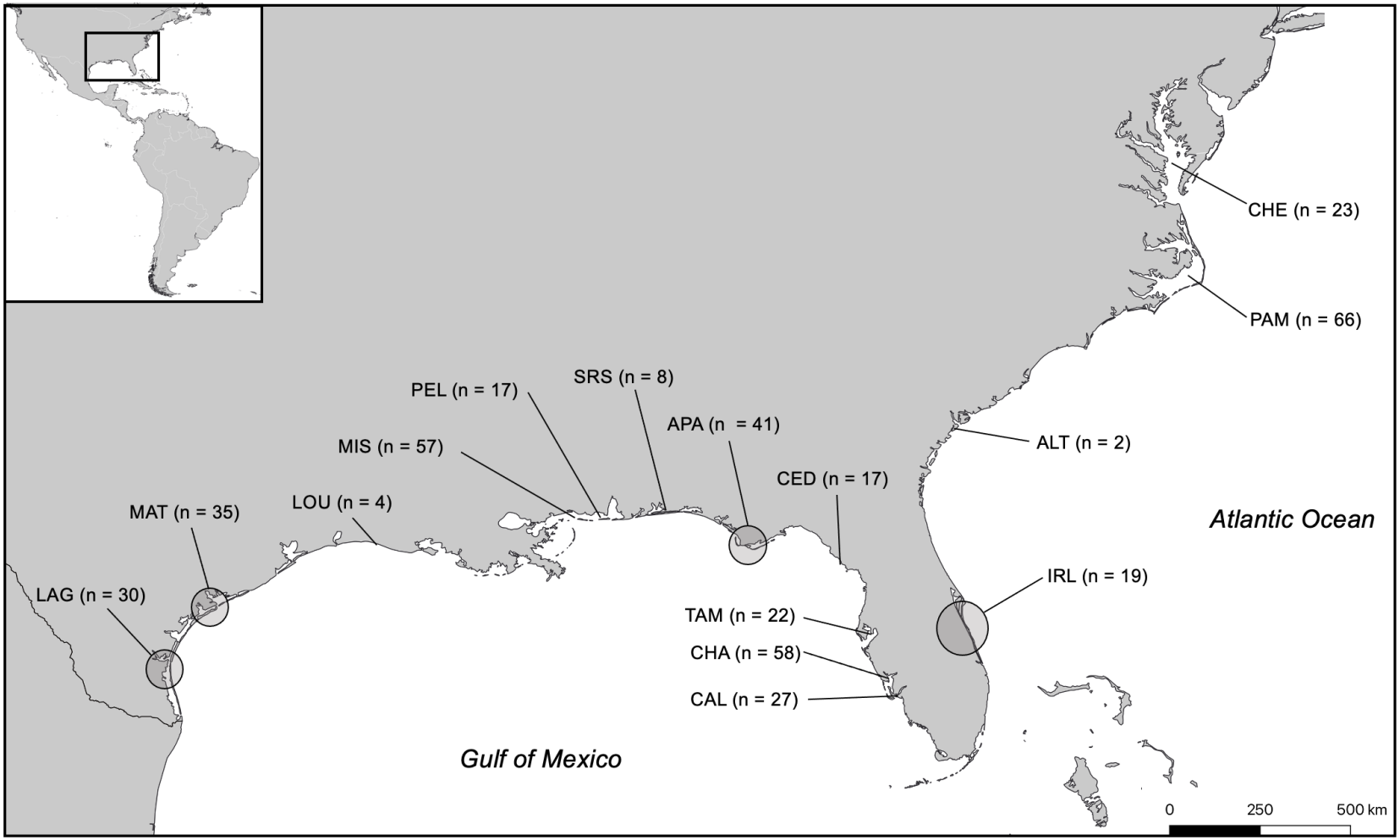

Figure 3.3 *Sample sizes of* Rhinoptera bonasus.

Abbreviated approximate sample locations (see Table 3.1). Samples from the southwestern Atlantic not depicted.

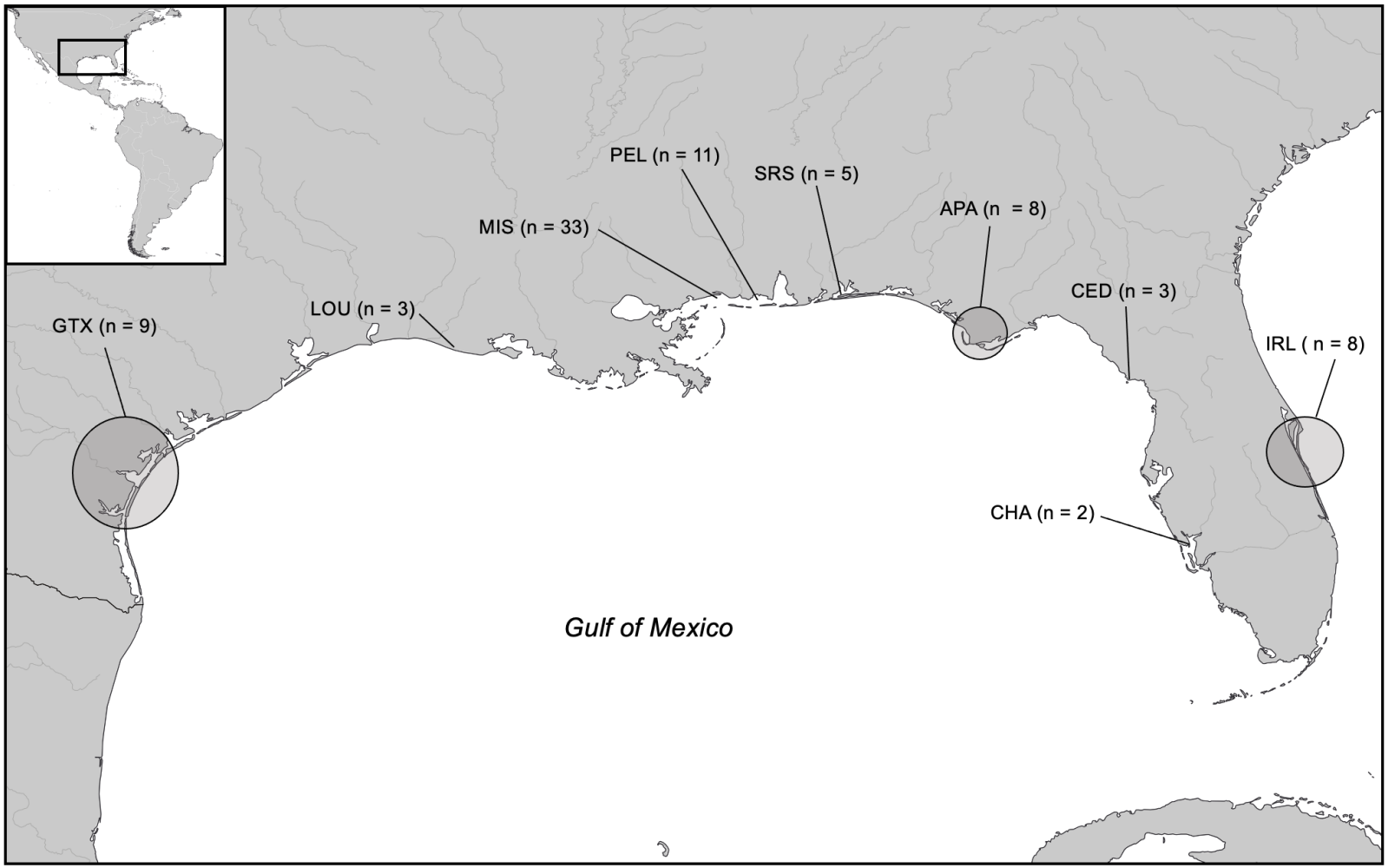

Figure 3.4 *Sample sizes of* Rhinoptera brasiliensis.

