MOLECULAR ECOLOGY AND EVOLUTION OF ELASMOBRANCH REPRODUCTIVE STRATEGIES

A Dissertation

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This dissertation meets the standards for scope and quality of
Texas A&M University-Corpus Christi and is hereby approved.

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ABSTRACT

Elasmobranchs are a diverse group of cartilaginous fishes consisting of sharks and batoids that exhibit a variety of reproductive strategies. Elasmobranch reproductive biology has been studied in the wild for many decades but molecular techniques have been used more recently to broaden understanding. Though polyandry has been demonstrated to be widespread, the benefits to females are unclear. Similarly, multiple species have been shown to re-use nurseries – which may increase juvenile survival – yet the impacts of this behavior on population structure require further study. Molecular studies using high-throughput sequencing can help to address knowledge gaps; however, the application of these techniques to study elasmobranchs is limited. Therefore, this dissertation examined elasmobranch reproductive strategies using high-throughput approaches.

The first chapter reviewed research on elasmobranch reproductive strategies and outlined how high-throughput data can help to address knowledge gaps. For the three other chapters, the blacktip shark (*Carcharhinus limbatus*) was studied to advance understanding of mate choice and nursery use and inform management. Chapter two assessed for MHC-associated mate choice. Evidence of assortative choice for *mhc1a* was observed in four of six litters but further study is needed to validate this observation. Chapter three examined the influence of philopatry on the genetic population structure of blacktip sharks using young-of-the-year sampled in United States waters. Regional philopatry by males and females has contributed to the formation of three genetically distinct units that closely align with fishing stocks. Furthermore, philopatry by females to environmentally heterogenous estuaries where offspring are born appears to have resulted in fine-scale adaptive structure within management units. Chapter four assessed the

genetic stock structure and movement of blacktip sharks sampled across the western North Atlantic Ocean to evaluate the potential for multinational fisheries management. The blacktip shark stock in the western Gulf of Mexico might straddle U.S. and Mexican waters, and stocks in Cuba and The Bahamas are much more genetically diverged compared with other stocks. Moreover, five blacktip sharks were determined to have moved across stock boundaries, but the majority of individuals were sampled in the region of their natal stock.

The research provides novel insights into elasmobranch reproductive strategies and is a basis for additional studies of mate choice and nursery use. There is preliminary evidence that MHC is involved in mate choice by blacktip sharks; however, additional research is necessary to examine the benefits and mechanisms associated with MHC-mediated choice. Further, there is evidence that female natal philopatry facilitates local adaptation to nursery conditions, but more research is required to provide direct evidence of this behavior and determine the distribution of putatively adaptive loci across the genome. Future studies should examine how mating systems and patterns of habitat use can generate, maintain, and disperse adaptive variation because this is vital for resilience to environmental change. Understanding disparities in abundance and dispersal potential between continental and insular populations would be particularly informative for management.

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CHAPTER I: INTRODUCTION

Reproductive Strategies

An individual's lifetime reproductive success is determined by the total number of viable offspring produced (Clutton-Brock, 1988). Only a subset of individuals succeeds in producing offspring that contribute to subsequent generations, so lifetime reproductive success often provides an approximate estimate of Darwinian fitness (Newton, 1989). Traits that influence fitness are subject to natural selection; at the same time, however, traits influencing reproductive success may be subject to sexual selection, and the two forces can work together or in opposition (Lindsay et al., 2019). Males usually contribute less to individual offspring than females and exhibit greater variance in reproductive success, and thus, are generally affected more by sexual selection than females (Bateman, 1948). Consequently, a considerable diversity of male and female reproductive strategies displayed before and after copulation has evolved in sexually reproducing organisms. Pre-copulatory strategies include advertisement, mate choice, and morphologies and behaviors that mediate copulation (Birkhead et al., 1993; Chapman et al., 2003; Whittle et al., 2000). By contrast, post-copulatory strategies include cryptic mate choice and parental investment via resource allocation to offspring development and care (Gasparini & Pilastro, 2011; Trivers, 1972). Allocation of resources to reproductive strategies can vary remarkably among species because fitness is optimized by balancing investment between reproduction and survival/growth (Stearns, 1989). Males and females can also adjust their allocation to different strategies within and among mating efforts based on perceived intrasexual competition and receptivity/quality of potential mates (Kelly & Jennions, 2011; Zeh & Zeh, 1997). Therefore, the combination of reproductive strategies exhibited by individuals determines

their lifetime reproductive success and ultimately contributes to variation in life history among species.

Elasmobranch Reproductive Biology and Conservation

Many of the life history and reproductive strategies of elasmobranchs (i.e., sharks and batoids) are distinct from those of most bony fishes (Musick, 2005). Elasmobranchs have relatively late ages-at-maturity and long life spans (Cortés, 2004), and while fecundities vary widely among species (one to several hundred), the number of eggs produced per reproductive effort is generally much smaller compared with other fishes (Daley et al., 2002; Holden, 1975). All elasmobranchs display internal fertilization and direct development of offspring – approximately 60% give live birth and 40% deposit egg cases (Conrath & Musick, 2012; Wourms, 1977). While mating is usually annual, females of some species reproduce every two years or more (Castro, 2000, 2009; Whitney & Crow, 2007), meaning only a fraction of females are available to mate each year. These traits – in addition to the use of mating sites – can lead to male-skewed operational sex ratios and intense competition among males to fertilize eggs, resulting in sexual conflict and polyandry that have been documented in many species (Carrier et al., 2004; Pratt Jr. & Carrier, 2001; Whitney et al., 2004).

Multiple species display fidelity to nursery habitats where offspring remain after birth (Castro, 1993; Chapman et al., 2009). Nurseries are thought to increase juvenile survival relative to other habitats by providing food resources and/or refuge from predators, but benefits may differ among species (Heupel et al., 2007, 2019). To date, nurseries have mainly been identified in bays and estuaries that are spatially discrete from habitats where adults are usually found (Feldheim et al., 2002; Froeschke et al., 2010; Heupel & Hueter, 2002). Thus, while elasmobranchs do not provide parental care post-partum, migration to parturition sites in or near

nurseries may require females to allocate resources in a similar manner. Furthermore, there is evidence that some females re-use their own nursery or neighboring nurseries in the region of their birth for parturition, a phenomenon known as female philopatry (Chapman et al., 2015). Philopatry likely constitutes a post-copulatory reproductive strategy to maximize fitness by delivering offspring in habitats that have facilitated reproductive success in previous generations, and might be particularly advantageous for species with low fecundities and large maternal investments (Hueter et al., 2005). Juveniles may also display fidelity to nurseries for the first few years of life – conditions in some nurseries are suitable for juveniles to remain year-round (Simpfendorfer & Milward, 1993) whereas others are used seasonally (Grubbs et al., 2007; Hueter et al., 2005). Patterns of movement to and from nurseries can therefore be used to understand the evolutionary and ecological impacts of reproductive strategies while identifying essential habitats to conserve.

Studies of reproductive biology can support the management of elasmobranch populations that are subject to exploitation and/or of conservation concern. Many of these species occupy higher trophic positions and are vital to maintaining the health and resilience of marine ecosystems (Heithaus et al., 2012; Heupel et al., 2014; Williams et al., 2018). Elasmobranchs also have economic value as resources for harvest and ecotourism, generating ~$1 billion in annual income globally (Cisneros-Montemayor et al., 2013; Dent & Clarke, 2015). However, according to the IUCN Red List of Threatened Species, elasmobranchs are among the most imperiled vertebrates on Earth (Dulvy et al., 2021). Understanding how mating systems and patterns of habitat use influence population dynamics can help to reduce overexploitation by determining the appropriate scale of management plans. Thus, studies of reproductive strategies

have the potential to improve the conservation status of elasmobranchs by informing sustainable management

Studying Elasmobranch Reproductive Strategies Using Molecular Techniques

While observations of mating behavior and tracking of individual movements have traditionally provided insights into elasmobranch reproductive strategies, molecular techniques offer an alternative set of approaches to validate these studies and broaden understanding. For example, though the behavior has been directly observed in few species, microsatellites have helped to demonstrate that polyandry is widespread among elasmobranchs (Chevolot et al., 2007; Portnoy et al., 2007; Saville et al., 2002). Microsatellites are short tandem repeats of nuclear DNA that are inherited biparentally, show high intraspecific variation, and facilitate assessments of relatedness (McDonald & Potts, 1997; O'Connell & Wright, 1997). Thus, these markers can be used to examine elasmobranch mating systems by estimating the minimum number of sires per litter and the degree of relatedness between mates (DiBattista et al., 2008). In addition, assessments of relatedness enable the identification of kin sampled in the same nurseries in successive reproductive efforts, which can be indicative of female fidelity/philopatry (Feldheim et al., 2014; Mourier & Planes, 2013). Furthermore, in combination with mitochondrial DNA that is inherited only from mothers, microsatellites have been used to show evidence of female philopatry in other species on a broader scale by comparing patterns of genetic population structure resulting from disparities in male and female-mediated gene flow (Daly-Engel et al., 2012; Karl et al., 2011; Pardini et al., 2001). However, microsatellites are generally unaffected by selection, and genotyping many individuals at these loci can be labor-intensive. Therefore, studies of reproductive strategies using more efficient molecular approaches with broader capabilities have the potential to further advance understanding.

High-throughput sequencing can enable relatively cost-effective approaches to study reproductive strategies by genotyping individuals at markers containing single nucleotide polymorphisms (SNPs). These approaches can improve studies of fidelity/philopatry by allowing for the detection of kin among many hundreds of individuals sampled in the same habitats (Feutry et al., 2017, 2020). Further, SNP genotypes can be used to examine how male and female philopatry impact patterns of gene flow by facilitating assessments of population structure. If sufficient resolution is present among populations, SNPs can also be used to detect movement between them by assigning individuals to putative populations of origin (Dimens et al., 2019). Moreover, in contrast to microsatellites, SNPs occur in coding and non-coding DNA and allow for assessments of local adaptation that might be associated with fine-scale female philopatry (Portnoy et al., 2015). Conversely, many individuals can be genotyped at a small number of markers with very high sequencing coverage (Lighten et al., 2014). This approach can be used to examine complex and highly variable genes that may influence mate choice by acting as indicators of mate quality and compatibility (Rekdal et al., 2019). While polyandry has been studied in elasmobranchs for decades, very little is known about how mate choice is exerted. Microsatellites remain a useful tool for studying mating systems by assessing for genetic polyandry and inbreeding/outbreeding, but these loci cannot be used to assess for evidence of mate choice by themselves. By contrast, high-throughput sequencing enables approaches that can efficiently generate millions of sequencing reads to facilitate the genotyping of multi-copy and highly polymorphic genes that might fulfill functional roles in elasmobranch mate choice.

The Blacktip Shark

The blacktip shark (*Carcharhinus limbatus*) is a typical coastal elasmobranch that is distributed throughout tropical and warm temperate waters where it is exploited by a variety of

fisheries (Compagno et al., 2005; Rigby et al., 2021). In United States waters of the western North Atlantic Ocean, males and females mature at five and six years, respectively, and can live for ~20 years (Baremore & Passerotti, 2013; Carlson et al., 2006). Though mating is annual, females give live birth to one to eight pups every two years with a 12-month gestation period that is followed by 12 months of resting to replenish lipid stores needed to produce eggs (Baremore & Passerotti, 2013). Blacktip shark mating behavior has not been documented; however, observations of females with severe injuries (150 mm long and 20 mm deep) show that males copulate by biting and holding females (Castro, 1996). This is consistent with mating behavior studied in other carcharhinids (Whitney et al., 2004) and suggests that mating can be costly to females. Furthermore, results from a molecular study demonstrate that blacktip shark litters are often sired by multiple males but the benefits of polyandry are unknown (Bester-van der Merwe et al., 2019).

In U.S. waters, blacktip shark females migrate to bays and estuaries in the late spring/early summer for parturition, and young-of-the-year remain there until the fall (Castro, 1996; Heupel et al., 2004). Some juveniles return to their natal site the following spring and repeat this seasonal migration for several years (Hueter et al., 2005). While it is unclear if females re-use the same habitats for parturition, assessments of genetic population structure using microsatellites and mitochondrial DNA suggest that females reproduce in the region of their birth (i.e., regional philopatry) but males often mate with females from other regions (Keeney et al., 2005). However, this signal of sex-biased dispersal could result from differences in the molecular markers used (Birky Jr et al., 1983). Therefore, further study is necessary to determine the spatial scale of female philopatry and the influence of male- and female-mediated gene flow on patterns of population structure.

Studying Elasmobranch Reproductive Strategies Using High-throughput Sequencing

Advances in molecular techniques based on high-throughput sequencing have improved understanding of many areas of evolutionary ecology and enabled a variety of approaches to study reproductive strategies. By examining coding and non-coding DNA, these approaches can be used to study mating systems and patterns of movement related to reproduction, providing insight into factors that influence reproductive success and population structure. However, the use of high-throughput data in studies of elasmobranchs is limited compared with other vertebrates. Hence, this dissertation focuses on understanding elasmobranch reproductive strategies using high-throughput techniques to advance understanding and inform management. Chapter two reviews two aspects of elasmobranch reproductive biology, identifies knowledge gaps requiring further study, and proposes molecular approaches that could be used to address them. Mate choice might be exerted by females through a variety of pre- and post-copulatory mechanisms that are yet to be examined. Elasmobranchs can display varying degrees of site fidelity and philopatry, but how these behaviors are mediated is unclear. Chapter three assesses the potential for MHC-associated mate choice in the blacktip shark by genotyping females, their offspring, and adults at microsatellites and MHC genes. MHC has been implicated in mate choice in a variety of vertebrates but its influence on elasmobranch mate choice has not been assessed. Chapter four examines the influence of philopatry on the genetic population structure of blacktip sharks sampled in U.S. waters. Regional philopatry by either sex can reduce gene flow across broad spatial scales and contribute to neutral population structure among management units. By contrast, philopatry by females to environmentally heterogeneous habitats that are used for parturition can lead to local adaptation and fine-scale adaptive structure within units. Chapter five broadens the assessment of neutral population structure by incorporating

samples from blacktip sharks captured throughout the western North Atlantic. Because this species is capable of moving vast distances, evaluating the potential for fishing stocks that straddle and/or mix across national boundaries is vital for sustainable management.

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CHAPTER II: EXAMINING THE REPRODUCTIVE BIOLOGY OF ELASMOBRANCHS USING HIGH-THROUGHPUT SEQUENCING

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Abstract

Elasmobranchs are a diverse group of cartilaginous fishes that display a variety of reproductive strategies. All species fertilize internally, but differences in morphology contribute to a diversity of mating behaviors. Although some elasmobranchs deposit offspring in egg cases, the majority are live-bearing, and nourishment is provided in a multitude of forms. None of these species provide parental care; however, maternal investment varies greatly during embryonic development, and females of many species invest additional energy by migrating to and delivering offspring in or near habitats that are thought to increase juvenile survival. Elasmobranch reproduction has traditionally been studied in the wild through observations and tracking but over the last few decades, molecular approaches have been used to support earlier studies while advancing understanding. High-throughput sequencing has led to more rigorous molecular approaches, collectively known as omics, which allow for large-scale and comprehensive assessments of DNA and RNA. Due in part to a shortage of genomic data, omics-based studies of elasmobranchs are limited compared with other jawed vertebrates but have the potential to further advance understanding of reproductive biology. The goal of this review is to highlight aspects of elasmobranch reproduction requiring further study and outline how omics can be used to address knowledge gaps.

Introduction

Cartilaginous fishes (Chondrichthyes) are an intriguing group for studies of reproductive biology because of their evolutionary history and contemporary diversity. Chondrichthyans

comprise a lineage with a fossil record originating in the Ordovician period, approximately 470 million years ago (mya; Andreev et al., 2015; Karatajūtė-Talimaa, 1998). Alongside osteichthyans (teleosts and tetrapods), chondrichthyans form one of two extant lineages of jawed vertebrates (Brazeau & Friedman, 2015). These species are unified by tesserate endoskeleton mineralization, internal fertilization, and direct development of offspring that are live-birthed or hatch from egg cases (Maisey, 1984; Wourms, 1977). More than 1,000 species across 16 extant orders have been described (Weigmann, 2016) and can be divided into two groups – elasmobranchs and holocephalans – that diverged approximately 415 mya (Coates & Sequeira, 2001; Inoue et al., 2010). Elasmobranchs are composed of sharks and batoids (skates and rays) which are estimated to have diverged approximately 370 mya (Heinicke et al., 2009; Sorenson et al., 2014).

Despite some conserved aspects of reproductive biology, chondrichthyans show a diversity of reproductive strategies. Female reproductive anatomy consists of paired ovaries and oviducts that often become asymmetrical and specialized (Wourms, 1977). Sperm are delivered via paired intromittent organs known as mixopterygia (i.e., claspers; Frey, 1995) and fertilization occurs within or close to oviducal glands that are found below the anterior oviducts (Hamlett et al., 1998; Hamlett & Koob, 1999). Extant holocephalans, known as chimaeras, display reproductive characters that are not found in other chondrichthyans. Female chimaeras have sperm receptacles, two uterine openings, and pre-pelvic abdominal slits; males have pre-pelvic claspers that are inserted into female pre-pelvic slits, and along with a club-like organ (the cephalic tenaculum), are used to hold females and induce copulation (Jones et al., 2005). Chimaeras primarily inhabit deep-water (up to 3,000 meters; Kyne & Simpfendorfer, 2007; Priede & Froese, 2013), making them particularly difficult to study. As a result, knowledge of

holocephalan reproductive biology is relatively limited in most species (see Jones et al., 2005 for an in-depth description of the reproductive biology of the elephant fish *Callorhinchus milii* Bory de Saint-Vincent 1823). Therefore, this review focuses on elasmobranchs.

Variation in morphologies among elasmobranchs appears to contribute to differences in copulatory behavior. Biting is common, but some species are more flexible and males may more easily access the cloaca by also wrapping their bodies around females (Pratt Jr. & Carrier, 2005). Many elasmobranchs deposit egg cases but the majority are live-bearing and the duration of embryonic development ranges from several months to two years (Capapé, 1993; Parsons, 1993; Wilson & Seki, 1994), and possibly longer (Hoff, 2008; McLaughlin & Morrissey, 2005; Tanaka et al., 1990). Fecundity also varies considerably: some species produce hundreds of young (Crow et al., 1996; Holden, 1975; Joung et al., 1996) while others birth one or two offspring per reproductive effort (Daley et al., 2002; Marshall et al., 2008; Notarbartolo-di-Sciara, 1987). Elasmobranchs do not provide parental care after birth or oviposition, but many species migrate to and release offspring in or near habitats that are thought to provide refuge and/or resources. These habitats vary in use and apparent benefits, and offspring may use them repeatedly during the first few years of life (Heupel et al., 2007).