Abbreviated approximate sample locations (see Table 3.1). Samples from the southwestern Atlantic not depicted.

3.2.2 Genetic methods

All tissue samples were stored in O-ring vials containing 95% ethanol at room temperature until DNA was extracted using the Qiagen® DNeasy® Blood & Tissue Kit (Hilden, Germany). DNA extractions were typically performed using 15 – 25 milligrams (mg) of tissue and the manufacturer's protocol, with the exception that tissue samples were digested overnight. Tissue samples that were <10 mg were eluted with a smaller volume of AE buffer (100 µl) to increase DNA yield concentrations. The success of DNA extractions was assessed by examining the quality and quantity of extracted DNA using gel electrophoresis and a spectrophotometer, respectively. Electrophoresis was performed on all extracted DNA using a 2% agarose gel stained with 10,000X Invitrogen SYBR™ Safe DNA stain and ran in a gel rig filled with 1X TBE buffer. DNA sample bandings were compared against a Lambda/Hind III DNA lane marker to approximate the size of the DNA fragments and was viewed on a ChemiDoc™ MP Imagining System (BioRad, USA).

3.2.2.1 Primer Design

Primers designed by McDowell and Fisher (unpubl. data) to target an 850-base pair (bp) portion of the mitochondrial NADH dehydrogenase subunit 2 (ND2) gene in *R. bonasus* were tested, but produced low quality, 'noisy' sequences (i.e., low, overlapping peaks) due to a dinucleotide microsatellite near base 500. A reverse primer was designed for this study to be used with McDowell and Fisher's forward primer, by aligning ND2 sequences from *R. bonasus* (GenBank accession no. JQ518923.1), *R. steindachneri*

(GenBank accession nos. JQ518921.1 and JQ518918.1), *R. jayakari* (GenBank accession no. KJ659875.1), and *R. javanica* (GenBank accession no. JQ518924.1) using CodonCode Aligner v 6.0.2 (CodonCode Corporation, Dedham, MA, USA) (see Chapter 2.3 for details).

3.2.2.2 Polymerase Chain Reaction

A polymerase chain reaction (PCR) was used to amplify a 442-base pair portion of the mitochondrial ND2 gene using a forward primer (RhinND2F1: 5'- GAA CCC YTT AAT CCT CTY CAT C - 3') and a reverse primer (RayND2R: 5'- GGA TTG ATA GTA CGC CTA TGG -3') (Weber et al., 2021). The PCR mixture consisted of 25 – 50 ng template DNA, 10 mM *Taq* buffer (Invitrogen), 1.5 mM MgCl2, 0.3 μM of each primer, 0.1 mM dNTPs, 1 U of *Taq* polymerase (Invitrogen) and PCR grade water for a final volume of 25 μL. The PCR cycling conditions included an initial denaturation at 95°C, 30 s annealing at 58°C, 30 s extension at 72°C, and a 5 min final extension at 72°C (Weber et al., 2021). In instances where a sample did not amplify using this protocol, the PCR was repeated with the addition of 0.1 μM Bovine Serum Albumin (BSA) (ThermoFisher) to improve specificity in amplification by reducing PCR inhibitors present in the extracted DNA.

Resultant amplicons were cleaned with 3 μL ExoSAP-IT (ThermoFisher, Waltham, Massachusetts, USA) and sequenced using an Applied Biosystems 3730XL DNA Analyser with BigDye Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA) in the forward and reverse direction (Weber et al., 2021) by

the University of Arizona Genetics Department. Sequencing followed the manufacturer's protocol apart from running all sequences for half reactions.

CodonCode v 9.01 (CodonCode Corporation, Dedham, Massachusetts, USA) was used to cut primer sequences from the end of raw sequences and call base pairs, resulting in a 442-base pair fragment for each individual, which was then aligned in the forward and reverse direction, creating a consensus sequence used in data analysis.

3.2.3 Data analysis

3.2.3.1 Species identification

Specimens caught in the northern Gulf of Mexico that had been identified as *R. bonasus* (n=10) and *R. brasiliensis* (n=10) from a previous study (Jones et al., 2017) were considered species representatives for analysis. Haplotypes were assigned a species based on which species representative they initially clustered with in CodonCode and were later confirmed by examination of the phylogenetic relationships between the haplotypes. The model of best fit was calculated to be Jukes-Cantor (Jukes & Cantor, 1969) using Bayesian Information Criterion (BIC) values (Darriba et al., 2012) in jModelTest 2 v 2.1.0.

3.2.3.2 Data analysis

Phylogenetic relationships between all samples were examined to determine species identification and haplotype assignment using a maximum likelihood method with 10,000 bootstrap replicates using the Jukes-Cantor substitution model with a 0.04

gamma correction in Mega v. 7 (Kumar et al., 2018) with the bat ray (*Myliobatis californica*) (NCBI accession no. KM364985) as an outgroup (see Figure 3.3). Relationships among haplotypes were estimated via maximum parsimony method (Polzin & Daneshmand, 2003) and visualized in a median-joining network in Network v 10.1.10 (Bandelt et al., 1999) (see Figure 3.4).

3.2.3.3 Population structure

Population structure analyses were run separately on 440 sequences of *Rhinoptera bonasus* and 88 sequences of *R. brasiliensis.* Small sample sizes from some localities (see Table 3.1) necessitated sample pooling for population-level analyses. Samples were pooled when N<30 per species at a location if samples were from neighboring localities within the same estuary system, and devoid of a phylogeographic barrier (i.e., the Mississippi River, the Gulf Stream, the Amazon River). Pooled samples were renamed with an abbreviation of the sample locations that were nested within the a larger sampling location (For *R. bonasus*: Baffin Bay, Texas' Gulf of Mexico, and Laguna Madre were pooled and renamed LAG+ (n = 30), Aransas Bay was pooled with Matagorda Bay (MAT+ = 35)), or pooled and renamed based on the sample location that contributed the largest number of samples to the pool (For *R. bonasus* Apalachicola Bay, Saint Joseph Bay, and Saint Andrew Bay were pooled, renamed APA+ (n = 41); For *R. brasiliensis*: Baffin Bay, Aransas Bay, Matagorda Bay, and Texas' Gulf of Mexico were renamed GMX+ (n = 9), Apalachicola Bay, Saint Joseph Bay, and Saint Andrew Bay were

renamed APA+ (n = 8)). Pooled samples end in a plus sign (+) to indicate multiple sampling sites are present within the abbreviated name.

An analysis of molecular variance (AMOVA) was used to test hypotheses of the spatial distribution of mtDNA variation in *Rhinoptera* spp. in the western Atlantic using ARLEQUIN v. 3.5 (Excoffier & Lischer, 2005). Samples were grouped based on each hypothesis and tested for variation within the groupings, among groupings within regions, and among regions. Based on previous genetic research (see Carney et al., 2017) and tagging studies (see Ogburn et al., 2018) on *R. bonasus* and the presence of the Gulf Stream, a phylogeographic barrier in many marine species (Palumbia, 1996), it was hypothesized that *Rhinoptera* spp. from GMX would be genetically differentiated from those of the NWA. Due to the physical distance (~7,000 km) and the presence of another known phylogeographic barrier (i.e., the Amazon River), it was also hypothesized that *Rhinoptera* spp. from the GMX would be genetically differentiated from those in the SWA. Within the GMX, it was hypothesized that *Rhinoptera* spp. would be genetically differentiated from the western GMX (wGMX) and the eastern GMX (eGMX) by the outflow from the Mississippi River.