Studies of elasmobranch reproduction have traditionally relied on anatomical descriptions, endocrine analysis, and observations and tracking of wild animals because most species are not amenable to breeding in captivity. Examination of dead specimens led to descriptions of reproductive anatomy, embryonic provisioning, and sperm storage (Wourms, 1977). Observations of multiple males attempting to mate with a single female demonstrated the potential for behavioral polyandry (Carrier et al., 1994; Chapman et al., 2003; Whitney et al., 2004) while tracking studies showed adults of some species return faithfully to areas

encompassing habitats for mating and parturition (Feldheim et al., 2002; Hueter et al., 2005; Pratt Jr. & Carrier, 2001).

Molecular approaches over the last two decades have corroborated earlier studies and revealed additional aspects of reproductive biology (reviewed in Portnoy & Heist 2012). Molecular studies have supported observations of behavioral polyandry by identifying litters sired by more than one male (multiple paternity or genetic polyandry) and allowed researchers to document the frequency of this phenomenon (Barker et al., 2019). Molecular approaches have also confirmed that females of multiple species give birth in the same habitats in successive breeding seasons (Feldheim et al., 2014; Feldheim et al., 2017; Mourier & Planes, 2013). More recently, advances in molecular techniques and high-throughput sequencing have led to a set of tools that enable the examination of hundreds to thousands of markers (e.g., genomics, transcriptomics, proteomics, etc.). Collectively referred to here as omics, these approaches have enhanced studies of vertebrate reproduction (Houston et al., 2020; Long, 2020; Van Dyke et al., 2014) but to date, the relative paucity of whole-genome assemblies (Table 2.1) has limited their use in studies of elasmobranchs.

This review focuses on the application of omics to study two aspects of elasmobranch biology that occur at the start and end of reproductive cycles: mate choice and nursery use. Each section contains a background of these reproductive strategies and reviews how molecular approaches have supplemented traditional methods to examine them. A combination of textbook chapters, review, and primary research articles sourced from Web of Science and Google Scholar, and studies cited therein are used to summarize and identify areas for further research. Studies of other vertebrates are then cited to guide investigative approaches incorporating omics tools to advance understanding of elasmobranch reproduction.

Mate Choice

All elasmobranchs fertilize internally, thus some coordination between mates is necessary for successful reproduction. Studies of elasmobranch copulation have documented males following and approaching females before inserting claspers, a behavior that involves specific body orientations and often biting (Clark, 1975; Nordell, 1994; Tricas & Le Feuvre, 1985). Across animal taxa, mating can incur costs due to increased vulnerability to predation (Rowe, 1994), disease transmission (Thrall et al., 2000), and physical harm (Chapman et al., 1995). In elasmobranchs, the biting of females during mating attempts can produce lesions and scars around the gills, pectoral and pelvic fins (Pratt Jr. & Carrier, 2001; Ritter & Amin, 2019). Also, claspers typically have spurs and spines that can cause wounds to cloaca and vaginal walls (Carrier et al., 2004). Consequently, mating can be particularly costly to female elasmobranchs and they exhibit behaviors ranging from active avoidance to encouragement (Pratt Jr. & Carrier, 2005).

While the costs of copulation appear high for females and multiple mating has been directly observed in only a few species (Carrier et al., 1994; Chapman et al., 2003; Whitney et al., 2004), the phenomenon has been widely documented using molecular approaches. Multiple paternity has been detected in all but two species in which more than one litter was examined (more than 30 species across seven orders; Lamarca et al., 2020) – thus, it appears that polyandry is ubiquitous among elasmobranchs. The frequency of genetic polyandry differs greatly among species and may be related to the capability of oviducal glands to store sperm. Frequencies range from predominant monandry (6.5% in velvet belly laternshark *Etmopterus spinax* L. 1758 Duchatelet et al., 2020) to complete polyandry (100% in nurse shark *Ginglymostoma cirratum* (Bonnaterre 1788) Heist et al., 2011), and can also vary within species, suggesting population

dynamics and environment may influence the female remating rate. In sandbar sharks *Carcharhinus plumbeus* (Nardo 1827), 85% of litters were multiply sired in the western North Atlantic Ocean (Portnoy et al. 2007), whereas only 40% were multiply sired in Hawaii (Daly-Engel et al., 2007). *Carcharhinus plumbeus* undergo seasonal migrations in the western North Atlantic and are thought to use specific mating sites (Grubbs et al., 2007), facilitating high encounter rates over a short mating period. In the Pacific Ocean, by contrast, *C. plumbeus* is more resident, mating is more protracted and widely distributed (Joung et al., 2004; Joung & Chen, 1995), and movement of fertilized ova down the reproductive tract may inhibit subsequent fertilization.

Despite extensive descriptive study, little is known about the contribution of mate choice to differences in patterns of genetic polyandry. Theoretically, polyandry should be favored when females reap direct or indirect benefits from multiple matings that outweigh associated costs (reviewed in Jennions & Petrie, 2000). Direct benefits increase female reproductive output, such as insurance against sperm limitation (Wedell et al., 2002), shared territory and parental care (Avise et al., 2002), and nuptial gifts which can be invested in egg production (Sakaluk et al., 2006) or somatic growth (Boggs & Gilbert, 1979). Indirect (genetic) benefits boost fitness by increasing the reproductive success of offspring (reviewed in Zeh & Zeh 2001). These include increased probability of fertilization by high-quality males (Birkhead et al., 1993; Madsen et al., 1992), compensation for poor-quality mates (Hasselquist et al., 1996; Kempenaers et al., 1992), bet-hedging to account for environmental variability and mate choice errors (Watson, 1991; Yasui, 2001), increased additive genetic variation of offspring (Landry et al., 2001), and insurance against genetic incompatibility (Zeh & Zeh, 1996, 1997).

Multiple studies have searched for evidence of direct or indirect benefits to polyandrous elasmobranchs without success (Boomer et al., 2013; Daly-Engel et al., 2010; Feldheim et al., 2004). Elasmobranchs do not provide nuptial gifts, parental care, or maintain pair bonds after copulation, and in most species, the number of eggs produced per reproductive effort is too small for sperm limitation. Thus, direct benefits are unlikely to explain the prevalence of polyandry. A study of *C. plumbeus* found no relationship between female reproductive output and the number of sires and determined that attempts to avoid genetic incompatibility could not sufficiently explain the high number of sires detected (Portnoy et al. 2007). In addition, a study of lemon sharks *Negaprion brevirostris* (Poey 1868) demonstrated that offspring from polyandrous litters did not have higher genetic diversity or survival as compared with offspring from monandrous litters (DiBattista et al., 2008a). In fact, juvenile survival was greater in individuals with more genetically similar parents (DiBattista et al., 2008a), suggesting polyandrous females did not benefit from mating with genetically dissimilar males. Genetic benefits cannot be discounted and require further investigation. Nonetheless, the lack of discernible benefits and apparent costs of mating led to the hypothesis that multiple paternity in some species could be a consequence of convenience polyandry (Portnoy et al., 2007, DiBattista et al, 2008b), whereby females engage in multiple matings because resistance is more costly than copulation (Thornhill & Alcock, 1983). Subsequently, studies have tended to rely on convenience polyandry and perpetuate the notion of aggressive, indiscriminate males copulating with coy and pliant females (Lyons et al., 2021). However, both males and females likely exhibit some form of mate choice. Thus, in combination with methods assessing for genetic polyandry, more sophisticated approaches are necessary to examine factors that influence male and female decisions to engage in copulation.

Molecular approaches that have been used to assess for genetic polyandry can also be used in studies of mate choice by examining if individuals are mating randomly. Non-random mating can be inferred if reproduction has occurred between individuals that are more (assortative mate choice) or less (disassortative mate choice) genetically similar to each other than they are to individuals randomly selected from the population. Relatedness between a pair of individuals can be estimated by inferring the percentage of alleles that descend from the same ancestral gene in the previous generation and are thus identical-by-descent (IBD; Blouin, 2003). Because unrelated individuals may also share alleles that are identical-by-state (IBS), estimating relatedness requires genotyping a representative sample of the population to characterize background genetic variation (Blouin, 2003), and many statistical methods to correct relatedness estimates using population-level allele frequencies have been developed (e.g., Wang, 2007). Also, individuals must be genotyped at a sufficient number of loci to provide adequate resolution for differentiating kin from unrelated individuals (Goudet et al., 2018). Genomic approaches using high-throughput sequencing can facilitate this by enabling researchers to genotype hundreds of individuals at thousands of loci spread throughout the genome.

Assessments of genome-wide relatedness can identify examples of assortative and disassortative mate choice resulting from inbreeding and outbreeding, respectively. However, the vast majority of genotyped loci will have no functional role in offspring viability or mechanisms of mate choice. Therefore, assessing mate choice requires examination of loci fulfilling putatively functional roles in mate discrimination (hereafter putatively functional loci; Galaverni et al., 2016; Richardson et al., 2005; Schwensow et al., 2008). To do this, it is essential to understand the behavioral and physiological processes mediating mate choice before and after copulation – known as pre- and post-copulatory mate choice, respectively.

Considering the diversity among species and limited observations of copulation, the degree to which elasmobranchs can exert pre-copulatory mate choice is unclear. Across most animal mating systems, it is generally assumed that females display a greater level of choice than males because they typically produce fewer and larger gametes, provide more parental investment, and take longer to become receptive to subsequent matings (Bateman, 1948; Hubbell & Johnson, 1987; Trivers, 1972). Therefore, males can more easily allocate investment to multiple matings and generally display greater variance in reproductive success (Collet et al., 2014; Fritzsche & Arnqvist, 2013; Jones et al., 2002; but see Gowaty et al., 2012). The ability of female elasmobranchs to choose mates before copulation likely varies among species due to differences in behavior. Multiple species exhibit sexual dimorphism in teeth (de Sousa Rangel et al., 2016; Kajiura & Tricas, 1996) and skin thickness (Crooks & Waring, 2013; Nakano & Stevens, 2008; Pratt Jr., 1979) that are associated with copulatory biting. Benthic species can ventilate via buccal or spiracular pumping and may exhibit distinct behaviors to obligate ram ventilators. Springer (1967) observed that males of larger carcharhinid species violently harassed females to invoke their cooperation. Because of their sharp teeth and inability to hold females by wrapping their bodies around them, as more flexible species often do (Castro et al., 1988; Dempster & Herald, 1961; Dral, 1980), mating attempts by these males can cause severe injuries. Therefore, for some species, the costs of injuries sustained by females during resistance could outweigh the potential costs of multiple mating and lead to convenience polyandry. Conversely, for other species in which mating produces less trauma, females might incur less damage during resistance and display more pre-copulatory mate choice.

The aggressive mating tactics of male elasmobranchs complicate female pre-copulatory mate choice and are thought to drive avoidance behaviors. Sexual segregation is commonly seen

in adults (Economakis & Lobel, 1998; Mucientes et al., 2009; Sims, 2005) and may enable females to rebuff male copulation attempts. Female scalloped hammerhead sharks *Sphyrna lewini* (Griffith & Smith 1834) form schools and compete for positions close to the center when approached by conspecific males (Klimley, 1987). Further, females can pivot and roll out of bites, shield their cloaca, and arch their body to prevent clasper insertion (Johnson & Nelson, 1978; Pratt Jr. & Carrier, 2001; Tricas et al., 1995). Male elasmobranchs may also display pre-copulatory choice to avoid depletion of sperm and seminal fluid proteins, but instances of copulation with immature females suggest this is limited (Farrell et al., 2010; Storrie et al., 2008). However, male mate choice via strategic sperm allocation cannot be ruled out and should be evaluated because males could vary their investment in ejaculates based on the perceived level of male competition and female quality (Dewsbury, 1982; Pizzari et al., 2003; Wedell et al., 2002).

Female elasmobranchs may use behavioral and olfactory cues to facilitate pre-copulatory mate choice (Pratt Jr. & Carrier, 2005). Johnson & Nelson (1978) first hypothesized the role of olfactory cues in elasmobranch courtship based on accounts of male blacktip reef sharks *Carcharhinus melanopterus* (Quoy & Gaimard 1824) closely following females with their snouts orientated towards the cloaca. Similar behaviors have been observed in other species (Klimley, 1980; Luer & Gilbert, 1985; Tricas, 1980), including myliobatiforms that release fluids to attract males (Chapman et al., 2003; Marshall & Bennett, 2010; Stevens et al., 2018). Elasmobranchs are renowned for their olfactory abilities (Dryer & Graziadei, 1993; Meredith et al., 2013) and secretions from females could serve as olfactory signals to advertise location and receptivity. Additionally, females may communicate a willingness to mate through behavioral cues like body

arching and pelvic fin flaring (Carrier et al., 1994; Clark, 1963; Luer & Gilbert, 1985), which could also increase the dispersal of olfactory signals (Gordon, 1993).

Olfactory signals that might be involved in pre-copulatory mate choice include major histocompatibility complex (MHC) proteins. These proteins are found in all jawed vertebrates, are encoded by highly polymorphic genes, and play a critical role in immune defense (Hedrick, 1994; Klein, 1986). T-cells recognize when peptides are presented by MHC proteins, and because each protein can bind a limited number of peptides, distinct MHC variants (loci and alleles at each locus) increase the range of peptides that can elicit an immune response (Janeway et al., 2001). The complementarity of peptides and specific MHC variants led to the hypothesis that degradation of self-proteins could produce peptides that bind to variants and communicate MHC composition by subsequently binding to olfactory receptors in a similar manner (reviewed in Ruff et al., 2012). The first evidence for olfactory signaling of MHC was documented in mice that were shown to discriminate individuals of different MHC genotypes from urine (Yamaguchi et al., 1981). Additional evidence for olfactory communication of MHC has since been uncovered in other rodents (Bard et al., 2000; Beauchamp et al., 1990; Singh et al., 1987), birds (Grieves et al., 2019; Leclaire et al., 2017), and three-spined sticklebacks *Gasterosteus aculeatus* L. 1758 (Aeschlimann et al., 2003; Reusch et al., 2001). Furthermore, peptides that compliment particular MHC binding regions attach to specific neurons of the mouse vomeronasal organ and produce distinct neurophysiological responses (Leinders-Zufall et al., 2004). This discovery indicated a mechanism by which secreted MHC protein-peptide complexes might enable mice to communicate MHC genotypes to potential mates, and similar mechanisms may be responsible for olfactory detection of MHC in other vertebrates.

Patterns of MHC-associated pre-copulatory mate choice vary widely among jawed vertebrates and instances have been documented in every class except Chondrichthyes (Kamiya et al., 2014). The majority of studies in mammals have focused on mice and follow a disassortative pattern (i.e., preference for MHC-dissimilar individuals; Potts et al., 1991; Roberts & Gosling, 2003; Yamazaki et al., 1976); however, evidence of assortative and disassortative mate choice has been documented in other mammals (Setchell et al., 2011; Sin et al., 2015; Sommer, 2005) as well as birds (Bonneaud et al., 2006; Freeman-Gallant et al., 2003; Juola & Dearborn, 2012). While some reptiles appear to choose mates disassortatively based on MHC (Han et al., 2019; Miller et al., 2009; Olsson et al., 2003), a study of salamanders found evidence of assortative mate choice (Bos et al., 2009). In addition, teleosts show a general pattern of disassortative pre-copulatory mate choice based on MHC (Forsberg et al., 2007; Landry et al., 2001; Neff et al., 2008), but in some cases, choice may be more complex (Milinski et al., 2005).

Assessing for evidence of MHC-associated pre-copulatory mate choice in elasmobranchs requires genotyping individuals at MHC genes to determine if a preference is exerted for MHC-similar or -dissimilar mates. Such assessments require genotyping parents or individuals that have been observed copulating, along with a sample of reproductively mature individuals to characterize MHC allele frequencies in the breeding population. If primers targeting peptide-binding regions of MHC genes can be designed, high-throughput sequencing can facilitate reliable genotyping of many individuals, enabling assessments of similarity between mates at genes putatively involved in mate choice and offspring viability (Dearborn et al., 2016; Grieves et al., 2019; Santos et al., 2018). Assortative or disassortative mate choice can be more robustly determined by examining loci like those encoding MHC because choice might reflect a preference for similar, dissimilar, or specific alleles that may be communicated to potential

mates. At the same time, examining loci not involved in mate choice would enable researchers to determine if variation at MHC is correlated with genome-wide similarity. This would indicate a mechanism to avoid inbreeding by using specific genes as a proxy of overall relatedness.

Preference tests, commonly used to assess pre-copulatory choice by observing behaviors of individuals when presented with different mates, could be conducted using captive elasmobranchs. Water that bathed individuals could be introduced to choice chambers housing females to assess if they prefer odors from unrelated or related males, and vice versa. Replicates of individuals with similar and dissimilar MHC genotypes could also be used to see if males and females choose assortatively or disassortatively based on MHC. The mechanisms facilitating pre-copulatory choice could be further investigated by manipulating sensory systems. Because MHC composition is hypothesized to be communicated via olfactory signals, responses could be compared between control subjects and those in which olfactory senses are blocked. In addition, synthetic peptides that communicate specific MHC variants could be used in preference tests to assess if males and females alter their behavior towards potential mates when presented with manipulated olfactory signals.

In addition to choosing mates before copulation, females can exert choice by skewing paternity after copulation, a phenomenon known as post-copulatory (cryptic) mate choice (Eberhard, 1996; Thornhill, 1983). Post-copulatory choice can reinforce preferences exerted during pre-copulatory choice or compensate for a limited ability to choose mates before copulation. The latter occurs in the internally fertilizing and live-bearing guppy *Poecilia reticulata* (Peters 1859) in which polyandry is common (in part) because of forced copulations by males (Magurran & Seghers, 1994; Pilastro & Bisazza, 1999; Viken et al., 2006). *Poecilia reticulata* females appear to engage in polyandry more readily after mating with close relatives

(Speechley et al., 2019), and may subsequently compensate for limited pre-copulatory choice by biasing fertilization in favor of sperm from unrelated males (Fitzpatrick & Evans, 2014). Several mechanisms enabling post-copulatory mate choice have been documented in vertebrates, such as controlling the number of sperm from different males that remain in the reproductive tract (Pizzari et al., 2004; Suarez & Pacey, 2006). For example, female feral chickens prefer to mate with socially dominant males, but cannot prevent insemination by subdominants, and therefore expel their ejaculates shortly after copulation (Birkhead & Pizzari, 2002; Pizzari & Birkhead, 2000).

Post-copulatory choice can also be exerted via interactions between sperm and the reproductive tract, some of which may be mediated by the immune system. In mammals, variation in oviduct cell responses to different sperm suggests the female reproductive tract can discriminate between sperm (Almiñana et al., 2014). A variety of proteins involved in cellular recognition are expressed on sperm cell membranes, including MHC (Dorus et al., 2010; Hedger, 2007), which could facilitate the recognition and inactivation of sperm from particular males via the binding of female antibodies (Ghaderi et al., 2011). Notably, MHC variants associated with olfactory receptors involved in pre-copulatory choice are also expressed on mammalian sperm and thus might also influence post-copulatory choice (Spehr et al., 2006). Moreover, evidence from birds that sperm from MHC-dissimilar males are favored by post-copulatory mechanisms (Løvlie et al., 2013), potentially via preferential sperm storage (Birkhead & Brillard, 2007; Pizzari et al., 2008), suggests MHC can influence mate choice after copulation. Interactions between sperm and ovarian fluid could also contribute to post-copulatory choice. Gasparini & Pilastro (2011) demonstrated that *P. reticulata* sperm velocity was significantly lower in a solution containing ovarian fluid from a sibling female as compared with that from an unrelated

female. A subsequent study demonstrated that significantly more offspring were sired by males with more similar MHC alleles, but genetic similarity at ten microsatellites was not significantly correlated with fertilization success (Gasparini et al., 2015).

Once sperm have traversed the reproductive tract and reached the egg, post-copulatory choice can occur based on gametic compatibility. An *in vitro* fertilization study of mice found that sperm from non-siblings fertilized more eggs than expected by chance compared to sperm from siblings (Firman & Simmons, 2015). A similar study mixed *G. aculeatus* sperm and eggs expressing a variety of MHC variants, and the resulting offspring exhibited an intermediate level of MHC diversity (Lenz et al., 2018). Both studies suggest oocyte-mediated post-copulatory mate choice, with the latter providing evidence for a mechanism of sperm selection based on MHC (Lenz et al., 2018). Such mechanisms may function to reduce inbreeding and hybridization, as suggested for a hermaphroditic tunicate in which self-fertilization is reduced via self-discriminating interactions between sperm and gene products in the vitelline coat surrounding the oocyte (de Santis & Pinto, 1991).

Even after fertilization, post-copulatory choice may occur via selective abortion and reallocation of maternal investment to more viable offspring (Burley, 1988; Zeh & Zeh, 1997). Evidence for this has been documented in mammals and birds (Cunningham & Russell, 2000; Hull, 1964) and may constitute an additional mechanism by which vertebrates bias paternity after copulation. Intriguingly, multiple studies of elasmobranchs have documented non-developing eggs or deformed embryos alongside healthy embryos (Castro, 2000; Parsons, 1993; Peres & Vooren, 1991), which may indicate selective abortion to reduce energetic investment in offspring sired by genetically incompatible males (Lyons et al., 2021).

While an array of mechanisms enabling post-copulatory mate choice has been described in vertebrates, to date, none have been examined in elasmobranchs, even though many aspects of their reproductive biology suggest the potential for post-copulatory choice (reviewed in Fitzpatrick et al., 2012). Post-copulatory choice predicts that polyandrous litters will be skewed towards preferred males (Eberhard, 1996), and reproductive skew has been documented in multiple elasmobranch species (Boomer et al., 2013; Nosal et al., 2013; Veríssimo et al., 2011). Although abortion is commonly observed, it is usually associated with capture-induced parturition (reviewed in Adams *et al.*, 2018), and only two studies have documented evidence of fetal resorption by elasmobranchs (Brown et al., 2020; Lesniak et al., 2013). Post-copulatory choice via selective abortion and reallocation of maternal resources could be evaluated by comparing aborted and healthy embryos at genes with immune functions, such as MHC. In addition, females of multiple species could exhibit post-copulatory choice by storing sperm in the terminal zone of oviducal glands (Hamlett et al., 1998). Although long-term sperm storage has advantages for highly migratory species with low frequencies of mate contact (Pratt, 1993), its occurrence in species that are not characterized by low contact frequency, such as bonnethead *Sphyrna tiburo* (L. 1758) and brownbanded bamboo sharks *Chiloscyllium punctatum* (Müller & Henle 1838) suggests other adaptive functions are involved (Bernal et al., 2015; Pratt, 1993). Signaling molecules associated with immune responses are expressed in the terminal zone of dusky smoothhound *Mustelus canis* (Mitchill 1815) oviducal glands and could facilitate the removal of sperm expressing particular proteins (Hamlett et al., 2005). Sperm that remain could then be released from storage bundles by secretion of hormones that stimulate ovulation (Hamlett et al., 2002). Activation of quiescent shark sperm has been demonstrated in laboratory experiments using ionic changes in solutions that reflect conditions of the female reproductive

tract (Minamikawa & Morisawa, 1996), suggesting physiological changes could release and activate sperm concurrent with ovulation.

Because pre-copulatory effects must be accounted for, assessing for post-copulatory mate choice in wild elasmobranchs would be very challenging, and carefully designed experiments involving captive animals are likely necessary. One approach would be artificial insemination using related and unrelated pairs, in addition to those with distinct genotypes for putatively functional loci like MHC. This would allow researchers to investigate different mechanisms that may be responsible for post-copulatory choice.

Studies of mate choice can advance understanding of elasmobranch mating systems and how they influence population dynamics. Mate choice studies can also be used to explore the role of molecular signals in social interactions because communication of genes like those encoding MHC may provide a proxy for overall relatedness and facilitate kin recognition. Social behaviors have been documented in multiple species (Guttridge et al., 2011; Heupel & Simpfendorfer, 2005; Jacoby et al., 2012), but correlations between genome-wide relatedness and similarity at specific genes have yet to be explored. There is evidence that mammals (Manning et al., 1992; Yamazaki et al., 2000), amphibians (Villinger & Waldman, 2008), and teleosts (Olsén et al., 1998; Rajakaruna et al., 2006) can discriminate kin from non-kin using MHC, and this may also be possible in elasmobranchs. Discrimination could potentially result in the formation of social groups based on kin, thus olfactory signals used in pre-copulatory mate choice might also help to maximize inclusive fitness (Hamilton, 1964).

Molecular approaches to examine pre- and post-copulatory mate choice in elasmobranchs will become more feasible as high-quality genome assemblies and sequence data for putatively functional genes become available. Examining MHC genes is technically challenging because

they are often multi-copy and have many highly variable alleles. Furthermore, advances in husbandry and artificial insemination have led to the successful captive breeding of elasmobranchs (Daly & Jones, 2017; Luer et al., 2007; Masuda et al., 2003, 2005; Wyffels et al., 2021). Therefore, future studies examining elasmobranch mate choice should endeavor to develop partnerships between academic and aquaria researchers.

Nursery Use

Although elasmobranchs do not provide parental care after delivering offspring, many species have large maternal investments in the form of migration to specific, often distant localities to give birth or release eggs. Habitats that have a greater mean contribution to adult recruitment, as compared to other habitats in which juveniles occur, are described as nurseries (Beck et al., 2001). Consistent with this description, Heupel et al. (2007) developed the following criteria to identify shark nursery habitats: i) juveniles have a higher mean density; ii) juveniles tend to remain in or return for extended periods of time; iii) juveniles use the habitat repeatedly across years. These criteria can also be applied to batoids and oviparous species whose offspring occupy specific habitats after hatching (Martins et al., 2018). However, oviposition can occur in geographically discrete locations (i.e., egg case nurseries; Hoff, 2016), so additional criteria have been developed to identify habitats i) with higher densities of hatched or unhatched egg cases in contact with substrates; ii) used by adults for oviposition repeatedly across years; iii) that newly hatched offspring immediately leave (Martins et al., 2018). It is important to note that variation in life histories among species means the costs and benefits associated with nurseries vary (Heupel et al., 2007; Knip et al., 2010), and the majority of elasmobranchs likely do not use nurseries (Carlson et al., 2008; Driggers III et al., 2008).

Elasmobranch nurseries have been studied for over 100 years and are hypothesized to enhance reproductive success by providing young-of-the-year (YOY) and juveniles with food resources and protection from predators (Castro, 1993; Meek, 1916; Simpfendorfer & Milward, 1993). Many nurseries have been identified in shallow waters of coastal regions, which are often warmer and more productive than adjacent deeper habitats (Beck et al., 2001; Castro, 1987). Nurseries are also typically discrete from habitats with higher densities of larger marine predators, including other elasmobranchs, so they may facilitate reduced predation rates, faster juvenile growth rates, and ultimately increased offspring survival (Branstetter, 1990).

There is some evidence that elasmobranchs benefit from nurseries because they provide refuge from predators. In The Bahamas, *N. brevirostris* give birth in coastal nurseries where offspring remain for up to three years (Gruber, 1988; Morrissey & Gruber, 1993). YOY and juvenile *N. brevirostris* are more abundant in shallow waters close to mangroves and their movement away from shore coincides with low tide (Guttridge et al., 2012). By contrast, adult *N. brevirostris* can occupy more diverse habitats at depths greater than 50 meters, and large juveniles move into nearshore areas during high tide (Cortés & Gruber, 1990; Guttridge et al., 2012). Cannibalism is common among elasmobranchs (Compagno et al., 2005), thus mangrove inlets are thought to shelter young *N. brevirostris* from predation by larger conspecifics and other species, contributing to a relatively low first-year mortality rate (44-61%; Gruber et al., 2001; Guttridge et al., 2012; Manire & Gruber, 1993). YOY and juvenile bull sharks *Carcharhinus leucas* (Valenciennes 1839) also use nurseries, including estuaries and rivers with salinities of 7-20 (Heupel & Simpfendorfer, 2008). These conditions are less tolerable for more stenohaline sharks and appear to result in low rates of juvenile mortality (approximately 23% in their first 18 months; Heupel & Simpfendorfer, 2011).

It should be noted that putative nurseries do not always facilitate low juvenile mortality. Competition for food and low caloric consumption were attributed to the poor energetic condition and growth of YOY *S. lewini* occupying Kāne'ohe Bay, Hawaii (Bush & Holland, 2002; Duncan & Holland, 2006). A large proportion of these individuals are thought to die from starvation within their first year (Lowe, 2002), suggesting that this area may simply be a parturition site rather than a nursery. Similarly, the presence of juvenile blacktip sharks *Carcharhinus limbatus* (Valenciennes 1839) in Terra Ceia Bay, a nursery in the eastern Gulf of Mexico, was not found to be correlated with food availability, and an estimated 61-91% of individuals experience natural mortality in their first six months (Heupel & Simpfendorfer, 2002). Therefore, predator avoidance may be a more important driver of nursery use than prey abundance (Heupel & Simpfendorfer, 2005; Heupel & Hueter, 2002), and additional studies are necessary to determine the benefits afforded by nurseries.

Among elasmobranch species for which nurseries have been identified, use varies in terms of duration and frequency. In tropical waters, temperatures do not substantially change with seasons, so suitable habitat is available year-round and juveniles may remain in nurseries for several years (Gruber, 1988; Simpfendorfer & Milward, 1993). By contrast, in subtropical and temperate regions, water temperatures fluctuate seasonally and encourage migration between summer nurseries and wintering grounds in deeper waters offshore or warmer waters at lower latitudes (Castro, 1993; Castro, 1996). For example, YOY *C. limbatus* born in Terra Ceia Bay in spring remain until they emigrate in the fall (Heupel, 2007; Heupel et al., 2004). The following spring, many return to the vicinity of the habitat in which they were born (hereafter natal site) and are thought to repeat this seasonal migration for the first three years of life (Heupel & Hueter, 2001; Hueter et al., 2005). Similar patterns of nursery use have been documented in *C.*

plumbeus born in Chesapeake Bay, Virginia, where YOY and juveniles migrate south before winter and return the following summer (Grubbs et al., 2007). As they age, however, frequencies of return migrations vary with sex. After the first six years, males stop returning and occupy offshore areas further south, whereas females continue to return for at least ten years, which may help to establish migration routes that they will later use to locate nurseries for parturition (Grubbs, 2010).

The tendency to return to specific localities, known as site fidelity (Speed et al., 2011), has been documented in over 30 elasmobranch species across 19 families and ten orders (reviewed in Chapman et al., 2015; Flowers et al., 2016). Detecting site fidelity is more challenging in deeper and pelagic habitats as compared with shallow bays and estuaries, thus additional studies are necessary to determine the diversity of species displaying this behavior and the types of habitat being used. Individuals returning to their natal site, as described above for juvenile *C. limbatus* and *C. plumbeus*, is known as natal site fidelity (Chapman et al., 2015). Many studies have also demonstrated that females of multiple species faithfully return to the same site to give birth, known as parturition site fidelity (Chapman et al., 2015; DiBattista et al., 2008b; Mourier & Planes, 2013). Natal and parturition site fidelity are distinct because the former refers to the return of individuals to the site in which they were born, whereas the latter describes the repeated use of a site by a female for parturition that is not necessarily her natal site. Interestingly, parturition site fidelity may extend across generations and result in females returning to their natal site, or neighboring sites in their natal region, to give birth.

Philopatry, derived from the Greek and Latin terms *philos* and *patria*, meaning "beloved" and "homeland", respectively, describes fidelity to a locality encompassing an individual's origin for reproductive purposes (Mayr, 1963; Pearce, 2007). Philopatry is therefore a form of fidelity,

but fidelity only constitutes philopatry if individuals are returning to or residing in their natal site or natal region for reproduction. Many elasmobranchs are highly mobile and capable of traveling hundreds or thousands of kilometers (Kohler & Turner, 2019), thus individuals may leave their natal region and return to reproduce. Females of multiple species are thought to return to or remain in their natal region for parturition, known as regional philopatry (e.g., Keeney et al., 2005; Pardini et al., 2001). Furthermore, some individuals may repeatedly return to their exact natal site to give birth, known as natal philopatry (Feldheim et al., 2014). Natal philopatry should not be confused with natal site fidelity because the latter does not involve the use of a site for reproduction (Table 2.2). For most elasmobranchs, it appears that females tend to be more philopatric while males disperse farther (Chapman et al., 2015; but see Blower et al., 2012). This phenomenon, known as sex-biased dispersal, has been documented in sharks and batoids (Phillips et al., 2017; Portnoy et al., 2010; Roycroft et al., 2019), is thought to be a consequence of discrepancies in maternal and paternal energetic investment in offspring (Perrin & Mazalov, 2000), and has important ramifications for genetic structure and population persistence (Portnoy et al., 2015).

Inferring site fidelity and philopatry in elasmobranchs has traditionally relied on tagging and telemetry studies that document individuals residing in or returning to specific localities (Edrén & Gruber, 2005; Heupel et al., 2004; Heupel & Hueter, 2001). In the recent past, however, philopatry has become an important component of population genetics studies because it has the potential to generate predictable patterns of genetic structure within and among populations (Heist, 2005; Hueter et al., 2005). While tags can provide real-time behavioral data, they may fall off or not report, and deploying sufficient replicates can be expensive (Arnold & Dewar, 2001; Donaldson et al., 2008; Musyl et al., 2011). Molecular methods can supplement

these approaches by studying more individuals over longer periods and in larger geographic areas. One molecular approach to infer female regional philopatry is to assess variation in genetic markers with different modes of inheritance. Mitochondrial DNA (mtDNA) is only inherited from mothers, in contrast with nuclear DNA (nDNA; e.g., microsatellites) which is inherited from both parents (Avise, 1994). If genetic differentiation in mtDNA among regions is larger and distinct in pattern from differentiation in nDNA, it may indicate sex-biased dispersal in which females are remaining in or returning to their natal region to reproduce (regional philopatry), while males are dispersing among regions (male-mediated gene flow). This mixed-marker analysis method has been used to infer regional philopatry in multiple elasmobranch species (Day et al., 2019; Karl et al., 2011; Tillett et al., 2012). It is important to note, however, that lineage sorting occurs at a rate approximately four times faster in mtDNA, as compared with nDNA, because mtDNA exists as one copy and is inherited only from the mother, whereas nDNA exists as two copies and is inherited biparentally (Birky Jr et al., 1983). Thus, differences in patterns of population structure between mtDNA and nDNA may not necessarily indicate sex-biased dispersal.

Another molecular approach to infer philopatry and site fidelity relies on developing individual genetic tags. Genotyping individuals at many polymorphic loci can provide sufficient resolution for genetic tags because there will be a negligible probability that different individuals have the same composite genotype across many loci (O'Reilly & Wright, 1995). Genetic tags work in a similar way to traditional physical tags, allowing the tracking and detection of individuals by resampling over time. Genetic tags can also be used to estimate relatedness between individuals, enabling identification of parents and offspring, full- and half-siblings, and with enough loci, potentially other relationships too. Genetic tags and kinship analysis have

revealed evidence of parturition site fidelity in *N. brevirostris*, *C. melanopterus*, and smalltooth sawfish *Pristis pectinata* (Latham 1794) by identifying females that have returned to the same sites to give birth (Feldheim et al., 2004, 2017; Mourier & Planes, 2013). Furthermore, genetic tags were used to uncover the first conclusive example of natal philopatry in elasmobranchs. Based on a composite genotype match at 11 polymorphic microsatellites, a gravid female *N. brevirostris* caught in Bimini, The Bahamas in 2008 was determined to have been previously captured in the same nursery as a two-year-old in 1995 (Feldheim et al., 2014). Although she was not encountered as a YOY, six of her siblings identified using kinship analysis were also captured in the same nursery in 1995, indicating this was her natal site. Further, in 2008, a YOY individual was caught less than 4 km from where the gravid female was caught, and kinship analysis revealed this to be her offspring (Feldheim et al., 2014).

Kinship analyses based on composite genotypes can be a reliable method to examine site fidelity and philopatry, but there are caveats to consider. Intensive sampling is often required to catch kin, which may not be tractable in wide-ranging populations that do not use discrete habitats. It is also vital to determine the nature of kin relationships once they are detected. Identifying YOY in the same natal site or region that their mother inhabited as YOY is strong evidence of philopatry, but inferring parturition site fidelity may not be as straightforward. Polyandry can lead to the detection of half-siblings in the same natal site within and across years. Because females deliver offspring to natal sites and appear more philopatric than males, half-siblings caught in the same site are often assumed to be maternally related. However, half-siblings could be paternally related and subsequent inferences of parturition site fidelity would not be fully supported. Although it is more likely that half-siblings share a mother than a father, additional analyses – such as comparing mitochondrial haplotypes – could be used to support

observations of parturition site fidelity based on the detection of half-siblings in the same natal site across years.

High-throughput sequencing can enhance studies of site fidelity and philopatry by enabling the simultaneous genotyping of large numbers of individuals at thousands of loci, thereby efficiently generating data with greater power to differentiate kin relationships. This is accomplished by identifying loci containing single nucleotide polymorphisms (SNPs) that are found throughout genomes in coding and non-coding regions (Allendorf et al., 2010; Luikart et al., 2003). SNP-based genotypes have been used to detect kin and recaptures in the speartooth shark *Glyphis glyphis* (Müller & Henle 1839) and northern river shark *Glyphis garricki* (Compagno, White & Last 2008), providing evidence of natal and parturition site fidelity at small spatial scales (i.e., a few hundred kilometers; Feutry et al., 2017, 2020). One genomic approach to produce SNP-based genotypes involves restriction enzymes that cut DNA at specific motifs and generate reproducible, reduced genomic datasets known as RAD libraries (Davey & Blaxter, 2010; Peterson et al., 2012). RAD libraries are generally limited by the number of individuals that can be included because they prioritize the genotyping of many thousands of loci at high coverage. However, reliable kin identification can be accomplished with several hundred loci, particularly if SNPs are phased into constituent microhaplotypes to produce polymorphic loci (Baetscher et al., 2018; Willis et al., 2017). Alternative techniques such as genotyping-in-thousands by sequencing (GT-Seq; Campbell et al., 2015) focus on genotyping many thousands of individuals at hundreds of highly informative loci, and are more suitable for identifying kin and recaptures within sites and among sites within regions. GT-Seq would therefore be highly applicable for long-term studies of site fidelity and philopatry, particularly in benthic and pelagic elasmobranchs that are more difficult to capture and track.