Overall patterns of genetic differentiation were examined for each species overall and between pooled sites in the GMX and the SWA, the GMX and the NWA, the SWA and the NWA combined with the GMX (hereafter ATL), and the wGMX from the eGMX using pairwise F_{ST} (Weir & Cockerham, 1984) and Φ_{ST} where N≥10 for *R. bonasus* and N≥5 in *R. brasiliensis* and calculated in ARLEQUIN v 3.5 (Excoffier & Lischer, 2005). The difference in sample size threshold was due to small sample sizes in *R. brasiliensis*

which would otherwise exclude key regions from analysis. The statistical significance of F_{ST} and Φ_{ST} were assessed in ARLEQUIN v. 3.5 with 10,000 permutations, and exact tests (Raymond & Rousset, 1995) with 100,000 steps in the Markov chain (Excoffier & Lischer, 2005). To avoid type I errors, a Bonferroni correction for multiple tests was used to calculate a new significance threshold (Rice, 1989).

3.2.3.4 Demographic history

Aspects of the demographic history of each of *R. bonasus* and *R. brasiliensis* were inferred via neutrality tests and mismatch distributions. Tajima's D (1989) and Fu's Fs (1997) were used to assess whether patterns of genetic diversity in defined populations were selectively neutral and/or at population expansion. The signature of a population expansion was also assessed using a mismatch distribution (Rogers & Harpending, 1992) in DnaSP v.6 (Rozas et al., 2017). A sum of squares deviations (SSD) with 1,000 bootstrap replicates was used to measure the goodness-of-fit of the observed distribution of nucleotide differences between individuals compared to expectations of population growth in ARLEQUIN v 3.5 (Excoffier & Lischer, 2005).

3.2.3.5 Genetic diversity

Levels of genetic diversity were calculated overall, and for each defined population for each of *R. bonasus* and *R. brasiliensis* calculated with a 0.04 gamma correction in ARLEQUIN v 3.5 (Excoffier & Lischer, 2005). The number of haplotypes,

haplotype diversity (*h*), and nucleotide diversities (π) were calculated in DnaSP v. 6 using the Jukes and Cantor method with a gamma correction value of 0.04 (Rozas et al., 2017).

3.3 Results

The 440 *Rhinoptera bonasus* samples yielded a minimum of 1 bp and maximum of 15 bp differences, or a sequence divergence of was 0.23% – 3.39%. From the 88 *R. brasiliensis* samples, there was a minimum of 1 bp and a maximum of 14 bp differences, or a sequence divergence of 0.23% – 3.16%. The minimum and maximum bp differences between the two species was 23 bp and 33 bp, respectively, or a sequence divergence of 5.20% – 7.47%.

There was a moderate level of evolutionary divergence within and between species (Figure 3.3). The *R. bonasus* haplotypes from the NWA formed one clade, and the haplotype (O4) from Suriname branched off from the NWA clade and showed notable divergence with low branch support (bootstrap value of 50%). The haplotypes from Brazil were nested within the ATL clade but showed longer branch lengths. The *R. brasiliensis* haplotypes formed two clades with low support (bootstrap value of 63%) for the branching between samples from throughout their range. As a similar pattern to *R. bonasus*, the *R. brasiliensis* haplotypes found in Brazil were nested within a clade from the NWA with low branch support (bootstrap value of 67%) (see Figure 3.3).

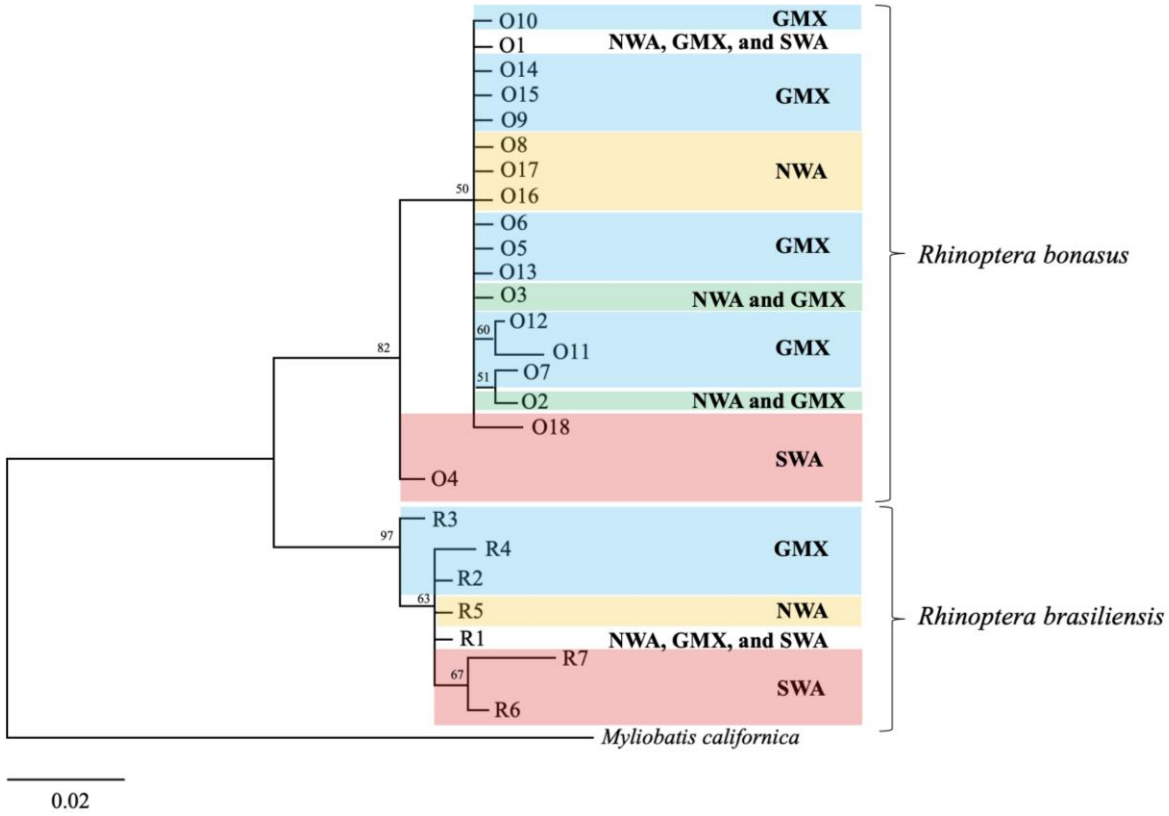

Figure 3.5 *Phylogeny of collected* Rhinoptera *spp. samples from the western Atlantic*

Maximum likelihood phylogeny based on a 442-base pair portion of the mitochondrial NADH dehydrogenase subunit 2 gene, where O represents *R. bonasus* and R denotes *R. brasiliensis* haplotypes. *Myliobatis californica* served as an outgroup. Bootstrap values are indicated at branching nodes where lengths of branches were proportional to the number of base pair substitutions per site.