How elasmobranchs navigate to nurseries is unclear, but studies of other vertebrates have provided clues. Salmonids and sea turtles can detect variation in angle and intensity of Earth's magnetic field and develop magnetic maps which are used to navigate across large distances (Lohmann et al., 2007; Quinn, 1980; Quinn & Brannon, 1982). Magnetic maps are acquired by sea turtles and salmonids early in life through a process known as geomagnetic imprinting (Lohmann et al., 2004; Putman et al., 2014). In salmonids and tuna, detecting variation in Earth's magnetic field may be accomplished via iron-based magnetite crystals in ethmoid tissue that transduce magnetic stimuli to the nervous system (Diebel et al., 2000; Walker et al., 1984; Walker et al., 1997). While it is unclear if elasmobranchs possess magnetite, they have a highly sensitive electrosensory system (Kajiura, 2001; Kajiura & Holland, 2002) and respond to magnetic changes (Kalmijn, 1974, 1978; Meyer et al., 2005; Newton & Kajiura, 2017). Detection of magnetic variation does not equate to the ability to use magnetic maps for navigation, but the direction and intensity of electrical current induced by movement through Earth's magnetic field could enable spatial orientation and travel along a bearing (Molteno & Kennedy, 2009; Newton & Kajiura, 2020; Paulin, 1995). In fact, behavioral trials conducted on *S. tiburo*, which migrate hundreds of kilometers and display site fidelity to specific estuaries (Driggers et al., 2014), demonstrated that individuals exposed to magnetic conditions resembling a location 600 km south of their capture site responded by orientating their movements northward (Keller et al., 2021). By contrast, individuals showed no orientation after exposure to magnetic conditions resembling their capture site nor a location 600 km north. These results suggest *S. tiburo* can extract spatial information from geomagnetic cues and provide evidence of orientation using magnetic maps that could facilitate site fidelity (Keller et al., 2021).

Magnetic maps might enable navigation to the vicinity of natal sites, but some salmonid species display fine-scale philopatry – returning from ocean gyres to specific stream branches – which requires greater precision than that conferred by variation in Earth's magnetic field (Quinn et al., 2006, 2012). Olfactory cues have been shown to guide salmonids upstream from the mouth of natal rivers to their exact spawning beds (Døving et al., 1985; Hasler & Scholz, 1983; Johnsen & Hasler, 1980). Experiments have also provided strong evidence that olfactory imprinting on the natal environment occurs during parr-smolt transformation and that biological molecules can act as olfactory cues during return migrations to locate natal sites where breeding occurs (Dittman et al., 1996; Nevitt & Dittman, 1998; Scholz et al., 1976; Yamamoto et al., 2010). Like salmonids, YOY of many coastal elasmobranchs reside in their natal site after parturition and olfactory imprinting could occur during this period. Gardiner et al. (2015) demonstrated that olfactory cues allow young *C. limbatus* to locate their nursery after displacement. Because the animals in this study were estimated to be less than three weeks old (Gardiner et al., 2015), if olfactory imprinting is occurring, it likely happens very early in life.

Omics tools have also allowed researchers to conduct preliminary investigations of the mechanisms facilitating philopatric behavior in salmonids. Transcriptomics – the study of RNA transcripts expressed by specific tissues – has been used to quantify gene expression in brain tissue of rainbow trout *Oncorhynchus mykiss* (Walbaum 1792) exposed to magnetic impulses. One study documented differential expression of a gene encoding a subunit of ferritin, a protein involved in intracellular iron storage (Fitak et al., 2017). The study also indicated that ferritin might play a role in magnetoreception by producing or repairing magnetite-based magnetoreceptors (Fitak et al., 2017), but additional research is necessary to support this. Similar experiments could be performed on elasmobranchs to examine gene expression in subjects

exposed to magnetic pulses to assess for evidence of magnetite-based magnetoreception. Transcriptomics may also be used to assess for differential expression of genes putatively associated with olfactory imprinting. A study of Atlantic salmon *Salmo salar* L. 1758 targeted a suite of olfactory genes and showed that nine were differentially expressed between two groups of the same age, in which one group had undergone parr-smolt transformation and the other had not (Madsen et al., 2019). Five olfactory genes were up-regulated in the post-transformation group, suggesting olfactory function is enhanced, perhaps indicating imprinting (Madsen et al., 2019). Studies using elasmobranchs born in captivity or caught in the wild shortly after birth could also assess for olfactory imprinting. Individuals could be stimulated with olfactory cues and placed in choice chambers to assess for positive chemotaxis, as compared with control subjects that were not stimulated. If evidence of olfactory imprinting is found, transcriptomics could be used to quantify differences in gene expression between stimulated and control subjects in subsequent experiments.

Nurseries are considered essential fish habitats for elasmobranchs because they are required to complete the lifecycle and are fundamental for survival, but philopatry may mean that some nurseries are more essential than others. If nurseries of highly philopatric species are fragmented or removed, it could lead to considerable declines in recruitment to adult populations unless other suitable habitats are used instead. Even if nurseries are subsequently restored, recovery of populations would be dependent on re-colonization by straying individuals, which may take a very long time (Hueter et al., 2005). Furthermore, because suitable habitat may be limited, determining the degree and scale of fidelity (e.g., none, parturition site fidelity, natal philopatry) and the navigational mechanisms enabling these behaviors are crucial for conservation. Identifying fidelity to nurseries will likely be more difficult in species that do not

use discrete nearshore habitats, thus genomic approaches may be particularly useful in elucidating how extensive nursery use is.

Philopatry to nurseries can influence genetic population structure and this is important to consider when delineating stocks that are subject to harvest. Regional and natal philopatry have the potential to reduce gene flow across a species' range and lead to distinct stocks at smaller spatial scales than expected based on the dispersal potential of the species (Harden Jones, 1968). Population dynamics of distinct stocks are largely determined by local demographics, rather than immigration and emigration, and therefore, applying the same fishing pressure across different stocks could lead to localized depletion and collapse (Cortés, 2004). Moreover, if nurseries increase survivorship at early life stages and are environmentally heterogeneous, the repeated use of specific nurseries across generations could result in local adaptation (Portnoy et al., 2015). It is therefore vital to evaluate the relative contribution of nurseries to adult recruitment and determine if particular habitats are sources of adaptive variation because this may facilitate persistence following environmental changes (Bowen & Roman, 2005).

Conclusions

Advances in molecular techniques and DNA sequencing have revolutionized studies of vertebrate reproduction. While a variety of approaches have helped to reveal the diversity of elasmobranch reproductive biology, omics have the potential to considerably enhance understanding of how reproductive strategies evolved and are impacting contemporary populations. Many of the approaches outlined in this review are currently feasible, and others will become more feasible as the abundance and diversity of elasmobranch genomic resources increase. Genome assemblies for species that complement those already assembled are needed to fill "taxonomic gaps" so an array of genomic resources can be made available. This will facilitate

studies of elasmobranch reproductive biology using high-throughput sequencing, but many of the approaches reviewed herein will also require collaborations across a range of disciplines. Interdisciplinary partnerships among researchers with expertise in elasmobranch biology and conservation should therefore be encouraged. Given the economic, ecological, and evolutionary value of cartilaginous fishes, the opportunities for future study are broad and exciting.

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Table 2.1. The number of vertebrate species with assembled genomes (NCBI, 2021).

Taxa	Number of Species with Assembled Genomes
Bony Fishes	626
Birds	515
Mammals	456
Reptiles	65
Amphibians	21
Chondrichthyans	10

Table 2.2. Descriptions of the degrees of site fidelity displayed by elasmobranchs.

	Description
None	No repeated use of a site
Natal site fidelity	Remaining in or returning to a site of birth
Parturition site fidelity	Repeated use of a site to give birth
Regional philopatry	Remaining in or returning to sites within a birth region for reproductive purposes
Natal philopatry	Remaining in or returning to a birth site to give birth

CHAPTER III: ASSESSING THE POTENTIAL FOR MHC-ASSOCIATED MATE CHOICE IN THE BLACKTIP SHARK (*CARCHARHINUS LIMBATUS*)

Abstract

Females generally exhibit a greater level of mate choice than males because they typically invest more energy per individual offspring. Females of many elasmobranch species can suffer injuries during mating and display traits to exert pre-copulatory choice. However, genetic polyandry is prevalent, suggesting females receive benefits that compensate for the apparent costs. While polyandry is unlikely to benefit females directly, genetic benefits are possible and may be accrued via post-copulatory choice, but evidence for this is lacking. In a variety of vertebrates, mate choice is influenced by the major histocompatibility complex (MHC), a cluster of highly variable genes encoding proteins with immune functions. MHC-based mate choice can confer genetic benefits by facilitating inbreeding avoidance and providing offspring with allelic combinations that optimize immunity. However, the potential for MHC to influence mate choice in elasmobranchs has not been assessed. Therefore, evidence of MHC-associated mate choice in the blacktip shark (*Carcharhinus limbatus*) was assessed by genotyping six mothers, each of their offspring, and adults at four microsatellites and two MHC genes (*mhc1a* and *b2m*). Microsatellite and *b2m* genotypes were used to estimate the minimum number of sires per litter and assess for inbreeding/outbreeding. Paternal *mhc1a* alleles in each litter were inferred using genotypes of mothers and offspring, and three metrics of similarity for observed maternal-paternal allele combinations were calculated. For each litter, simulations were used to compare the observed value for each metric to the distribution of values expected under random mating. For four litters, each similarity metric was greater than the upper confidence limit determined by simulations, indicating females successfully reproduced with males carrying

mhc1a alleles more similar to their own than expected based on random mating. Though the results require validation, they provide a basis for future studies of the genetic benefits and mechanisms facilitating mate choice in elasmobranchs.

Introduction

Mate choice involves the preferential allocation of reproductive investment to members of the opposite sex (Edward, 2015). Because members of both sexes have finite resources to invest (Dewsbury, 1982), a degree of choice is expected to be exerted by both sexes to maximize fitness. However, differences exist between the sexes in terms of the size and number of gametes produced per reproductive effort and across a lifetime (i.e., anisogamy). Males produce many small gametes and can typically engage in more matings, and thus display greater variation in reproductive success (Bateman, 1948). Females, by contrast, tend to produce fewer and larger gametes, typically contribute more to post-copulatory investments (e.g., embryonic nourishment and parental care), and can take longer to become receptive to additional matings (Hubbell & Johnson, 1987; Schärer et al., 2012; Trivers, 1972). Consequently, while sex-specific reproductive investment can vary dramatically among species, females are generally considered to invest more in individual offspring and therefore exert a greater level of mate choice (Janicke et al., 2016).

Mate choice might be particularly important for females of many elasmobranch species (e.g., sharks) because mating can be costly (Pratt Jr. & Carrier, 2005). Males use their jaws to bite and hold females while inserting copulatory organs (claspers) with sharp hooks and spurs, often resulting in external and internal injuries (Carrier et al., 2004; Pratt Jr. & Carrier, 2001; Springer, 1967). Studies have also documented multiple males biting and holding a single female while attempting to copulate, which could exacerbate injuries (Carrier et al., 1994; Chapman et

al., 2003; Whitney et al., 2004). Nevertheless, the evolution of behaviors to facilitate avoidance (Gordon, 1993; Klimley, 1987; Tricas, 1980) and morphologies such as thicker skin and denser dermal denticles around the areas that males bite (Crooks & Waring, 2013; Pratt Jr., 1979) enable females to thwart some unwanted mating attempts. These traits are the result of sexually antagonistic co-evolution – a series of evolutionary changes that results when there is conflict between the sexes over the ideal mating rate (Lessells, 2006) – and indicate that females can exert some pre-copulatory choice. However, molecular approaches over the last two decades have revealed that genetic polyandry (i.e., multiple sires for a single litter) is ubiquitous among elasmobranchs (reviewed in Lamarca et al. 2020), suggesting females may accrue benefits from multiple matings that outweigh the perceived costs. Direct benefits increase reproductive output (e.g., insurance against infertile males) whereas genetic benefits increase reproductive success (fitness) by providing offspring with "good" and/or compatible alleles (Andersson, 1994; Jennions & Petrie, 2000; Neff & Pitcher, 2005). Thus, direct benefits are gained at fertilization but genetic benefits can continue after and may be under strong selection when pre-copulatory choice is limited (Lindsay et al., 2019).

Several studies have assessed for direct/genetic benefits of polyandrous mating in elasmobranchs but none have found evidence (Boomer et al., 2013; Daly-Engel et al., 2010; DiBattista et al., 2008; Feldheim et al., 2004). One study (Portnoy et al., 2007) proposed that the lack of discernible benefits could indicate that polyandry occurs when female resistance to mating is more costly than acceptance (i.e., convenience polyandry; Thornhill & Alcock, 1983). Subsequently, convenience polyandry has been invoked repeatedly to explain observations of multiply sired litters in a variety of species (e.g., Barker et al., 2019; Byrne & Avise, 2012; Griffiths et al., 2012), inadvertently shifting the focus away from potential benefits and

mechanisms of female choice (Lyons et al., 2021). Most elasmobranchs produce too few eggs to experience sperm limitation so direct benefits are unlikely; however, genetic benefits are plausible and require further study. Many elasmobranchs store sperm in oviducal glands (where fertilization occurs; Hamlett et al., 2005; Pratt, 1993) and display extensive variation in sperm morphology (Jamieson, 2005; Rowley et al., 2019), indicating paternity may be influenced by post-copulatory processes (e.g., sperm competition and cryptic female choice; Fitzpatrick et al., 2012). Indeed, if females can weaken pre-copulatory choice, they might accrue genetic benefits by increasing sperm competition and exerting post-copulatory choice (Lindsay et al., 2019).

The major histocompatibility complex (MHC) is a cluster of highly polymorphic genes found only in jawed vertebrates that has been shown to be important in mate choice in many species (Kamiya et al., 2014; Kaufman, 2016; Tregenza & Wedell, 2000). Though the mechanisms remain unclear (Ziegler et al., 2010), an individual's MHC composition can be communicated to prospective mates via olfactory signals (Leinders-Zufall et al., 2004; Yamaguchi et al., 1981) and expression on gametes (Fernandez et al., 1999; Ziegler et al., 2005). Thus, MHC has the potential to influence both pre- and post-copulatory choice (Lenz et al., 2018; Strandh et al., 2012). The diversity of MHC (>200 alleles per locus in humans; Janeway et al., 2001) means that unrelated individuals are unlikely to share alleles. Therefore, mate choice based on MHC can mediate genetic benefits by reducing the deleterious effects of inbreeding (Brown & Eklund, 1994). Furthermore, MHC genes encode cell-surface glycoproteins that comprise a vital component of the adaptive immune system (Klein, 1986). As a result, individuals may exert choice for mates with MHC alleles that complement their own and gain genetic benefits by providing offspring with optimal immune function (Milinski, 2006). The genetic benefits of mate choice for MHC have been studied in a variety of vertebrate species

(Bos et al., 2009; Landry et al., 2001; Olsson et al., 2003; Potts et al., 1991); however, the potential for MHC to influence elasmobranch mate choice has not been assessed.

Although MHC has been studied in elasmobranchs for decades (Bartl & Weissman, 1994; Kasahara et al., 1992; Ohta et al., 2000), genotyping large numbers of individuals is a significant challenge due to multiple gene copies and many highly variable alleles (Babik, 2010). One to two copies of the classical MHC class I gene (*mhc1a*) are present in elasmobranchs (Simona Bartl & Nonaka, 2014). The first two exons encode the peptide-binding domain (Okamura et al., 1997; Wu et al., 2021), the most variable region of the protein that presents cell-derived peptides and has been implicated in mate choice (Milinski, 2006; Ruff et al., 2012). The protein is stabilized by a non-covalent subunit encoded by a single copy gene (beta-2-microglobulin; *b2m*) that is less variable than *mhc1a* (Bjorkman et al., 1987). Notably, the structure and function of the MHC class I protein are highly conserved among jawed vertebrates (Ohta et al., 2002; Okamura et al., 1997; Wu et al., 2021). Therefore, assessments of *mhc1a* similarity between mothers and sires might provide insight into elasmobranch mate choice. However, accurate genotyping is essential and requires a lot of sequence data to discriminate among putative alleles, pseudogenes, and artifacts. Some of the first studies of genetic polyandry in elasmobranchs genotyped mothers and offspring at MHC genes by cloning and sequencing bacterial colonies (Ohta et al., 2000; Saville et al., 2002), a labor-intensive procedure not suitable for large-scale studies (Babik, 2010). By contrast, high-throughput sequencing provides a more efficient approach that allows hundreds of individuals to be genotyped at high coverage, facilitating a reliable and reproducible characterization of MHC diversity (Lighten et al., 2014a).

Therefore, the potential for MHC-associated mate choice was assessed for the blacktip shark (*Carcharhinus limbatus*) by genotyping individuals at the second exon of *mhc1a* using

high-throughput sequencing. Tissue samples were collected opportunistically from gravid females, their offspring, and adults, and their genotypes used to infer patterns of mating. All individuals were initially genotyped at four neutral microsatellite loci and *b2m* to estimate the minimum number of sires per litter and assess for inbreeding/outbreeding. Next, the paternal *mhc1a* alleles were inferred for each litter, and three similarity metrics for observed maternal-paternal allele combinations were calculated. Simulations were then used to assess for evidence of MHC-associated mate choice by comparing the observed metrics of *mhc1a* similarity to those expected under random mating.

Methods

Fin clips were collected from six gravid female blacktip sharks and their offspring following incidental mortalities that occurred during sampling efforts. Fin clips were also collected from 114 male and female blacktip sharks that were observed or estimated to be adult based on length-at-age and maturity data (Carlson et al., 2006). Fin clips were immersed in 20% DMSO-0.25M EDTA NaCl-saturated buffer (DMSO; Seutin et al., 1991) or ethanol, in which case they were transferred into DMSO, and all samples were stored at room temperature until DNA extraction. All sharks were sampled in the eastern Gulf of Mexico from 2012 to 2020 and were considered to constitute a single population (Keeney et al., 2005; Swift et al., 2022). High molecular weight genomic DNA was extracted from fin clips using Mag-Bind® Blood and Tissue DNA Kits (Omega Bio-Tek) or the phenol-chloroform protocol (Patrinos et al., 2017).