A common *R. bonasus* haplotype (O1) occurred in 93.18% of samples, while the common *R. brasiliensis* haplotype (R1) occurred in 88.63% of samples. In *R. brasiliensis*, two haplotypes (R6, R7) were found only in Brazil and in *R. bonasus*, two haplotypes (O4 and O18) were found in only in Suriname and Brazil, respectively. The NWA had six haplotypes of *R. bonasus*, three of which were unique to the region, while *R. brasiliensis* had only two haplotype representatives in the NWA, one of which was unique. The GMX had fourteen haplotypes of *R. bonasus*, eleven of which were unique

to the region, whereas *R. brasiliensis* had five haplotypes, four of which were unique. Both *R. bonasus* and *R. brasiliensis* had three haplotypes in the SWA and two of each were unique (Tables 3.4 & 3.5).

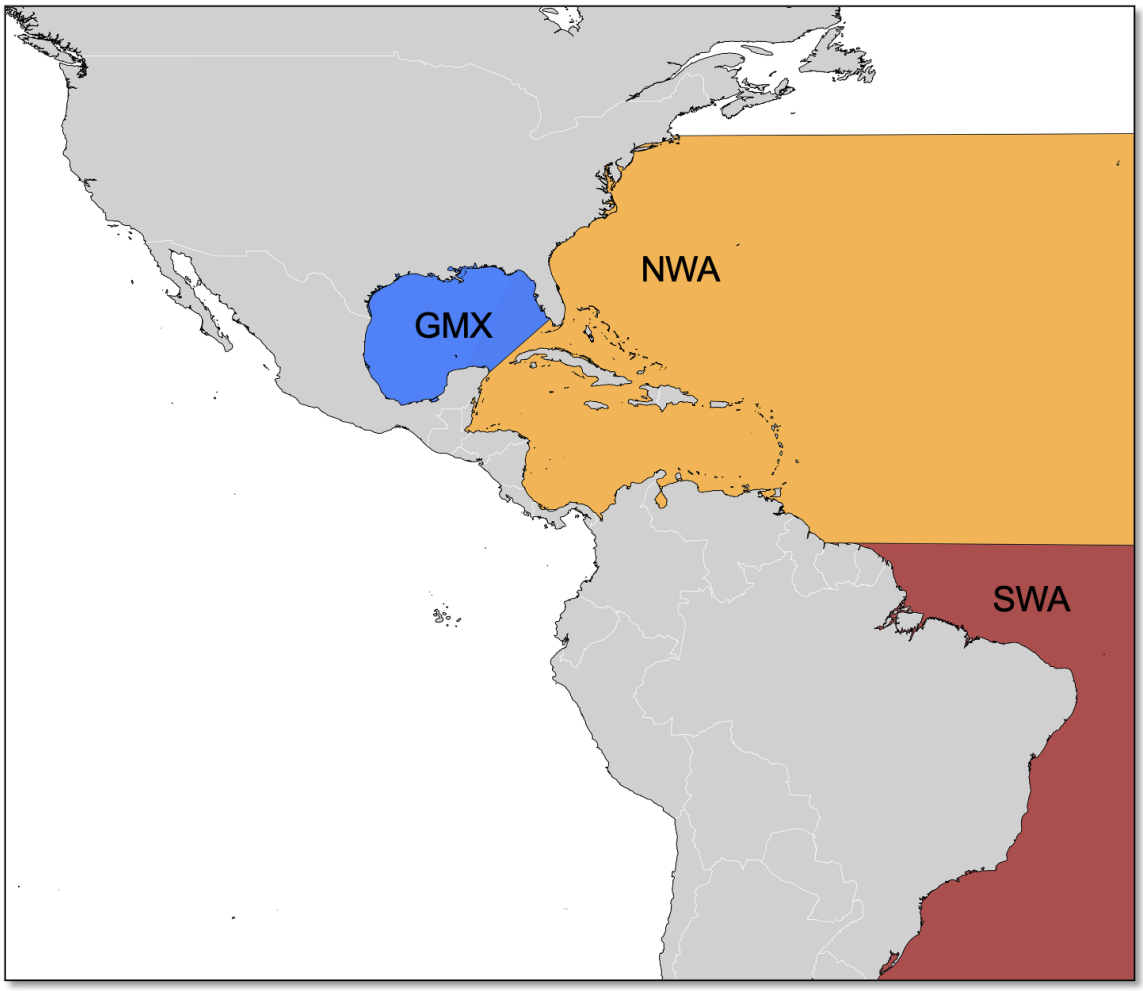

Figure 3.6 *Designation of three sampling regions in the western Atlantic.*

The western Atlantic divided into three regions: northwestern Atlantic (NWA), Gulf of Mexico (GMX), and southwestern Atlantic (SWA). Samples that fall within the highlighted portion were assigned to that region for data analysis. Colors correspond to the haplotype network.

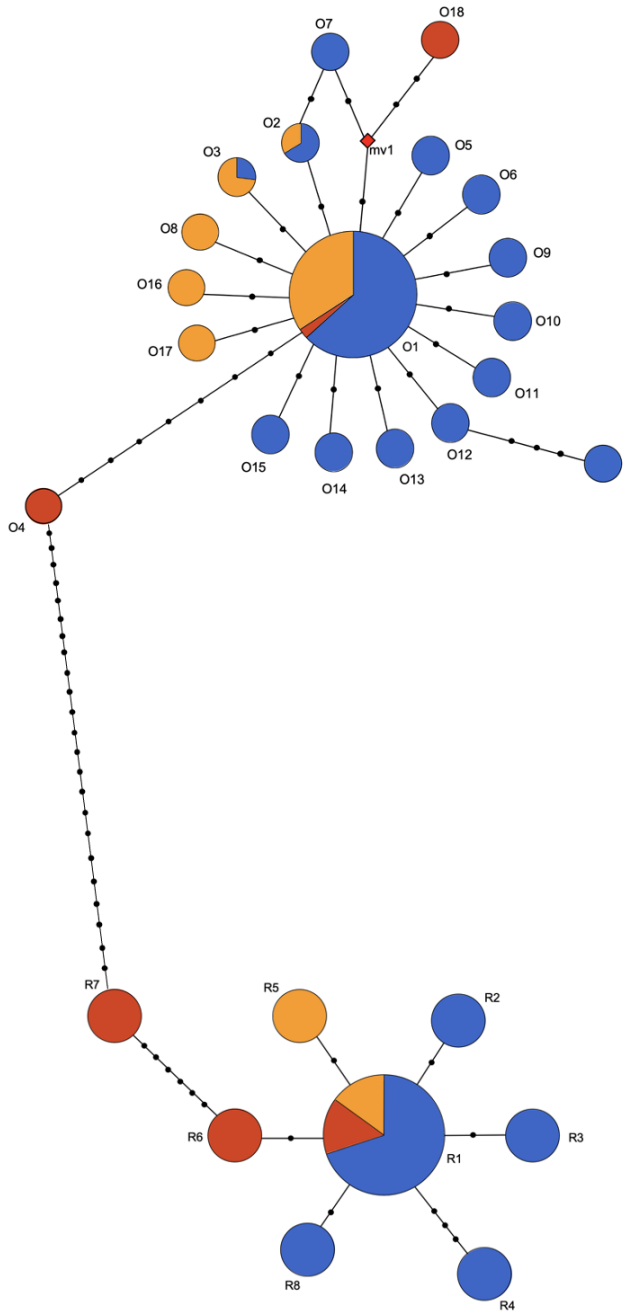

Figure 3.7 *Evolutionary relationships among haplotypes of Rhinoptera spp.*

Based on a 442-base pair portion of the mitochondrial DNA NADH dehydrogenase subunit 2 gene in Rhinoptera bonasus (O) and Rhinoptera brasiliensis (R) from the northwestern Atlantic (orange), the Gulf of Mexico (blue) and the southwestern Atlantic (red). Each circle represents a Rhinoptera spp. haplotype where the colors represent the three difference regions examined in this study (NWA, GMX, and SWA). Each black dot along the branching lines represents an unsampled haplotype.

3.3.2 Population structure

Rhinoptera bonasus

High overall F_{ST} and Φ_{ST} values indicated high levels of population structure for *R. bonasus* within their range (Figure 3.2). Genetic differences among groupings (i.e., the SWA and the ATL) resulted in a high level of variance (95.68%) (for more grouping results see Table 3.9). Supporting this result, pairwise comparisons between Suriname and all other sampling locations were significantly differentiated from one another (Table 3.5). A reason for the strong differences between Suriname and the rest of the samples may be due to a single unique haplotype and no shared haplotypes in Suriname.

Rhinoptera brasiliensis

Low but statistically significant F_{ST} and Φ_{ST} values indicate genetic differentiation for *R. brasiliensis* between the NWA and GMX (see Table 3.7). Samples from Brazil were statistically different from samples from Mississippi Sound (Table 3.3). Genetic differences among groupings (i.e., the NWA v the GMX v the SWA; the NWA v the GMX; wGMX v eGMX; ATL v SWA) had a low and statistically insignificant level of variance. A reason for the discrepancy between the pairwise values and levels of variance could be a result of low sample size from Brazil (N=9), where 77.78% of samples shared the most common haplotype (R1) found throughout the species range (see Table 3.5).

Table 3.2 *Overall pairwise F_{ST} and Φ_{ST} of* R. bonasus.

Data derived from the NADH subunit 2 mitochondrial gene from pooled sample locations where N >10. F_{ST} (above the diagonal) and Φ_{ST} (below the diagonal).

	SUR	LAG	MAT	MIS	PEL	APA	CED	TAM	CHA	CAL	IRL	PAM	CHE
SUR	-	**0.8621***	**1.0000***	**0.8893***	**1.0000***	**0.8596***	**0.9284***	**0.8830***	**0.8908***	**1.0000***	**0.9309***	**0.9012***	**0.9411***
LAG	**0.9724***	-	0.0422	-0.0060	0.0074	-0.0042	-0.0210	-0.0195	-0.0055	0.0294	-0.0190	-0.0037	-0.0099
MAT	**1.0000***	0.0052	-	0.0167	0.0000	0.0487	0.0460	0.0516	0.0158	0.0000	0.0396	0.0076	0.0190
MIS	**0.9834***	0.0026	-0.0017	-	-0.0062	-0.0018	-0.0179	-0.0098	-0.0039	0.0091	-0.0249	-0.0072	-0.0125
PEL	**1.0000***	-0.0206	0.0000	-0.0216	-	0.0169	0.0000	0.0093	-0.0068	0.0000	-0.0033	-0.0130	-0.0138
APA	**0.9790***	-0.0039	0.0311	0.0117	0.0027	-	-0.0087	-0.0071	0.0068	0.0372	-0.0328	0.0012	0.0020
CED	**0.9898***	-0.0009	0.0460	0.0013	0.0000	0.0143	-	-0.0243	-0.0178	0.0284	-0.0293	-0.0188	-0.0237
TAM	**0.9827***	-0.0023	0.0220	0.0081	-0.0122	0.0190	-0.0029	-	-0.0262	0.0356	-0.0223	-0.0079	-0.0131
CHA	**0.9836***	0.0091	-0.0021	0.0089	-0.0218	0.0274	0.0015	-0.0238	-	0.0084	-0.0170	-0.0052	-0.0126
CAL	**1.0000***	-0.0359	0.0000	-0.0079	0.0000	0.0211	0.0284	0.0095	-0.0082	-	0.0234	0.0011	0.0071
IRL	**0.9901***	-0.0269	0.0396	-0.0142	-0.0033	-0.0357	0.0001	-0.0025	0.0008	0.0234	-	-0.0260	-0.0238
PAM	**0.9744***	-0.0022	-0.0104	-0.0060	-0.0283	0.0065	-0.0109	-0.0023	0.0030	-0.0157	-0.0200	-	-0.0155
CHE	**0.9915***	-0.0049	0.0190	-0.0005	-0.0138	0.0173	0.0024	0.0007	-0.0005	0.0071	0.0015	-0.0100	-

*denotes a *P* value below 0.05

Table 3.3 *Overall pairwise F_{ST} and Φ_{ST} of* R. brasiliensis.

	MUC	MAT	MIS	PEL	SAN	APA	IRL
MUC	-	0.0161	**0.0767***	0.1066	0.0161	-0.0847	0.0160
MAT	0.0462	-	-0.0279	0.0000	0.0000	0.6279	0.0000
MIS	**0.1136***	-0.0593	-	0.0143	-0.0279	-0.0057	-0.0095
PEL	0.0584	0.0234	-0.0140	-	0.0000	0.7881	0.0931
SAN	-0.0492	-0.0778	-0.0812	0.0000	-	0.6279	0.0000
APA	0.0175	-0.0141	-0.0354	0.0000	0.0000	-	0.3249
IRL	0.0260	0.0004	-0.0112	0.0420	-0.0687	0.0000	-

Data derived from the NADH subunit 2 mitochondrial gene from pooled sample locations where N >10. F_{ST} (above the diagonal) and Φ_{ST} (below the diagonal).

* denotes a *P* value below 0.05

Table 3.4 *Haplotype distribution table of* R. bonasus.

	SWA		GMX									NWA			
Haplotype	MUC	SUR	LAG+	MAT+	MIS	PEL	APA	CED	TAM	CHA	CAL	IRL	ALT	PAM	CHE
O1	1	-	27	35	53	17	37	16	20	54	27	17	2	62	22
O2	-	-	-	-	-	-	3	-	-	-	-	1	-	1	-
O3	-	-	-	-	-	-	-	-	1	2	-	-	-	-	-
O4	-	**11**	-	-	-	-	-	-	-	-	-	-	-	-	-
O5	-	-	-	-	-	-	-	-	1	1	-	-	-	-	-
O6	-	-	**1**	-	-	-	-	-	-	-	-	-	-	-	-
O7	-	-	**1**	-	-	-	-	-	-	-	-	-	-	-	-
O8	-	-	-	-	-	-	-	-	-	-	-	-	-	**1**	-
O9	-	-	-	-	-	-	-	**1**	-	-	-	-	-	-	-
O10	-	-	-	-	-	-	**1**	-	-	-	-	-	-	-	-
O11	-	-	-	-	**2**	-	-	-	-	-	-	-	-	-	-
O12	-	-	-	-	**1**	-	-	-	-	-	-	-	-	-	-
O13	-	-	**1**	-	-	-	-	-	-	-	-	-	-	-	-
O14	-	-	-	-	-	-	-	-	-	**1**	-	-	-	-	-
O15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**1**
O16	-	-	-	-	-	-	-	-	-	-	-	-	-	**1**	-
O17	**1**	-	-	-	-	-	-	-	-	-	-	-	-	-	-
O18	-	-	-	-	-	-	-	-	-	-	-	-	-	**1**	-
TOTAL	2	11	30	35	57	17	41	17	22	58	27	18	2	66	23

Top row has three regions abbreviated southwestern Atlantic (SWA), Gulf of Mexico (GMX), northwestern Atlantic (NWA). The second row has abbreviated sampling locations, with a plus sign (+) indicating a location where samples were pooled. Highlighted sampling locations indicate a location where unique haplotypes were found. Unique haplotype rows are highlighted. Bolded values are the number of unique haplotypes in each sampling location/region. A dash (-) denotes no representatives of that haplotype at that sampling location.