Four microsatellite loci were amplified for each individual using GoTaq PCR Master Mix (Promega) and pairs of primers designed by Keeney & Heist (2003). The 5’ end of each forward primer was tailed with an 18-base pair (bp) M13 sequence (TGTAAAACGACGGCCAGT; Applied Biosystems™) that complements the sequence of fluorescent dyes (i.e., 6-FAM, VIC,

PET; ThermoFisher Scientific), enabling the binding of dyes used to estimate fragment size. Each locus was amplified and dye attached using polymerase chain reactions (PCR) in a total volume of 10 µl consisting of 1 µl genomic DNA, 1x green buffer, 0.1% tween, 1.5 mM magnesium chloride ($MgCl_2$), 0.2 mM dNTPs, 0.3 µM fluorescent dye, 0.1 µM forward primer, 0.3 µM reverse primer, and 0.5 units of *Taq* polymerase. Amplification started with two minutes at 94 °C and ended with 1 minute at 72 °C, between which there were 35 cycles at 94 °C for 15 seconds, 58-64 °C for 15 seconds (Table 3.1), and 72 °C for 30 seconds. To confirm amplification, 5 µl of each PCR product was mixed with 2 µl of gel red and electrophoresed through 1% TAE-agarose gels, then visualized using ultraviolet light. Samples that showed amplification were cleaned with AMPure XP beads (Beckham Coulter) and 70% ethanol and eluted in 10 µl molecular grade water. Cleaned products were diluted 1:5 with water and fragment sizes assessed by electrophoresing through an ABI 3730xl DNA Analyzer using a fluorescently labeled size standard (GeneScan™ 600 LIZ™).

For each microsatellite locus, alleles sizes were estimated by eye using Peak Scanner Software (v2.0 Applied Biosystems). To minimize the effects of allele dropout, each individual scored as a homozygote was re-amplified and assessed a second time. Each mother and offspring were assessed twice using independent rounds of PCR, regardless of the initial score. If the first two allele scores for any individual were inconsistent, the individual was re-amplified and assessed again until the same score was observed at least twice. Raw allele lengths were binned into allele size classes using an automated approach implemented in TANDEM (Matschiner & Salzburger, 2009), and any samples with allele lengths that differed from the motif were removed and re-assessed. Evidence of scoring error due to stuttering, large allele dropout, and null alleles was evaluated using MICRO-CHECKER (van Oosterhout et al., 2004). Finally,

conformance to the expectations of Hardy-Weinberg and linkage equilibrium were assessed using GENEPOP (Raymond & Rousset, 1995; Rousset, 2008).

Primers amplifying MHC genes of the blacktip shark or closely related species were not available prior to this study. Thus, primers were designed for the second exons of *b2m* and *mhc1a* by aligning available shark sequences and finding conserved regions. For *b2m*, sequences for the sandbar shark (*Carcharhinus plumbeus* (Carcharhinidae; Carcharhiniformes); accession number: GQ865622), banded houndshark (*Triakis scyllium* (Triakidae; Carcharhiniformes); HQ634972), and nurse shark (*Ginglymostoma cirratum* (Ginglymostomatidae; Orectolobiformes); GQ865623) were acquired from GenBank (Sayers et al., 2022). For *mhc1a*, sequences for the banded houndshark were downloaded from GenBank (AF034316) and compared to transcriptome sequences of five carcharhinid sharks that were inferred to have functional roles associated with MHC class I (Swift et al., 2016). To ensure that primers amplified the correct loci in a sufficient sample of the study population, amplified products were sequenced using Sanger sequencing and compared to those downloaded from GenBank. Primers were re-designed and PCR conditions optimized until the same fragments were consistently amplified in each blacktip shark.

Forward and reverse primers for each MHC gene were tailed with universal 5' TruSeqHT sequences complimenting iTru primers so that each amplicon (i.e., amplified gene-specific sequences for an individual) could be uniquely indexed, enabling the pooling and sequencing of all amplicons in a single library (Glenn et al., 2019). An initial round of PCR with gene-specific primers was used to amplify and tag the fragments of interest. The second round of PCR was used to incorporate specific index sequences using iTru5 and iTru7 primers, resulting in amplicons with unique combinations of i5 and i7 indices. Both rounds of PCR used GoTaq PCR

Master Mix. The first PCR was run in a total volume of 25 µl with 1 µl genomic DNA, 1x green buffer, 0.1% tween, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µM forward primer, 0.4 µM reverse primer, and 1 unit of *Taq* polymerase. The first PCR began with two minutes at 95 °C, ended with 2 minutes at 72 °C, between which there were 30 cycles at 95 °C for 45 seconds, 56 °C for 45 seconds, and 72 °C for 2 minutes. PCR products were stored at 4 °C and amplification success was assessed using electrophoresis. All samples were then cleaned and eluted in 20 µl of water. The second PCR used all products remaining after electrophoresis in a total volume of 40 µl, with 1x green buffer, 0.1% tween, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.4 µM iTru5 and iTru7 primer, and 1.5 units of *Taq* polymerase. The second PCR began with two minutes at 98 °C and ended with 5 minutes at 72 °C, between which there were 20 cycles at 98 °C for 30 seconds, 56 °C for 30 seconds, and 72 °C for 1 minute. PCR products were stored at 4 °C and subsequently electrophoresed through 1% TAE-agarose gels to confirm successful amplification of larger fragments as compared with the first PCR, indicating the incorporation of index sequences. All samples were then cleaned, eluted in 20 µl of water, and the concentration of each sample was quantified using AccuBlue High Sensitivity dsDNA Quantitation Kit (Biotium). To minimize variation in sequencing coverage among amplicons, DNA yields were standardized and pooled. Two libraries were prepared, and both were shipped to the Field Museum of Natural History on dry ice (-80 °C) where they were sequenced on a MiSeq System (Illumina). The first library (paired-end 250 bp) was used to generate sequence data to genotype *b2m* and assess levels of coverage required to genotype *mhc1a*. A second library (paired-end 300 bp) was subsequently prepared to genotype *mhc1a* and replicates for *b2m*.

Because poor sequence quality can result in inaccurate genotyping, raw reads were assessed for quality using FASTQC (Andrews, 2010) and trimmed to remove bases with a Phred

score < 30 using TRIMMOMATIC (Bolger et al., 2014). Sequences were then demultiplexed into amplicons, forward and reverse reads merged, and coverage (read depth) for each unique sequence variant within an individual quantified using AMPLISAS (Sebastian et al., 2016). Putative alleles were discriminated from artifacts generated during PCR and sequencing following the degree-of-change method (Lighten et al., 2014a). Briefly, this method assumes that real alleles amplify at considerably greater sequencing depths than artifacts and that sequences of sufficient quality show an inflection in the relative depth of variants within an individual that can be used to identify putative alleles (Lighten et al., 2014a). Variants were initially filtered to remove those contributing to <1% of reads within an individual and all remaining variants were aligned using CLUSTAL OMEGA (Sievers et al., 2011). Alignments were imported into BIOEDIT (Hall, 1999), inspected by eye, and nucleotide similarity matrices generated so that additional artifacts (i.e., poorly aligned sequences) could be flagged and removed. Among the remaining sequence variants, putative alleles were identified by manually inspecting the relative depth of variants within and among individuals. For each gene, putative alleles were aligned with the sequences used to design primers so that intron and exon sequences could be identified. To assess for coding differences at *mhc1a*, 5' and 3' ends were trimmed so that the alignment was in the correct reading frame and started at the first base of the second exon. The alignment was also inspected to ensure it did not contain stop codons which could indicate pseudogenes.

For each litter, the minimum number of sires was assessed using the microsatellite and *b2m* genotypes of mothers and offspring. Genotypes were initially inspected to ensure that all offspring shared at least one allele at each marker with their mother. The minimum number of sires per litter was first estimated by counting the number of paternal alleles for each gene. The minimum number of sires was further assessed for each litter by reconstructing paternal

genotypes using allele frequencies for the breeding population and genotypes of mothers and offspring, as implemented in GERUD (v2.0; Jones, 2005) and COLONY (v2.0.6.8; Jones & Wang, 2010). COLONY was executed with the following parameters: male and female polygamy, without inbreeding and cloning, dioecious and diploid, full-likelihood with very high likelihood precision, and very long run length.

To assess for evidence of inbreeding/outbreeding, the mean parental similarity was estimated for each litter by calculating the internal relatedness (IR) of each offspring using their genotypes and population allele frequencies for microsatellites and *b2m*, as implemented in an Excel Macro produced by Amos et al. (2001). Internal relatedness uses population allele frequencies to measure parental similarity and is centered around zero, with highly negative and positive values indicating relative outbreeding and inbreeding, respectively (Amos et al., 2001). In contrast to *mhc1a, b2m* is not expected to influence mate choice and was therefore included in assessments of inbreeding/outbreeding.

To assess for evidence of non-random mating associated with *mhc1a*, three metrics of similarity were calculated for each litter using observed maternal-paternal allele combinations. For each individual in a litter, an allele was assigned to be paternal even if it was also present in the mother (i.e., the offspring was homozygous for the maternal allele). The first two metrics – nucleotide and amino acid similarity (%) – were calculated using BIOEDIT. The third metric – functional similarity – is based on five categories that summarize physicochemical properties of amino acids: hydrophobicity (z_1), steric bulk (z_2), polarity (z_3), and electronic effects (z_4 and z_5; Sandberg et al., 1998). To calculate functional similarity, every amino acid was assigned a score for each of the five categories; each amino acid in a maternal allele was then compared to the corresponding amino acid in a paternal allele, and the differences in score were summed across

all five categories to define a distance. Finally, the summed distances were averaged across all amino acids for all allele combinations to produce a metric of functional dissimilarity (d_f), which was then converted into a functional similarity score ($s_f = (100-d_f)/100$). For each similarity metric, the mean value for a given litter was compared to a distribution of 10,000 simulated mean values produced by combining each observed maternal allele with *n* alleles drawn using population allele frequencies, where *n* is the number of paternal alleles observed in a given litter. Evidence of non-random mating was assessed for each litter by comparing the observed value to the distribution of expected values for each metric. All figures were generated in R using *ggplot2* (Wickham, 2016).

Results

Four litters consisted of six offspring and the other two litters had four and five offspring. All mothers and offspring and 69-86 adults were genotyped at each microsatellite locus (Table 3.1). When microsatellite genotypes were assessed for all individuals, there was no evidence of null alleles, large allele dropout, or scoring errors due to stuttering. After excluding genotypes of offspring, there was no evidence of deviation from Hardy-Weinberg or linkage equilibrium. Seven to 35 alleles were observed per microsatellite locus (Table 3.1). All offspring had at least one maternal allele and the number of paternal alleles ranged from one to five per locus per litter.

Forward and reverse reads for *b2m* replicates that were sequenced in the second library were trimmed so that all reads for this locus were 250 bp in length. In total, 48,791 unique *b2m* variants were identified among 92,129 reads, with 1-21,547 reads and 1-14,207 unique variants per individual. However, after removing variants with <1% relative depth within an individual, only 101 remained. An additional 17 variants were removed based on alignment inspection and nucleotide similarity (< 90%). After manually inspecting the remaining 84 variants for relative

depth within and among individuals, five were identified as putative alleles; each was present in 13-55 individuals (allele frequencies: 0.07-0.30), with minimum and maximum relative depths of 2.78 and 50.00% within an individual, respectively. The five alleles differed by one to four single nucleotide polymorphisms (SNPs) and only one allele coded for a distinct amino acid sequence, differing at a single base position. All mothers and offspring and 69 adults were genotyped at *b2m* (depth: 22-2740 reads). All offspring shared at least one allele with their mother and each litter contained two to four paternal alleles.

The reverse reads and the last 55 bp of forward reads for *mhc1a* had poor quality (Phred score < 30). Thus, the reverse reads were discarded and forward reads cropped to 245 bp. More than 700,000 unique *mhc1a* variants were identified among 4,683,147 reads, with 5,430-66,645 reads and 2,599-10,591 unique variants per individual. Only 268 variants remained after removing those with <1% relative depth within an individual; 18 more were removed based on nucleotide similarity (< 75%) after alignment inspection. The remaining 250 variants were manually inspected for relative depth within and among individuals, and 89 were identified as putative alleles. The minimum and maximum relative depths were 2.67% and 70.81% within an individual. After trimming the 5' and 3' ends, *mhc1a* alleles were 207 bp in length, contained no stop codons, and encoded 69 amino acids; 85 alleles (95.5%) coded for unique amino acid sequences. All mothers and offspring and 97 adults were genotyped at *mhc1a* (depth: 2028-39,146 reads). Each individual had one to four alleles (Figure 3.1), indicating the presence of at least two copies of this gene. In total, 239 alleles were observed across 136 individuals (mean: 1.76). The most common allele was present in 21 individuals (allele frequency: 0.088) and 44 alleles were present in only one individual (allele frequency: 0.0042). Notably, seven offspring (21.2%) across three litters did not share an allele with their mother, suggesting null alleles were

present. The number of paternal alleles per litter ranged from three to eight; 59 alleles found among adults were not present in the offspring and four alleles present in offspring were not found in adults.

Except for one litter (three), GERUD and paternal allele counts produced consistent estimates of the minimum number of sires, with each litter having at least two or three sires (Table 3.2). However, COLONY produced estimates that were inflated by one to two sires per litter (Table 3.2); full-sibling inclusive (0.06-1; mean: 0.80) and exclusive (0.04-0.92; mean: 0.43) probabilities were highly variable, indicating difficulty in distinguishing between full- and half-siblings. Mean IR by litter ranged from -0.083 to 0.15 (Table 3.3), suggesting offspring were not produced by inbreeding or outbreeding. For the first two litters (six offspring each), the observed values for each similarity metric fell within the distribution produced by simulations (Figure 3.2), suggesting random mating with respective to *mhc1a*. By contrast, for the other litters (four to six offspring each), the observed values for each metric were greater than 97.5% of simulated values (Figures 3.3 and 3.4), indicating females successfully reproduced with males displaying *mhc1a* alleles that were more similar (on average) to their own than would be expected under random mating.

Discussion

Combinations of *mhc1a* maternal-paternal alleles observed in four of six litters are more similar than would be expected under random mating. Further, assessments of parental similarity using microsatellites and *b2m* indicate the inferred pattern of non-random mating is not a result of inbreeding. Therefore, there is evidence that blacktip sharks have greater reproductive success with mates carrying similar *mhc1a* alleles. While the results should be interpreted with caution

due to limitations such as null alleles, the study provides a foundation for future research on the genetic benefits and mechanisms mediating MHC-associated mate choice in elasmobranchs.

Genetic polyandry was observed for each of the six litters examined. Estimates of the minimum number of sires using GERUD and paternal alleles counts were mostly consistent, but COLONY produced estimates that were greater by one or two sires. Though genetic polyandry is prevalent among elasmobranchs, the rate observed here (100%) has been documented in just four other species when more than two litters were examined (Barker et al., 2019; Chevolot et al., 2007; Green et al., 2017; Heist et al., 2011). The high rate of genetic polyandry detected in this study was likely facilitated by the high diversity of three microsatellites (> 20 alleles each). By contrast, lower rates (55-71%) were observed in another study that examined genetic polyandry in blacktip sharks from the western Indian Ocean using 14 litters and less variable microsatellites that were developed for other species (Bester-van der Merwe et al., 2019). Across elasmobranchs, there are many examples of intraspecific variation in rates of genetic polyandry (Byrne & Avise, 2012; Chabot & Haggin, 2014; Lage et al., 2008; Veríssimo et al., 2011), but the disparity observed for blacktip sharks could also reflect differences in the molecular markers used.

Five alleles with low nucleotide and amino acid diversities were shown for *b2m*, in contrast to *mhc1a*, for which more than 80 alleles encoding unique amino acid sequences were observed. Twenty blacktip sharks (15%) were found to have three or four *mhc1a* alleles, indicating copy number variation (CNV) resulting from gene duplication and deletion events (Sebat et al., 2004). Many MHC genes have experienced multiple duplication/deletion events (Kulski et al., 2002) and extensive CNV may occur within and among populations (Siddle et al., 2010; Traherne, 2008). Because of individual fitness benefits, an intermediate number of MHC

alleles is expected to be observed within populations (Nowak et al., 1992; Woelfing et al., 2009), as seen in Lighten et al. (2014b). By contrast, 40% of individuals in this study displayed just one *mhc1a* allele. This observation, in addition to the lack of a maternal allele for seven offspring, suggests null alleles are present. Therefore, the results likely underestimate *mhc1a* diversity and further study is needed to describe *mhc1a* CNV and allelic diversity in blacktip sharks. Nonetheless, the observed gene diversities are fairly consistent with other studies of MHC in elasmobranchs. A single *b2m* locus was observed for sandbar and nurse sharks, with the former species displaying two alleles differing by two SNPs (Chen et al., 2010; Ohta et al., 2011). Also, 29 *mhc1a* alleles encoding unique amino acid sequences were found in 22 banded houndsharks, with 16 and six individuals displaying two and three alleles, respectively (Okamura et al., 1997).

Evidence of assortative mate choice for *mhc1a* was found in four litters (67%) each consisting of four to six offspring. For two litters (six offspring each), the observed value for each similarity metric was lower than the mean simulated value but fell within the 95% confidence interval (Figure 3.2). For the other litters, by contrast, the observed value of each metric was greater than the upper confidence limit calculated using simulations (97.5%; Figures 3.3 and 3.4). Thus, four litters showed evidence of significant deviation from expectations of random mating in favor of mates with more similar alleles. Furthermore, the preference for similarity was consistent across nucleotide, amino acid, and functional metrics, but this may not always be the case. The degenerate nature of the genetic code means that changes in nucleotide sequence can result in the same amino acid sequence (i.e., synonymous mutations; Kimura, 1968). Conversely, nonsynonymous mutations (usually at the first or third codon base) code for different amino acids (e.g., missense mutations) or premature stop codons (i.e., nonsense mutations) and can drastically alter protein structure (Kimura, 1980). However, some missense

mutations do not lead to appreciable differences in protein function if alternative amino acids have similar physicochemical properties (Kimura, 1968; Stone & Sidow, 2005). Therefore, when evaluating evidence of MHC-associated mate choice, studies should focus on functional differences between alleles – rather than differences in nucleotide and amino acid sequence – because functional properties determine the range of peptides that can be bound to the protein.

Studies of polyandrous teleosts provide insight into the genetic benefits that may mediate MHC-assortative mate choice in blacktip sharks. Significant correlations in siring success and MHC similarity were demonstrated for the guppy (*Poecilia reticulata*; Gasparini et al., 2015) and Atlantic salmon (*Salmo salar*; Yeates et al., 2009). Populations of these species are highly genetically structured (Barson et al., 2009; Garant et al., 2000) and capable of hybridization (Garcia-Vazquez et al., 2001; Russell et al., 2006), so preference for mates with similar MHC alleles could confer genetic benefits by preserving co-adapted allele complexes (Hendry et al., 2000; Yeates et al., 2009). Assortative mate choice may be particularly beneficial for Atlantic salmon because this species displays philopatry and adaptation on a localized scale (Garcia de Leaniz et al., 2007), and hybridization/outbreeding might reduce fitness associated with pathogen resistance in natal environments (Yeates et al., 2009). Similar benefits could mediate assortative mate choice in the blacktip shark. Hybridization has been demonstrated between multiple elasmobranch species (Barker et al., 2019; Cruz et al., 2015; Marino et al., 2015), including two species of blacktip shark (i.e., *Carcharhinus tilstoni* and *C. limbatus*; Morgan et al., 2012). Moreover, the population of blacktip sharks studied here displays fidelity to nurseries used for parturition and fine-scale genetic structure indicative of local adaptation (Swift et al., 2022). Therefore, preference for males with similar MHC alleles could reduce

hybridization/outbreeding and provide genetic benefits via the maintenance of locally-adapted allele complexes.

Studies examining MHC-associated choice in elasmobranchs have broad implications for vertebrate mating systems. Evidence of mate choice mediated by MHC has been documented in every group of jawed vertebrates except elasmobranchs (Kamiya et al., 2014), but it is unclear which mechanisms of choice are common to these taxa. Though they fertilize internally and externally (respectively), ovarian fluids of the guppy and Atlantic salmon influence siring success by impacting sperm motility and velocity (Gasparini & Pilastro, 2011; Yeates et al., 2013). Because siring success for both species is also influenced by MHC similarity, it appears that ovarian fluid can differentially affect sperm based on MHC expression in species with highly distinct reproductive modes. Elasmobranch mate choice could be influenced by female reproductive fluid as well, potentially by increasing the motility of sperm stored in oviducal glands concurrent with ovulation. These species are a vital group for examining the evolution of mate choice because they constitute the lineage (Chondrichthyes) sister to all other jawed vertebrates (Osteichthyes). Therefore, future research examining how mate choice is mediated by elasmobranchs could help to determine the array of mechanisms used by vertebrates.

This research provides a basis for further study but caveats should be addressed to corroborate the results. First, primers amplifying *mhc1a* must be re-designed to reduce the prevalence of null alleles. Genotypes were called using 76% of the second exon (273 bp) because the reverse reads and 3’ ends of forward reads had poor quality, so revised primers should aim to generate overlapping sequences of higher quality that cover the entire exon. Primers that anneal with greater efficacy will also allow for amplification of sufficient DNA using a lower number of PCR cycles, reducing the incidence of artifacts (Lenz & Becker, 2008). To further mitigate

artifacts, amplicon data for replicate individuals should be generated using separate PCR and sequencing runs (e.g., Million & Lively, 2022; Smallbone et al., 2021). This will enable genotype validation by assessing sequence similarity of replicates within and among libraries, which will increase precision by verifying putative alleles using independent amplicons. Together, these improvements will enhance the reliability of the mate choice assessment and help to describe MHC allelic diversity and CNV within the population. However, the influence of MHC on mate choice versus differential embryo survival cannot be disentangled using samples collected in the wild. This would require artificial insemination of captive elasmobranchs and an assessment of embryonic mortality while controlling for post-copulatory effects (e.g., sperm competition). Therefore, collaborations between researchers and aquaria provide exciting opportunities for future studies of mate choice.

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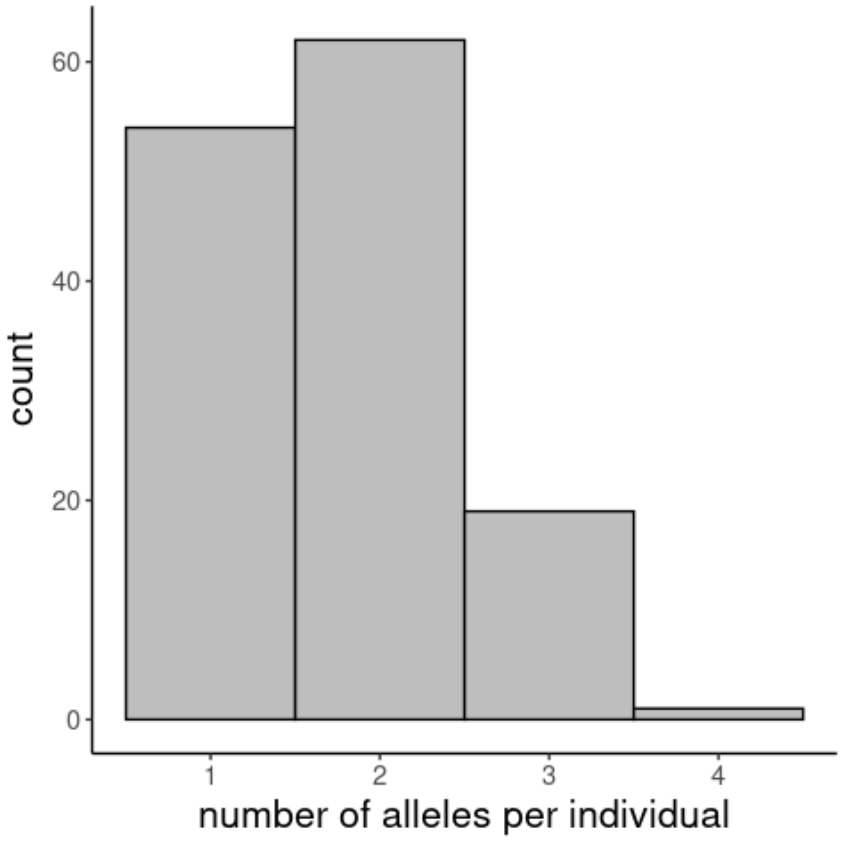

Figure 3.1. The number of *mhc1a* alleles observed per blacktip shark.

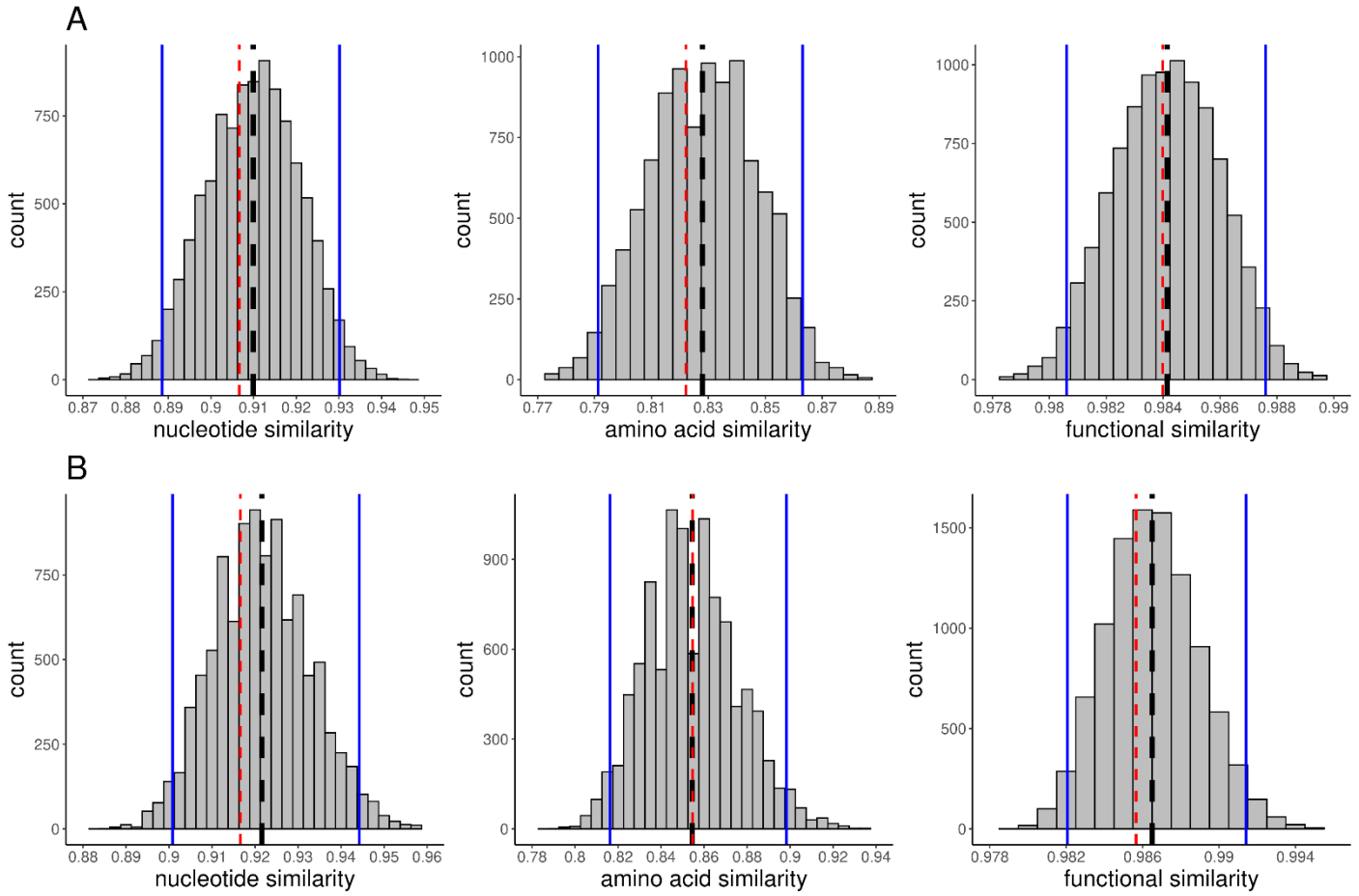

Figure 3.2. Distributions of expected values of *mhc1a* similarity for maternal-paternal allele combinations calculated using 10,000 simulations. Mean expected values are denoted by black dotted lines and confidence limits (2.5% and 97.5%) are denoted by solid blue lines. The observed values for each metric are denoted by dotted red line and fall within the confidence intervals, suggesting random mating. A) Litter 1. B) Litter 2.

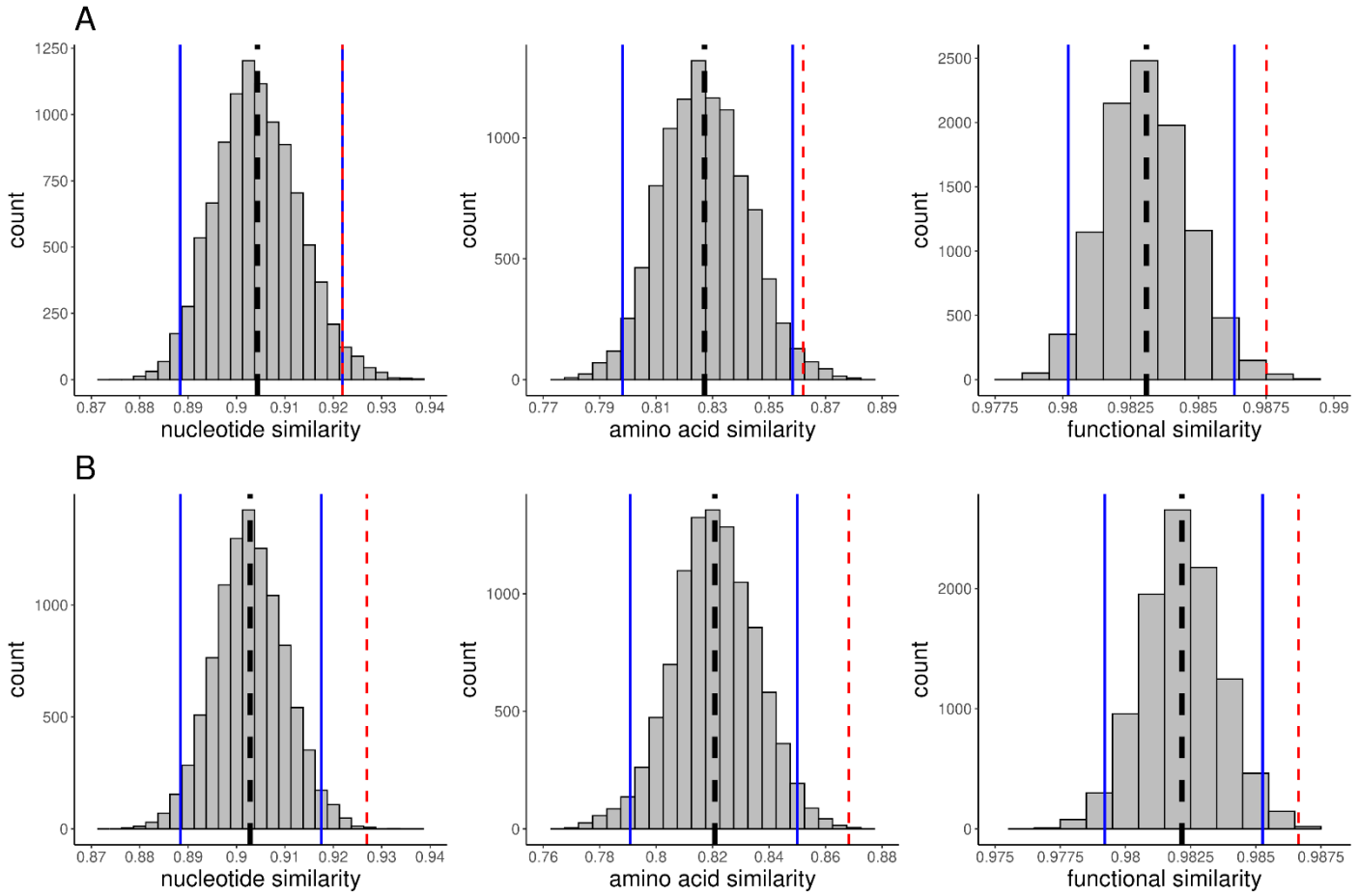

Figure 3.3. Distributions of expected values of *mhc1a* similarity for maternal-paternal allele combinations calculated using 10,000 simulations. Mean expected values are denoted by black dotted lines and confidence limits (2.5% and 97.5%) are denoted by solid blue lines. Observed values for each metric are denoted by dotted red line and fall outside of the upper confidence limit, suggesting non-random and assortative mating. A) Litter 3. B) Litter 4.

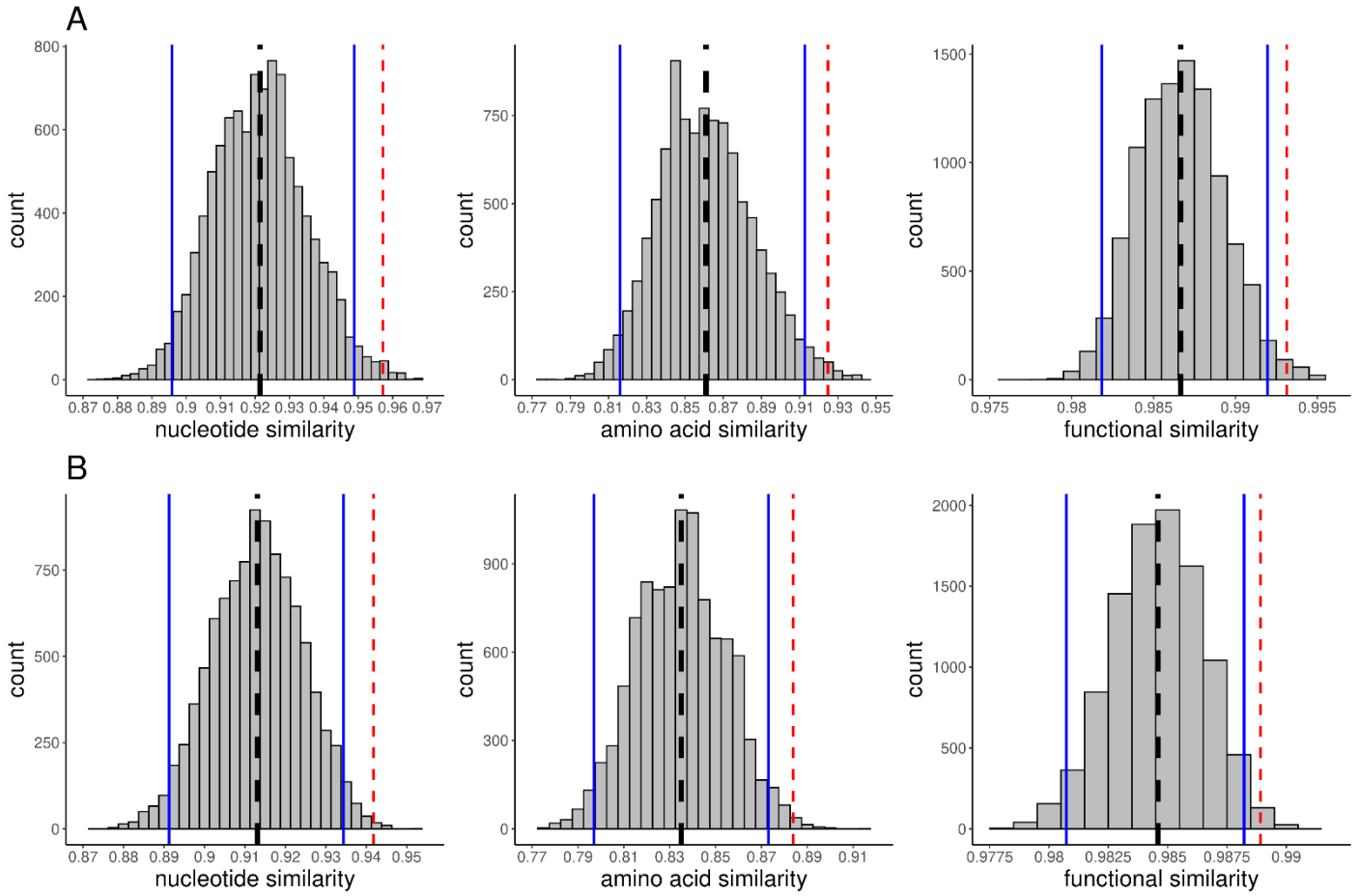

Figure 3.4. Distributions of expected values of *mhc1a* similarity for maternal-paternal allele combinations calculated using 10,000 simulations. Mean expected values are denoted by black dotted lines and confidence limits (2.5% and 97.5%) are denoted by solid blue lines. Observed values for each metric are denoted by dotted red line and fall within the confidence intervals, suggesting non-random and assortative mating. Litter 5. B) Litter 6.

Table 3.1. Annealing temperatures, allele size ranges, numbers of samples genotyped, and numbers of alleles observed for four blacktip shark (*Carcharhinus limbatus*) microsatellites.

Microsatellite	Annealing Temperature (°C)	Allele Size Range (bp)	Samples Genotyped	Alleles Observed
cli-7	64	211-283	115	35
cli-111	60	120-176	108	25
cli-108	58	152-166	125	7
cli-13	62	282-338	111	22

Table 3.2. Numbers of offspring and minimum numbers of sires estimated for each litter using three methods: paternal allele count, GERUD, and COLONY.

Litter	Number of Offspring	Minimum Number of Sires Estimated		
		Paternal Allele Count	GERUD	COLONY
1	6	3	3	4
2	6	2	2	4
3	6	2	3	4
4	6	3	3	5
5	4	2	2	3
6	5	2	2	3

Table 3.3. Mean internal relatedness value for each litter.

Litter	Mean Internal Relatedness Value
1	-0.083499293
2	0.124130769
3	-0.032156639
4	-0.070490738
5	0.149870304
6	0.076180632

CHAPTER IV: PHILOPATRY INFLUENCES THE GENETIC POPULATION STRUCTURE OF THE BLACKTIP SHARK (*CARCHARHINUS LIMBATUS*) AT MULTIPLE SPATIAL SCALES

This chapter was submitted to *Molecular Ecology*.