Table 3.5 *Haplotype distribution map of* R. brasiliensis.

	SWA	GMX								NWA
Haplotype	MUC	MAT+	GLA	MIS	PEL	SAN	APA	CED	CHA	IRL
R1	7	8	3	26	11	5	8	2	1	7
R2	-	-	-	2	-	-	-	-	1	-
R3	-	-	-	2	-	-	-	1	-	-
R4	-	1	-	3	-	-	-	-	-	-
R5	-	-	-	-	-	-	-	-	-	**1**
R6	-	-	-	-	-	-	-	-	-	-
R7	**1**	-	-	-	-	-	-	-	-	-
R8	**1**	-	-	-	-	-	-	-	-	-
TOTAL	9	9	3	33	11	5	8	3	2	8

Top row has three regions abbreviated southwestern Atlantic (SWA), Gulf of Mexico (GMX), northwestern Atlantic (NWA). The second row has abbreviated sampling locations, with a plus sign (+) indicating a location where samples were pooled. Highlighted sampling locations indicate a location where unique haplotypes were found. Unique haplotype rows are highlighted. Bolded values are the number of unique haplotypes in each sampling location/region. A dash (-) denotes no representatives of that haplotype at that sampling location.

Table 3.6 *Pairwise comparison between regions for* R. bonasus.

	NWA (N=106)	GMX (N=304)	SWA (N=2)
NWA	-	0.6061 (P = 0.0631)	0.7719 (P = 0.1351)
GMX	**0.8470*** (P = 0.0000)	-	0.0030 (P = 0.1351)
SWA	**0.8368*** (P = 0.0270)	0.0030 (P = 0.0901)	-

Pairwise F_{ST} (above diagonal) and Φ_{ST} (below diagonal) values calculated for *Rhinoptera bonasus* for the northwestern Atlantic (NWA), northern Gulf of Mexico (GMX), and southwestern Atlantic (SWA) for a 442-bp portion of the ND2 mitochondrial gene. Statistically significant values are bolded and found when $P < 0.05$. An asterisk (*) denotes statistical significance.

Table 3.7 *Pairwise comparison between regions for* R. brasiliensis.

	NWA (N=8)	GMX (N=74)	SWA (N=9)
NWA	-	0.0307 (P = 0.1081)	-0.0467 (P = 0.9910)
GMX	**0.1407*** (P = 0.0451)	-	-0.0207 (P = 0.3694)
SWA	0.0160 (P = 0.3423)	-0.0105 (P = 0.2162)	-

Pairwise F_{ST} (above diagonal) and Φ_{ST} (below diagonal) values calculated for *Rhinoptera bonasus* for the northwestern Atlantic (NWA), northern Gulf of Mexico (GMX), and southwestern Atlantic (SWA) for a 442-bp portion of the ND2 mitochondrial gene. Statistically significant values are bolded and found when $P < 0.05$. An asterisk (*) denotes statistical significance.

Table 3.8 *Overall analysis of molecular variance (AMOVA) for* Rhinoptera *spp.*.

Species	Variance among populations	Variance within populations	Overall F_{ST}
R. bonasus	12.63%	87.37%	0.1263* (P = 0.0068)
R. brasiliensis	7.78%	92.22%	0.0778 (P = 0.0763)

All samples pooled into the three regions NWA (northwestern Atlantic), GMX (Gulf of Mexico), and SWA (southwestern Atlantic) in one grouping. An asterisk (*) denotes statistical significance.

Table 3.9 *Hypotheses tested with an AMOVA for* R. bonasus.

Regions	Variance among regions	Variance among groups within regions	Variance within groups	Overall F_{ST}
NWA v GMX v SWA	**75.70%*** (P = 0.0020)	**8.87%*** (P = 0.0010)	**15.44%*** (P = 0.0000)	**0.8456*** (P = 0.0000)
NWA v GMX	-0.07% (P = 0.6364)	-0.01% (P = 0.4233)	100.08% (P = 0.4712)	-0.0081 (P = 0.4172)
wGMX v eGMX	-0.31% (P = 0.7380)	0.60% (P = 0.1672)	99.71% (P = 0.1838)	0.0029 (P = 0.1838)
ATL v SWA	**95.98%*** (P = 0.0108)	**1.34%*** (P = 0.0059)	**2.68%*** (P = 0.0000)	**0.9598*** (P = 0.0000)

Northwestern Atlantic (NWA; N = 106), Gulf of Mexico (GMX; N = 304); western Gulf of Mexico (wGMX; N = 64); eastern Gulf of Mexico (eGMX; N = 239); western Atlantic (ATL; N = 410); southwestern Atlantic (SWA; N = 22). Regions that are compared (verses) are denoted with a “v”. An asterisk (*) denotes statistical significance.

Table 3.10 *Hypotheses tested with an AMOVA for* R. brasiliensis.

Regions	Variance among regions	Variance among groups within regions	Variance within groups	Overall F_{ST}
NWA v GMX v SWA	8.65% (P = 0.1026)	-3.12% (P = 0.5571)	94.47% (P = 0.3998)	0.0553 (P = 0.3998)
NWA v GMX	-0.61% (P = 0.4663)	-1.21% (P = 0.5503)	101.82% (P = 0.5562)	-0.0183 (P = 0.5562)
wGMX v eGMX	-2.74% (P= 0.9560)	-0.86% (P = 0.4809)	103.60% (P = 0.5269)	-0.0360 (P = 0.5269)
ATL v SWA	14.82% (P = 0.0890)	-2.97% (P = 0.5103)	88.15% (P = 0.3979)	0.1185 (P = 0.3979)

Northwestern Atlantic (NWA; N = 8), Gulf of Mexico (GMX; N = 74); western Gulf of Mexico (wGMX; N = 42); eastern Gulf of Mexico (eGMX; N = 32); western Atlantic (ATL; N = 82); southwestern Atlantic (SWA; N = 9). Regions that are compared (verses) are denoted with a "v". An asterisk (*) denotes statistical significance.

3.3.3 Genetic diversity

Within the ATL, *R. bonasus* had low levels of haplotype diversities (0.0660 – 0.0360) and moderate levels of nucleotide diversities (0.1132 – 0.1303). In the SWA, *R. bonasus* had high levels of both haplotype (0.6667) and nucleotide diversity (3.0000). In terms of haplotype diversity, *R. brasiliensis* had low levels in the GMX (0.0810), moderate in the NWA (0.2500), and high in the SWA (0.7778). Nucleotide diversities in *R. brasiliensis* were moderate in the ATL (0.2500 – 0.4709) and high in the SWA (1.7222). Diversity was consistently high in both species in the SWA.