Abstract

Understanding how the interactions of microevolutionary forces generate genetic population structure of exploited marine species is vital to the implementation of management strategies that facilitate persistence in changing environments. Philopatry displayed by many coastal shark species can impact gene flow and selection and has direct implications for the spatial scales of management plans. Here, a reduced representation genomic approach was used to assess the genetic structure of the blacktip shark (*Carcharhinus limbatus*) in United States waters of the Atlantic Ocean (Atlantic) and Gulf of Mexico (Gulf). More than 400 young-of-the-year from 11 geographic samples were genotyped at 4,368 SNP-containing loci. F_{ST}-outlier and environmental association methods identified 70 loci putatively under selection, enabling separate assessments of neutral and adaptive structure. Neutral structure revealed three genetically distinct units in the Atlantic, eastern Gulf, and western Gulf that align with regional fishing stocks. Heterogeneity at loci putatively under selection associated with temperature and salinity was observed among samples within each Gulf unit, and structured individuals by latitude, suggesting local adaptation. Multiple pairs of siblings were identified in the same habitat across timescales corresponding with female reproductive cycles, indicating that females re-use habitats for parturition, which has the potential to facilitate the sorting of adaptive variation among neighboring habitats. The results demonstrate the differential impacts of microevolutionary forces at varying spatial scales and highlight the importance of conserving

essential habitats to maintain sources of adaptive variation that can buffer species against the effects of climate change.

Introduction

Genetic population structure is determined by differences in the distribution of alleles among contemporary populations that result from the interactions of microevolutionary forces (Laikre et al., 2005). Because genetic drift and gene flow influence allele frequencies on a genome-wide scale, selectively neutral loci exhibit patterns of variation that can be used to understand historical and contemporaneous demographic processes (Luikart et al., 2003). By contrast, selection acts upon variation at specific genes and/or genomic regions and often produces patterns of genetic structure distinct from those observed at neutral loci (Gagnaire et al., 2015; Nielsen, 2001). Disentangling these patterns is especially informative for the management of exploited species. While neutral structure can inform the designation of management units (Waples et al., 2008), loci under selection can be used to identify patterns of local adaptation across heterogeneous environments found within management units (Nielsen et al., 2009). Furthermore, as the adaptive potential of individual populations can facilitate the persistence of species confronted with environmental change, understanding levels of gene flow among and within units is also critical (Bowen & Roman, 2005; Garant et al., 2007).

Examining the interplay of microevolutionary forces is challenging in marine systems because they are open and more difficult to study than many terrestrial systems. Marine environments feature fewer physical barriers to gene flow and barriers that exist are often cryptic (Grummer et al., 2019; Palumbi, 1994). In addition, marine species typically exhibit weak population structure that is difficult to detect (Waples, 1998) due to high fecundity, the potential for long-distance dispersal (via adults and/or larvae), and large effective population sizes that

reduce the magnitude of genetic drift (Poulsen et al., 2006). However, large population sizes and high fecundities both provide more opportunities for mutation and increase the efficacy of selection relative to drift (Allendorf et al., 2010; Cormack et al., 1990). Further, many species have broad geographic ranges and are distributed across heterogeneous environments, increasing the potential for local adaptation (Bernatchez, 2016). Therefore, selection acting upon a small number of loci with a wide range of effect sizes can lead to fine-scale adaptive structure while neutral processes produce weaker, genome-wide structure across broader geographic scales (Gagnaire & Gaggiotti, 2016; Hoey & Pinsky, 2018).

Life history characteristics of elasmobranchs (i.e., sharks, skates, and rays) have an important role in shaping observed patterns of genetic structure. In contrast to many bony fishes and marine invertebrates, elasmobranchs mature late, have long life spans, and produce relatively few offspring within and across reproductive efforts (Conrath & Musick, 2012). Frequently, this leads to smaller effective sizes that are more coupled to census sizes (Portnoy et al., 2009). Though elasmobranchs lack a dispersive larval stage, they retain the potential for high levels of gene flow because they can move vast distances during juvenile and adult life stages (Kohler & Turner, 2019). However, females of many species display fidelity to specific habitats where they give birth or deposit eggs (Chapman et al., 2015; Flowers et al., 2016). This behavior can extend across generations and result in females delivering offspring in their region of birth (i.e., regional philopatry; Pardini et al., 2001) and even the same habitat in which they were born (i.e., natal philopatry; Feldheim et al., 2014).

Philopatry by females is common among coastal shark species that give birth in bays and estuaries where offspring may remain for extended periods (Heupel et al., 2007; Karl et al., 2011; Keeney et al., 2005). Female regional philopatry has the potential to limit gene flow

mediated by females compared with males, and evidence for this has been documented in multiple species based on discrepancies in maternally- and biparentally-inherited DNA (reviewed in Phillips et al., 2021). Because coastal sharks are heavily exploited around the world (Dulvy et al., 2017), understanding how philopatry influences neutral genetic structure by impacting gene flow is vital for delineating management units that will promote persistence. Furthermore, parturition sites are environmentally heterogeneous (Bethea et al., 2015; Matich et al., 2017) and newborn sharks are subject to higher rates of mortality relative to other life stages (Heupel & Simpfendorfer, 2002; Lowe, 2002; Manire & Gruber, 1993). Therefore, natal philopatry could drive selection for locally adapted phenotypes and lead to fine-scale adaptive structure (Portnoy et al., 2015; Portnoy & Heist, 2012). This scenario would have further implications for management because parturition sites harboring novel adaptive variants may require individually tailored policies.

The blacktip shark (*Carcharhinus limbatus*) is a coastal shark species with a circumglobal distribution in tropical and warm temperate latitudes that is harvested for meat, fins, and liver oil (Compagno et al., 2005; Rigby et al., 2021). In U.S. waters, blacktip sharks are found throughout the Gulf of Mexico and along the Atlantic coast from Florida to Massachusetts, where they are targeted by commercial and recreational fisheries (Castro, 1996; SEDAR, 2018, 2020). Males and females mature after five and six years (respectively) and females produce one to eight pups (an average of four) every two years (Baremore & Passerotti, 2013; Natanson et al., 2019). Blacktip sharks are highly migratory and females move into bays and estuaries in the spring/early summer to give birth (Castro, 1996; Hueter & Tyminski, 2007). Young-of-the-year (YOY) remain in parturition sites until the fall of their birth year and migrate south and/or offshore when water temperatures decrease (Castro, 1996; Heupel, 2007; Heupel et al., 2004),

with many returning to the vicinity of their parturition site the following spring (Hueter et al., 2005).

Based in part on population genetics studies, NOAA Fisheries currently manages blacktip sharks as two stocks in the Atlantic and Gulf, but the Gulf stock is split into two subregions, with the dividing line through Mobile Bay, Alabama (SEDAR, 2018, 2020). An assessment of genetic structure using YOY sampled in parturition sites from Texas, Florida (Gulf coast), and Georgia/South Carolina found significant differences with mitochondrial DNA but not with eight nuclear-encoded microsatellites, suggesting female regional philopatry (Keeney et al., 2005). However, the discordance between nuclear and mitochondrial data could also be due to limited resolution (i.e., too few loci) or insufficient time to accrue differences (Whitlock & McCauley, 1999). Because it is vital to accurately characterize blacktip population structure and adaptive potential to inform appropriate management and avoid loss of genetic variation resulting from localized depletion, a reassessment of population structure is warranted.

Therefore, the genetic structure of blacktip sharks in U.S. waters of the Atlantic Ocean and Gulf of Mexico was examined using a reduced representation genomic approach. The sampling design targeted YOY within or just outside parturition sites during their spring-fall residency to ensure that structure reflected differences among reproductive units. By examining thousands of loci spread throughout the genome, a higher resolution assessment of genetic structure at nuclear-encoded loci is possible, and the data can be used to identify siblings captured in the same habitats across years, a pattern indicative of parturition site fidelity by females. Moreover, by screening for loci putatively under selection, the approach facilitates an assessment of the influence of genetic drift, gene flow, and selection in structuring genomic

variation, providing a means to identify habitats harboring adaptive variation that may be vital for the species' persistence.

Methods

Fin clips were collected from 519 blacktip sharks captured within or near 11 estuaries (geographic samples) off the Atlantic coast of the United States (hereafter Atlantic) and throughout the northern Gulf of Mexico (hereafter Gulf). The three geographic samples in the Atlantic were along the coast of South Carolina. In the Gulf, there were three samples along the west coast of Florida, one on the coast of Alabama, and four along the coast of Texas. Mobile Bay, AL straddles the 88th meridian which separates the eastern and western blacktip shark Gulf stock subregions (NMFS, 2006) – due to its proximity to samples from Florida, this sample was considered part of the eastern Gulf region (Figure 4.1).

Fin clips were immersed in 20% DMSO-0.25M EDTA NaCl-saturated buffer (DMSO, Seutin et al., 1991), or ethanol and then transferred into DMSO, and stored at room temperature until DNA extraction. All sharks were captured between March and November 2012-2019. Sex, pre-caudal, fork, total, and/or stretch total lengths, and location of capture (latitude and longitude) were recorded for each individual. If one or more length measurements were not recorded, a customized R script (v3.6.0; R Development Core Team, 2008) was used to assign missing values based on published relationships among length measurements (Carlson et al., 2006). Approximately 47% of sampled individuals were observed as YOY based on the presence of an umbilical scar (Castro, 1993) and the rest were estimated as YOY using fork length (< 593 mm) if sampled in the Atlantic (Ulrich et al., 2007) or total length (< 800 mm) if sampled in the Gulf (Parsons & Hoffmayer, 2007). Based on observations that YOY blacktip sharks in the Atlantic and Gulf remain in or near their parturition site into the autumn months of their first

year of life (Castro, 1996; Heupel et al., 2004), all individuals were assumed to have been sampled in their parturition site.

High molecular weight genomic DNA was extracted from fin clips using either Mag-Bind® Blood and Tissue DNA Kits (Omega Bio-Tek) or phenol-chloroform extraction (Patrinos et al., 2017). A modified version of double digest restriction-site associated DNA sequencing (ddRAD; Peterson et al., 2012) was used to prepare genomic libraries containing individuals spread among geographic samples and sequenced across 11 lanes on an Illumina HiSeq 4000 system (paired-end 150 bp). A separate library consisting of 27 individuals sampled across Atlantic and Gulf locations was prepared using the same protocol and sequenced on a single Illumina MiSeq lane (paired-end 300 bp). Raw sequences were demultiplexed using *process_radtags* (Catchen et al., 2011) and quality trimmed. MiSeq reads were assembled into a reference of contiguous sequence alignments (i.e., contigs) representing putatively single-copy (orthologous) loci using the DDOCENT pipeline (v2.8.7; Puritz et al., 2014) to map and improve genotyping efficiency of HiSeq data. Read mapping and single nucleotide polymorphism (SNP) calling were performed for HiSeq reads from each library using the DDOCENT pipeline.

Raw SNPs were filtered using VCFTOOLS (v0.1.14; Danecek et al., 2011) and R functions in a customized workflow, following practices laid out in O'Leary et al. (2018). Retained loci had a minimum mean depth of 18 and were called in at least 90% of individuals, 50% of a given library, and 80% of individuals in a given sample. Loci were also filtered for allele balance, mapping quality, the ratio of reference vs. alternate allele, consistency of scoring in forward and reverse directions, proper pairing, depth/quality ratio, and excess heterozygosity to remove potential paralogs and other technical artifacts. Individuals with > 20% missing data or excessively low F_{IS} (i.e., less than the first quartile minus 1.5x interquartile range) were

removed because very low F_{IS} is indicative of cross-contamination. Haplotypes were generated by collapsing SNPs on the same contig using *rad_haplotyper* to produce a dataset of multi-allelic SNP-containing loci (hereafter loci; Willis et al., 2017).

Technical replicates were included within and across multiple libraries and their composite genotypes compared to characterize locus-specific genotyping errors. Replicates were confirmed by assessing relatedness between each pair of individuals using the dyadic likelihood estimator (Milligan, 2003) executed in *related* (Pew et al., 2015). Loci with systematic genotyping error and one individual from each replicate pair were removed, along with monomorphic loci. To minimize genotype inconsistencies across libraries (i.e., library effects), individuals were grouped by library and BAYESCAN (Fischer et al., 2011; Foll et al., 2010; Foll & Gaggiotti, 2008) executed to identify and remove loci contributing to significant differences among libraries.

To identify full- and half-siblings, pairwise relatedness was assessed using Wang's estimator corrected for sample size (Wang, 2002) in *demerelate* (Kraemer & Gerlach, 2017). Because female blacktip sharks are thought to display regional philopatry (Keeney et al., 2005) and relatedness analysis used to confirm technical replicates already screened for siblings sampled between regions, *demerelate* was executed for each region separately (i.e., Atlantic, eastern Gulf, and western Gulf). For each region, 1,000 pairs of simulated full- and half-sibling relationships were generated using empirical allele frequencies. To identify full- and half-siblings, minimum relatedness threshold values were set after trimming the lowest 1% of simulated values to reduce instances of false positives. Removal of randomly sampled siblings from population genetics datasets can bias estimates of allele frequencies used for subsequent analyses, but so can the inclusion of siblings that are non-randomly sampled (Waples &

Anderson, 2017). Therefore, full- and half-siblings were considered non-randomly sampled if both individuals were captured in the same estuary on the same day, in which case one individual from each pair was removed for all subsequent analyses.

Three methods were used to assess for F_{ST} outlier loci putatively under directional selection with individuals grouped by geographic sample. The first approach, implemented in *OutFLANK* (Whitlock & Lotterhos, 2015), identifies F_{ST} outliers based on an inferred distribution of neutral F_{ST} after trimming the lowest and highest 5% of F_{ST} values, thus avoiding implicit assumptions of population structure and demography. The second method generates a null distribution of F_{ST} for neutral loci using a Bayesian approach implemented in BAYESCAN (Fischer et al., 2011; Foll et al., 2010; Foll & Gaggiotti, 2008). This approach assumes an island model whereby allele frequencies in each group are correlated through a common ancestral gene pool. BAYESCAN was executed with prior odds of 1,000 and a burn-in of 200,000 iterations; 25 pilot runs of 5,000 iterations were used to tune MCMC parameters and following 30,000 sampling iterations with a thinning interval of 50, significance was evaluated using a *q*-value of 0.05. Finally, the FDIST method (Beaumont & Nichols, 1996), implemented in ARLEQUIN (v3.5.2.2; Excoffier & Lischer, 2010), identifies loci with elevated F_{ST} for simulated background heterozygosity under two models: an island model and a hierarchical island model in which samples in the Atlantic and Gulf were grouped. For both models, 50,000 simulations were executed, 100 demes were simulated per group, and significance was evaluated using α of 0.05 corrected for multiple comparisons (Benjamini & Hochberg, 1995).

To examine the effects of space and environment on genetic structure, correlations among genomic variation, spatial position, and environmental parameters were assessed using redundancy analysis (RDA), as implemented in *vegan* (Oksanen et al., 2018). RDA is a

constrained ordination method based on multivariate regression that models how linear combinations of explanatory variables explain variation at a series of linear response variables, thereby enabling the identification of loci that co-vary with multivariate predictors (Legendre & Legendre, 2012). This approach is particularly useful when applied to genomic datasets because it does not rely on assumptions of equilibrium between microevolutionary forces, which are inherent components of F_{ST}-based analyses, and thus provides an alternative approach to assess population structure while screening for loci putatively under selection (Forester et al., 2018). The genomic dataset was transformed into a set of response variables consisting of allele counts. Two sets of explanatory variables were then produced: one describing the relative spatial position of individuals, and the second summarizing environmental differences and similarities among sample locations. Unique locations were assigned to each individual by jittering latitude and longitude for individuals caught in the same sampling effort, as implemented in *geoR* (Ribeiro & Diggle, 2001). To generate the spatial matrix, Moran's eigenvector maps (MEMs) were calculated using *adespatial* (Dray et al., 2019) based on coastal distances estimated between all sample locations using *gdistance* (Van Etten, 2017). The environmental matrix encompassed a wide range of variables for coastal locations, accessed from the MARSPEC (Sbrocco & Barber, 2013) and BIO-ORACLE (v2.2; Assis et al., 2018; Tyberghein et al., 2012) databases using *sdmpredictors* (Bosch & Fernandez, 2021).

For each explanatory matrix, forward model selection using the adjusted R^2 and significance testing (999 permutations; $\alpha < 0.01$) was used to identify the combination of variables that best explained genomic variation (Blanchet et al., 2008). Because collinearity is likely among environmental variables, model selection prohibited the inclusion of variables with variance inflation factor (VIF) > 3. When sampling is spatially uniform, the scales of genetic

structure described by MEMs can be estimated using periods of sine waves (Borcard & Legendre, 2002). To do this for each selected MEM, sine waves with periods ranging from 1 to 5,000 km were produced and Akaike Information Criteria (AIC) used to determine waves with the optimal periods that fitted.

The significance of each axis for both selected RDA models was assessed using 999 permutation tests with α of 0.05. To visualize the differential effects of space and environment on genetic structure, two biplots depicting the clustering of individuals according to selected variables were produced using the approach outlined by Forester et al., (2018). However, when identifying loci putatively under selection, it is important to disentangle spatial and environmental signals (Hoban et al., 2016). Therefore, partial RDA (pRDA), in which the linear effects of one set of variables are adjusted by accounting for covariables (Capblancq & Forester, 2021), was used to identify alleles most strongly correlated with environmental differences adjusted for spatial position. Allele loadings should form a normal distribution in which alleles at the center show no relationship with environmental variables, while those with loadings in the tails are strongly associated, and may therefore be considered putatively under selection (Forester et al., 2018). Environmentally-associated loci were defined using a function that sets thresholds three standard deviations from the mean (equivalent to a two-tailed *p*-value of 0.0027; Forester et al., 2018). The significance of the full pRDA model and each axis was assessed using 999 permutation tests with α of 0.05.

Allele frequencies of neutral and adaptive loci are shaped by different sets of interactions among microevolutionary forces and may provide for distinct patterns of genetic structure (Luikart et al., 2003). Therefore, loci flagged as being under selection by either of the F_{ST} outlier methods or determined to be environmentally associated using pRDA were designated as

putatively adaptive. The data was then divided into adaptive and neutral (i.e., all other loci) datasets.

For neutral and adaptive datasets, hierarchical locus-by-locus AMOVA was performed using ARLEQUIN, with *F*-statistics calculated as weighted means of locus-specific values to account for uneven levels of missing data among loci (Weir & Cockerham, 1984). Samples were grouped as Atlantic and Gulf, with significance assessed ($\alpha < 0.05$) by permuting individuals among samples 10,000 times, and by bootstrapping the data 20,000 times to create 95% confidence intervals. Single level, locus-by-locus AMOVA was also executed for Atlantic and Gulf samples separately, with significance assessed as above. Subsequently, *post-hoc* estimates of locus-by-locus pairwise F_{ST} between samples were calculated using ARLEQUIN, with 95% confidence intervals produced and significance assessed as above but corrected for multiple comparisons (Benjamini & Hochberg, 1995). To prevent randomly sampled siblings detected within estuaries from distorting patterns of genetic structure, one individual from each pair was removed before executing AMOVA and pairwise F_{ST} tests.