3.3.4 Demographic history

Tests of neutrality were statistically significant within individual sites within the NWA and the GMX in both *R. bonaus* (e.g., pooled Laguna Madre, Mississippi Sound, pooled Apalachicola Bay, Tampa Bay, Charlotte Harbor within the GMX, and Pamlico Sound within the NWA; see Table 3.11) and *R. brasiliensis* (pooled Matagorda Bay; see Table 3.12). Within regions, *R. bonasus* in the NWA and GMX had high significant negative *D* and F_S values (Table 3.13), which is consistent with the NWA and the GMX being separate populations. Within regions, *R. brasiliensis* did not show any significant values, which may support the finding of genetic homogeneity (Table 3.14).

Table 3.11 *Results of neutrality tests for* R. bonasus *from pooled regions.*

Water body	Abbreviated region name	Sample size	No. of polymorphic sites	*h*	Sum of Squares	π	Tajima's *D*	*P*	Fu's *Fs*	*P*
Northwestern Atlantic	NWA+	106	6	0.0660	0.9445	0.1132	**-2.0063**	**0.0010**	**-3.9718**	**0.0040**
Gulf of Mexico	GMX+	305	11	0.0360	0.8798	0.1303	**-2.1066**	**0.0000**	**-22.1933**	**0.0000**
Southwestern Atlantic	SWA+	2	3	0.6667	0.5000	3.0000	0.0000	0.3490	1.0986	0.6591

The presence of a plus sign (+) after the three-letter abbreviation of a region name indicates the samples were pooled, *h* denotes haplotype diversity, π denotes nucleotide diversity and *P* values are

considered significant if >0.05, NS indicates a value that is not statistically significant. Bolded values indicate statistical significance. An asterisk (*) denotes statistical significance.

Table 3.12 *Results of neutrality tests for* R. brasiliensis *from pooled regions.*

Water body	Abbreviated region name	Sample size	No. of polymorphic sites	*h*	Sum of Squares	π	Tajima's *D*	*P*	Fu's *Fs*	*P*
Northwestern Atlantic	NWA+	8	1	0.2500	0.7812	0.2500	-1.0548	0.3865	-0.1820	0.8740
Gulf of Mexico	GMX+	74	6	0.0810	0.7999	0.4709	-1.4596	0.4102	-0.7170	0.4333
Southwestern Atlantic	SWA+	9	7	0.7778	0.6296	1.7222	-1.4780	0.6482	1.4498	0.1002

The presence of a plus sign (+) after the three-letter abbreviation of a region name indicates the samples were pooled, *h* denotes haplotype diversity, π denotes nucleotide diversity and *P* values are considered significant if >0.05, NS indicates a value that is not statistically significant. An asterisk (*) denotes statistical significance.

3.4 Discussion

This study provides the broadest and most wide-reaching sampling scheme of any *Rhinoptera* spp. study to date, encompassing the breadth of the range of each species, which may account for the detection of *R. brasiliensis*. The study identified *R. bonasus* to be more common (found 83% of the time) than *R. brasiliensis* (found 17% of the time), though this may have been due to gaps in sampling spread which excluded the southern Gulf of Mexico and southern Brazilian waters where *R. brasiliensis* may have a stronger presence. This study also provides the first estimation of patterns of genetic differentiation in *R. bonasus* throughout their entire range and is the first study to date to examine population structuring of *R. brasiliensis.* Supported by a previous study (see Carney et al., 2017), *R. bonasus* in the northwestern Atlantic and Gulf of Mexico showed a signature of genetic differentiation, indicating they should be considered separate stocks. However, the findings presented here require more extensive sampling throughout the southern Gulf of Mexico and southern Brazilian waters.

3.4.1 Population structure

Rhinoptera bonasus

This study found population structuring between the NWA and the GMX, and the NWA and the SWA in *R. bonasus* which supported two of the hypotheses of this study. Despite relatively small sample sizes in the SWA (N<20; see Table 3.1), *R. bonasus* showed high levels (95.98%) of significant variance between the NWA to the GMX, and

the NWA to the SWA, which indicated strong population structuring and suggests possible barriers to dispersal (i.e., the Gulf Stream, and the Amazon River/isolation by distance). This finding was supported by high pairwise F_{ST} and Φ_{ST} values between the NWA, the GMX, and the SWA. Additionally, the majority (90.91%) of individuals from the SWA had unique haplotypes. These results indicate there are barriers to gene flow for *R. bonasus* between the NWA and the GMX (i.e., the Gulf Stream Barrier), and the NWA and the SWA (possibly isolation by distance or the Amazon River). The population structure between the NWA and the GMX suggests there is a lack of female mediated gene flow across this region, and that the NWA, the GMX, and the SWA may constitute separate populations. Significant pairwise differences of *R. bonasus* within each of the NWA and GMX may have been masked by the presence of completely unique haplotypes from the SWA.

The finding of population structure between the NWA and the GMX in *R. bonasus* is supported by Carney et al., (2017), where statistically significant genetic structure was identified in *R. bonasus* between the Chesapeake Bay/Pamlico Sound estuary systems (N=149) and Tampa Bay, Florida (N=40). Carney used a 540-bp fragment of Cytochrome B (Cyt B), while here I used a 442-bp fragment of the NADH subunit 2 (ND2) gene. The difference in gene choice does not appear to have altered the findings of this study, even though ND2 generally has a faster rate of mutation in elasmobranchs compared to Cyt B (Schwartz & Maddock, 2002; Naylor et al., 2012). The samples for Tampa Bay in Carney et al., (2017) were collected during fall and winter**,** whereas samples in this study were collected throughout spring, summer, and fall.

Because sampling occurred only in the overwintering period in Tampa Bay in Carney et al., (2017), it is possible that a resident or overwintering population was sampled rather than a migratory population. Residency is typically defined as the presence of individuals for longer than 12 months (see Chapman et al., 2015), which has been documented in batoids (Campbell et al., 2012; Corcoran et al., 2013; Cerutti-Pereyra et al., 2014; White et al., 2014). Poulakis et al., (2013) noted the possibility of resident populations of *R. bonasus* from an estuary in relatively close proximity to Tampa Bay (Charlotte Harbor, approximately 100 km away), evidenced by the presence of *R. bonasus* found throughout all seasons. This finding was also supported by a tracking study by Collins et al., (2007) which found some cownose rays stayed within the Charlotte Harbor estuary system throughout the year. An overwintering population sampled in Tampa Bay and found to be genetically distinct from the NWA could suggest population structuring between the GMX and the NWA, as suggested in Carney et al., 2017. Another explanation is a lack of sampling in the estuaries between the Chesapeake Bay/Pamlico Sound estuary systems and Tampa Bay resulted in an artificial signature of population structure. Common haplotypes between the Chesapeake Bay/Pamlico Sound estuary systems and Tampa Bay, differences in sampling seasons between the two regions, and combined with low levels of significant population structure (33.68% variance among regions) from the Carney et al., may have resulted in artificial population structure. The results found in Carney et al., and this study are comparable to those found in *R. steindachneri* in the Pacific Ocean (Sandoval-Castillo & Rocha-Olivares, 2010), which found high levels of

significant population structure within the Gulf of California due to behavioral mediated movement using the ND2 gene.

The high levels of vagility in *R. bonasus* has been well noted, and indicates the group is both capable and has a history of traversing common phylogeographic barriers in the western Atlantic. Schwartz (1965) hypothesized *R. bonasus* migrated from the most northern portion of their range (New England, USA) to the SWA, inferred from the presence of *Rhinoptera* spp. in Venezuela that had been tagged in Chesapeake Bay. The movement of *R. bonasus* from Chesapeake Bay to Venezuela indicates at least some rays were able to traverse both the Gulf Stream and the outflow from the Mississippi River and indicates the possibility of gene flow throughout the northern Atlantic.

Rhinoptera brasiliensis

There was not strong evidence of population structure for *R. brasiliensis* between the GMX and the SWA, which may have been an artifact of small sample sizes. Pairwise comparisons indicated low but significant levels of genetic differentiation between one sampling site in the GMX (e.g., Mississippi Sound) and the SWA (e.g., Brazil), which may indicate population structure that was not detected due to small sample sizes. A lack of significant pairwise differences between sites in the NWA and the GMX could suggest a single population, while pairwise differences between sites in the GMX and SWA could suggest a similar pattern of population structure found in *R. bonasus* over the same spatial scale. Small sample sizes in the SWA had a large proportion (77.78%) of the most common *R. brasiliensis* haplotype (R1), which may be an indication of gene flow

and could be an explanation for a lack of significant population structure. There were no significant values of variance among regions to support the population structure hypotheses described in this study (see Table 3.10). However, a low level of variance (14.82%) was nearly significant ($P = 0.0890$) between the NWA+GMX and the SWA. Small sample sizes (N<10) of *R. brasiliensis* throughout their range (see Table 3.4) may have impeded the statistical significance of the AMOVA, and although the results do not meet the significance threshold, should not be discounted.

3.4.2 Genetic diversity

Levels of genetic diversity in both species were considered low to moderate in terms of elasmobranch diversity estimates throughout the NWA and GMX (Heist, 2004; Sandoval-Castillo et al., 2014; Hoelzel et al., 2006; Dominques et al., 2017), but high within the SWA. A study examining *R. steindachneri* in the Pacific found similar results of low nucleotide and haplotype diversities within regions that were found to be one population (e.g., the NWA and the GMX). The results are consistent with population structure assignment distinguishing the NWA and GMX, and the SWA to be separate populations, as evident by the overt differences in genetic diversity.

3.4.3 Demographic history

The demographic history inferred from the data in this study indicate an evolutionarily recent population expansion took place for both species (see Figure 3.7). A negative Tajima's *D* and Fu's *Fs* value combined with a unimodal mismatch distribution support a population expansion for *R. bonasus* in the NWA and the GMX. The haplotype

network (see Figure 3.7) combined with the neutrality test values for both species indicate samples from the SWA are more different than those found in the NWA and the GMX, a finding which supports the population structure in *R. bonasus* and indicates the nearly significant finding of population structure between the NWA and the GMX, and the SWA in *R. brasiliensis* should be examined further. Additional research (e.g., sampling, SNPs, tagging) are needed to fully refine population structure in for both species in the northwestern Atlantic.

3.4.4 Conservation implications

The life history of *Rhinoptera* spp., specifically the low fecundity and ease with which they are seen and caught, makes them highly susceptible to anthropogenic forces and necessitates accurate management and conservation. Exhibiting philopatry and population structure between regions may have resulted in region-specific adaptions and differences in biology, life history, behavior, or vagility, which would be important to consider in the management of each species. The delineation of each species' spatial scale is needed to define management units prior to management and conservation decisions being made.

The results presented in this study suggest population structure between the NWA, the GMX, and the SWA. A population structure in these regions would affect studies examining biology, life history, and genetic assessments of *Rhinoptera* spp. used to inform management and conservation efforts. Given the NWA, the GMX, and the SWA are considered here as separate populations, researchers within the northwestern

Atlantic should collaborate to study *Rhinoptera* spp. within the entirety of their range, which would include the southern Gulf of Mexico, Central America, and Brazil.

CHAPTER IV – CONCLUSIONS

4.1 Main findings

This research used molecular tools to assess the population genetics of two species of cownose rays found within the western Atlantic. It was also the first study to include samples from the breadth of both species' range, revealing novel insights into their ecologies. Within the northwestern Atlantic (NWA) and northern Gulf of Mexico (GMX), this study was the first to examine population genetics of Brazilian cownose ray, *Rhinoptera brasiliensis.* This study documented the first detection of *R. brasiliensis* along the eastern coast of Florida (e.g., Indian River Lagoon), extending its known range by over 1,500 km (Weber et al., 2021). This finding was especially important for ongoing population studies that will be used to inform conservation and management decisions for the sympatrically occurring American cownose ray, *R. bonasus*.

This study failed to detect matrilineal gene flow, based on a 442-bp portion of the NADH subunit II mitochondrial gene in *R. bonasus* between the northwestern Atlantic, the Gulf of Mexico, and the southwestern Atlantic.

This study noted high levels of population structure between the NWA and the GMX, and the NWA and the SWA in *R. bonasus* and an indication of genetic differentiation on the same spatial scale for *R. brasiliensis*.

4.2 Caveats of the data and future directions

There are a number of caveats to consider when interpreting the main findings of this research. Firstly, data presented here were from a 442-bp region of a single gene (e.g.,

ND2), of the maternally inherited mtDNA. This fragment contained 31 and 23 polymorphic sites in *R. bonasus* and *R. brasiliensis*, respectively, which should have been sufficient to detect population structure, unless populations are very recently diverged. To more fully refine population structure in *Rhinoptera* spp., future studies should strive for genomic approaches that utilize both mtDNA and nuclear DNA (nDNA), (e.g., mitogenomes and single nucleotide polymorphisms (SNPs)). More powerful datasets may reveal population structure at finer spatial scales that detected in this study, and the combination of mtDNA and nDNA datasets would allow for the identification of potential hybrids and assessments of male vs. female gene flow. In this study, sample collection was largely opportunistic and was limited by not including the winter season in most sampling locations, which obscured the detection of overwintering populations. Sampling sites were also primarily off the coastal area and did not include individuals that were offshore on the continental shelf, which may have produced samples from migrating individuals that may have been migrating through. Samples were collected opportunistically, so the information on sex and age was in some cases absent (e.g., Texas samples), which limited data analyses.

In the future, increased sample numbers from each location including members of both sex and maturity level will be needed. Extensive sampling throughout the southern Gulf of Mexico, Central American and throughout the southwestern Atlantic will be needed to understand the connection between the Gulf of Mexico and the southwestern Atlantic more fully. Samples need to be collected throughout the year to encompass the overwintering period in all sampling locations as well. Although the maternally inherited

ND2 gene is useful in detecting population structure, the male contribution is not considered with this approach.

Conservation efforts should prioritize further investigation into the biology, population dynamics, and genetic structure throughout the entirety of *Rhinoptera* spp.'s range, including understudied areas (e.g., the southern Gulf of Mexico, the Caribbean, and Central America). Sampling should include all seasons to detect potential residency (instead of excluding winter sampling which is common in elasmobranch studies), focusing on varied gillnet sizes and including rivers feeding into estuary systems to catch young-of-the-year and juvenile *Rhinoptera* spp.. Genetic marker selection should be considered in terms of comparability to past studies (e.g., ND2, Cyt B, COI) and to incorporate the highest statistical power (e.g., SNPs). Using mtDNA and nuclear DNA (nDNA) would give insight to both female and male mediated gene flow and result in more robust findings.

A consensus among studies on a species is ideal when defining management units, however the results here raise more questions on the genetic structure of *Rhinoptera* spp. and will require more investigation to confidently assign management units.

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