Genetic diversity at neutral and putatively adaptive loci was estimated for each geographic sample based on Nei's gene diversity (H_e; Nei, 1978) and rarified allelic richness (A_r) using *hierfstat* (Goudet, 2005) and *poppr* (Kamvar et al., 2014). For each diversity metric, differences among samples were assessed using Friedman's rank-sum test ($\alpha < 0.05$), and Wilcoxon signed-rank tests were used to assess for *post-hoc* pairwise differences ($\alpha < 0.05$), with *p*-values corrected for multiple comparisons (Benjamini & Hochberg, 1995). The effective number of breeders (N_b) was estimated for each sample and region using the linkage disequilibrium method (Hill, 1981) implemented in NEESTIMATOR (v2.1; Do et al., 2014), with 0.02 used as the lowest allele frequency. In addition to point estimates, 95% confidence intervals

were estimated using the jackknife approach. All figures were generated in R using *ggplot2* (Wickham, 2016).

Results

The minimum values of relatedness used to identify siblings – as determined by simulations – were 0.42 for full-siblings and ranged from 0.18-0.2 by region for half-siblings. No siblings were identified in the Atlantic. Non-randomly sampled siblings included one pair in Terra Ceia Bay (eastern Gulf) and a group of six full- and half-siblings in San Antonio Bay (western Gulf). Randomly sampled siblings were only detected in the eastern Gulf, including a pair of half-siblings sampled in Terra Ceia Bay and Apalachicola Bay in May 2018, and 19 pairs of full- and half-siblings captured in Terra Ceia Bay within and across years. Notably, three pairs of half-siblings were sampled two years apart and two pairs were sampled four years apart. After an individual from each non-randomly sampled sibling pair was removed, 419 individuals genotyped at 4,368 loci containing 6,709 SNPs (1.54 SNPs and 2.39 alleles per locus on average) were retained for subsequent analyses.

For individuals grouped by geographic location, one F_{ST} outlier was detected by *OutFLANK* but none were detected by BAYESCAN or ARLEQUIN.

Ten MEMs describing spatial differences among individuals were generated using the matrix of coastal distances and the first two were chosen by model selection: MEM1 (adjusted R^2 = 0.001285; $p < 0.01$) and MEM2 (adjusted R^2 = 0.001852; $p < 0.01$). The full spatial RDA model and both axes were significant ($p < 0.001$). While individuals clustered along MEM1 as Atlantic and Gulf, MEM2 divided Gulf individuals into eastern and western clusters. Individuals from Mobile Bay – which straddles the boundary between the eastern and western Gulf stock units – grouped predominantly with eastern Gulf individuals. Sine waves with periods of 2,060

km and 1,675 km were determined by AIC to be the best fits for MEM1 and MEM2, respectively (Figure 4.2). Model selection chose two environmental variables with VIF < 3 (Table 4.1): minimum annual sea surface temperature (adjusted $R^2 = 0.00133$; $p < 0.01$; Feldman & McClain, 2010) and mean sea surface salinity in June (adjusted $R^2 = 0.001948$; $p < 0.01$; Antonov et al., 2010). The full environmental RDA model and both axes were significant ($p < 0.001$). Similar to the spatial RDA, linear combinations of the two environmental variables structured Atlantic and Gulf individuals separately along the first axis. However, in contrast to clusters produced by spatial RDA, on the second axis, Mobile Bay individuals clustered with those from the western Gulf (Figure 4.3). The full pRDA model (i.e., the effect of temperature and salinity adjusted by MEMs 1 and 2) and both axes were significant ($p < 0.05$). Allele loadings followed a normal distribution and 70 environmentally-associated loci (1.6%) were identified, including the locus determined to be an F_{ST} outlier by *OutFLANK*. The environmentally-associated loci and F_{ST} outlier were removed to produce putatively adaptive (70 loci) and neutral datasets (4,298 loci).

For the neutral dataset, AMOVA detected significant heterogeneity among groups (Atlantic and Gulf; $p < 0.0001$) and among samples within groups ($p < 0.05$; Table 4.2). For the adaptive dataset, by contrast, heterogeneity was observed among samples within groups ($p < 0.0001$) but not among groups ($p = 0.2228$; Table 4.2). Based on single-level AMOVA, significant heterogeneity was observed within the Gulf for the neutral and adaptive datasets (F_{ST}: 0.0004 and $p < 0.05$; F_{ST}: 0.0060 and $p < 0.0001$, respectively), but not in the Atlantic (F_{ST}: 0.0003; $p = 0.2075$; F_{ST}: 0.0021 and $p = 0.2310$, respectively). At neutral loci, *post-hoc* estimates of locus-by-locus F_{ST} between samples were statistically significant ($p < 0.05$; after correction) for 87.5% of Atlantic-Gulf comparisons. Within the Gulf, significant comparisons were limited to the most eastern and the four western Gulf samples. Although estimates of pairwise F_{ST} were

much larger at putatively adaptive loci, statistically significant comparisons were fewer and predominantly observed between Gulf samples with the greatest latitudinal differences (Figure 4.4; Table 4.3).

Gene diversity (H_e) differed significantly among the 11 samples for neutral and putatively adaptive datasets ($p < 0.0001$). For the neutral dataset, estimated H_e was lowest in Port Royal Sound (0.1531; Atlantic) and significantly smaller ($p < 0.05$) than all samples except St. Helena Sound (Atlantic); estimated H_e was greatest in San Antonio Bay (0.1580; western Gulf) and significantly greater ($p < 0.05$) than all but three samples (i.e., Mobile Bay, Corpus Christi Bay, and Waccasassa Bay; all Gulf). For the adaptive dataset, estimated H_e was lowest in Waccasassa Bay (0.1612; eastern Gulf) and significantly greater in Mobile Bay (0.2324; eastern Gulf) than all other samples ($p < 0.001$). Allelic richness (A_r) estimates also differed significantly among samples for both datasets ($p < 0.0001$) and similar patterns were observed. For the neutral dataset, estimated A_r was lowest in Port Royal Sound (2.815; Atlantic) and significantly lower ($p < 0.05$) than three samples (i.e., Mobile Bay, San Antonio Bay, and Galveston Bay; all Gulf); estimated A_r for the neutral dataset was greatest in San Antonio Bay (2.853; western Gulf) and significantly greater ($p < 0.05$) than six samples. For the adaptive dataset, estimated A_r was lowest in Terra Ceia Bay (2.961; eastern Gulf) and significantly greater in Mobile Bay (3.646; eastern Gulf) than all other samples ($p < 0.001$).

While finite upper and point N_b estimates were obtained for only one and three samples, respectively, lower N_b estimates were obtained for all but one sample (i.e., Galveston Bay; western Gulf; Table 4.4). By sample, lower estimates were generally smaller in the eastern Gulf (299-1,074) compared with the Atlantic (1,378-2,505) and western Gulf (784-5,261). In addition, finite point and lower N_b estimates (respectively) were obtained for each region and were smaller

in the eastern Gulf (1,833 and 3,148) than in the Atlantic (4,482 and 15,167) and western Gulf (5,338 and 44,094).

Discussion

The results of this study highlight how philopatry can influence genetic population structure at multiple spatial scales by impacting both gene flow and selection. Neutral genetic structure indicated that blacktip sharks in the U.S. Atlantic Ocean and Gulf of Mexico constitute three genetically distinct units with little to no gene flow between them. Structure within Gulf units at putatively adaptive loci correlated with variation in sea surface temperature and salinity suggested local adaptation. Instances of parturition site fidelity were documented and if this behavior extends across generations (i.e., natal philopatry), it could contribute to the observed patterns of adaptive structure by facilitating selection for locally adapted phenotypes.

Results from neutral loci indicate that blacktip sharks in the Atlantic and Gulf are genetically distinct. The first MEM of the spatial RDA clustered Atlantic and Gulf individuals separately and indicated that the scale of structure is ~2,000 km (Figure 4.2), approximately the distance between Bulls Bay (the most northern Atlantic sample) and Mobile Bay (the middle of the Gulf). Significant genetic structure between these groups was also observed using hierarchical AMOVA and *post-hoc* estimates of F_{ST} between samples. In addition, estimates of H_e and A_r for the neutral dataset were lower in the three Atlantic samples than in all but one Gulf sample. The finding of genetically distinct units in the Atlantic and Gulf is consistent with differences in blacktip mitochondrial DNA (Keeney et al., 2003, 2005) and life history traits such as maximum length and growth rate (Carlson et al., 2006). This observation is also consistent with studies of other marine fishes (Gold et al., 2009; Leidig et al., 2015; Seyoum et al., 2017), including coastal sharks (Dimens et al., 2019; Portnoy et al., 2015; Portnoy et al.,

2016), and aligns with the Florida Vicariance Zone where constriction of the continental shelf from Miami to West Palm Beach has reduced nearshore habitat (Avise, 1992; Neigel, 2009). Consequently, suitable parturition sites for coastal sharks are lacking in southeastern Florida and may dissuade female movement across the vicariance zone. Although gene flow via males should be less impacted, tagging data suggest that male blacktip sharks do not move between the Atlantic and Gulf either (Kohler & Turner, 2019). Thus, additional factors are likely limiting connectivity.

Significant neutral genetic structure was also found within the Gulf, but not the Atlantic. YOY blacktip sharks occupy U.S. Atlantic estuaries from northern Florida to southern North Carolina (Castro, 1996; McCallister et al., 2013), so the lack of observed structure in the Atlantic could be due to limited spatial sampling. For the Gulf, single-level AMOVA indicated heterogeneity, and differences in pairwise F_{ST} were observed between the most eastern and the four western samples (Figure 4.4), which could reflect an isolation-by-distance effect (Laikre et al., 2005). However, the spatial RDA indicated the scale of structure is ~1,600 km – similar to the distance between Corpus Christi Bay and Terra Ceia Bay (~1,500 km) – and clustered individuals into eastern and western groups, with individuals from Mobile Bay predominantly associating with the eastern Gulf (Figure 4.2). This division aligns with a biogeographic break in the northern Gulf (McClure & McEachran, 1992), centered on an area of transition from carbonate sediments in the east to mostly terrigenous sediments in the west (Neigel, 2009). Low salinity outflows from the Mississippi and Atchafalaya rivers to the west of Mobile Bay could act as a barrier to gene flow for blacktip sharks, as suggested for other stenohaline sharks in the Gulf (Portnoy et al., 2014), as well as a variety of marine species around the world (Rocha, 2003; Volk et al., 2021). The pattern of neutral structure highlighted by this study has been observed in

multiple marine fishes in the northern Gulf (Karlsson et al., 2009; Portnoy et al., 2014; Seyoum et al., 2018). In particular, the results are similar to those of a genomic assessment of red drum (*Sciaenops ocellatus*; Hollenbeck et al., 2019), which do not give live birth but display spawning site fidelity to estuaries to which juveniles recruit after the larval period (Lowerre-Barbieri et al., 2019; Matlock, 1990). This is in contrast with the patterns seen in genomics studies of two species that spawn offshore, red snapper (*Lutjanus campechanus*; Portnoy et al., 2021) and southern flounder (*Paralichthys lethostigma*; O'Leary et al., 2021), and suggests that habitat use may be an important predictor of genetic structure for fishes of the Gulf of Mexico.

A previous assessment of blacktip shark genetic structure found differences among the Atlantic, eastern, and western Gulf in mitochondrial DNA – but not nuclear DNA – and the authors hypothesized that this reflected female regional philopatry and male-mediated gene flow (Keeney et al., 2005). Here, the findings of neutral heterogeneity among these regions at nuclear-encoded loci suggest males may display similar philopatric behavior or that male-mediated gene flow is insufficient to homogenize allele frequencies. In addition, spatial RDA and estimates of pairwise F_{ST} are consistent with the idea that straying by females occurs mostly among neighboring parturition sites, as suggested by other studies of coastal sharks (Duncan et al., 2006; Keeney et al., 2003). Taken together, this suggests that philopatry by both males and females has contributed to the formation of genetically distinct eastern and western Gulf units that align well with the current blacktip Gulf stock subregions defined by NOAA Fisheries. Although blacktip sharks in the Gulf are currently assessed as a single stock, there are distinctly unequal landing quotas in the eastern (37.7 metric tons) and western Gulf (347.2 metric tons; NMFS, 2021). This is notable because a disparity of similar magnitude was observed between eastern (1,833 and 3,148) and western Gulf (5,338 and 44,094) lower and point estimates of N_b

(respectively; Table 4.4), providing further evidence for genetically distinct units (Waples, 2010).

Significant genetic structure at putatively adaptive loci was observed on a more localized scale in the Gulf. Differences in pairwise F_{ST} were observed between samples within each Gulf unit, and comparisons between samples with the greatest environmental differences included the greatest F_{ST} values, indicating local adaptation. Environmental RDA structured individuals by latitude based on minimum annual temperature and mean salinity in June, and in contrast to spatial RDA, Mobile Bay individuals clustered with those from Texas (Figure 4.3). This corresponds with a transition in environmental conditions and a break in the coastal shark assemblage of the northern Gulf, as described by multiple studies (Bethea et al., 2015; Drymon et al., 2020). Estimates of H_e and A_r for the adaptive dataset were also significantly elevated in Mobile Bay, and this could be related to the spatial and temporal environmental heterogeneity that characterizes this estuary (Kim & Park, 2012; Orlando Jnr et al., 1993). However, Mobile Bay is proximal to a marine-suture zone (Portnoy & Gold, 2012) and greater diversity could also reflect contact between the eastern and western Gulf.

While the lack of a suitable reference genome precludes assessments of putative function, aspects of blacktip shark biology provide potential explanations for the fine-scale adaptive structure observed here. Adaptive differences associated with minimum annual temperature could reflect temporal variation in YOY migration out of parturition sites when waters cool in the fall. Sea surface temperatures in Gulf estuaries are colder seasonally in the north than in the south and can vary considerably due to a variety of climatic factors. A gradient exists along the western Gulf coast because temperature differences are predominantly influenced by seasonal heat flux and river discharges (Portela et al., 2018), whereas differences along the Gulf coast of

Florida appear less stark by comparison. Blacktip sharks born in Terra Ceia Bay were thought to remain until late October to late November, with emigration following dramatic decreases in water temperature (1.5-2°C) to approximately 21°C (Heupel, 2007). However, some remain until January and others return in February (Gardiner unpublished data), suggesting a longer duration of residency in this estuary. If there is a fitness cost to a shorter residency period, local adaptation could lead to individuals born in estuaries further north being more tolerant of lower temperatures. However, blacktip shark emigration from an Atlantic coast estuary (i.e., Bulls Bay, South Carolina) also coincides with ~21°C but occurs in early to mid-October (Castro, 1996). Therefore, it appears that similar temperature changes stimulate emigration, and blacktip sharks born in more northern Gulf estuaries should migrate earlier in the year when those temperatures are reached. This is observed along the Texas coast where YOY blacktip sharks are found in Corpus Christi Bay till mid-November (Matich et al., 2021), weeks after they have emigrated from Galveston Bay (Matich and Texas Parks and Wildlife unpublished data). Likewise, the species is mostly absent in Mobile Bay after October (Parsons & Hoffmayer, 2007). A similar but reverse trend in migratory timing occurs in Atlantic salmon (*Salmo salar*) that leave nurseries in the spring/summer (Hodgson & Quinn, 2002; Hvidsten et al., 1998), with individuals from southern habitats migrating weeks before those born further north because the temperatures that stimulate emigration are reached earlier (Otero et al., 2014; Vollset et al., 2021).

Salinities also vary among Gulf estuaries and adaptive differences associated with mean salinity in June – just after the peak period of parturition (March-May; Baremore & Passerotti, 2013) – could indicate local adaptation based on salinity tolerance. Peninsular Florida estuaries are relatively saline because they receive little freshwater inflow compared with those to the west – as a result, conditions are mainly influenced by precipitation. Conditions are less saline in the

Florida panhandle due to lower evaporation rates and freshwater discharge from the Apalachicola, Chattahoochee, and Flint rivers that flow into Apalachicola Bay (Orlando Jnr et al., 1993). Mobile Bay is relatively hyposaline because of the large freshwater influx via the Mobile River (Orlando Jnr et al., 1993), and June salinities in Texas estuaries are similar to Mobile Bay (consistent with environmental RDA) because precipitation is greatest in May (TexasET, 2022). Also, the major river systems (e.g., Mobile, Mississippi, Rio Grande) that drain into the Gulf are distributed from Alabama to the border with Mexico (USGS, 1990). Nonetheless, a salinity gradient exists along the Texas coast because estuaries in the north receive hyposaline waters from the central Gulf via westerly currents, while isolated freshwater pulses lead to more saline conditions in the south (Orlando Jnr et al., 1993). Consequently, blacktip sharks born in estuaries on the lower Texas coast may experience higher salinities, consistent with the conditions at which individuals have been captured in Corpus Christi Bay (mean: 25.0-33.4) and Galveston Bay (mean: 16.1-22.3; Matich et al., 2017). By contrast, the species has been captured in Mobile Bay at 11 (mean: 18.7 ± 0.55; Parsons & Hoffmayer, 2007) and is usually found at a wide range of salinities in Florida estuaries (20-40; Bethea et al., 2009; Heupel et al., 2003).

It should be noted that the environmental data sources available provide insufficient resolution to describe environmental variation within estuaries. The MARSPEC and BIO-ORACLE databases reflect coastal conditions for which differences are predominantly driven by latitude, and consequently, environmental heterogeneity among the geographic samples is underestimated. Additionally, the environmental measurements are unable to account for habitat use by blacktip sharks because these individuals are highly mobile, use only a subset of the available estuarine habitat, and change locations with environmental conditions (Froeschke et al.,

2010). Even so, the environmental RDA shows clear latitudinal gradients in both the eastern and western Gulf. Thus, the results may reflect local adaptation to conditions that are not described by the environmental data but also vary with latitude in each region.

Five pairs of half-siblings were captured two and four years apart in Terra Ceia Bay (eastern Gulf), accordant with the proposed two-year reproductive period of female blacktip sharks (Baremore & Passerotti, 2013). This implies that five females re-used the habitat for parturition. While evidence of parturition site fidelity was not observed in other estuaries, it is important to note that all randomly sampled siblings captured within a year in the same estuary were detected in Terra Ceia Bay, and N_b estimates indicate that the number of breeders using this habitat is very small relative to all other samples (Table 4.4). Thus, blacktip sharks may exhibit parturition site fidelity to additional estuaries, but the behavior may be easier to detect in Terra Ceia Bay because there is a higher probability of catching siblings. Females that re-use the same estuary for parturition display a strong degree of habitat fidelity, but for this behavior to constitute philopatry, the estuary that is re-used must be the habitat in which females were born (i.e., natal philopatry). Multiple studies have demonstrated that sharks can navigate to their place of birth (Edrén & Gruber, 2005; O'Gower, 1995; Sundström et al., 2001), including blacktip sharks (Gardiner et al., 2015; Heupel et al., 2003), and while natal philopatry has been speculated to occur in this species (Hueter et al., 2005), the behavior has been demonstrated only in the lemon shark (*Negaprion brevirostris*) in Bimini, The Bahamas (Feldheim et al., 2014). This was possible because lemon sharks in Bimini are easily captured in a nearly exhaustive manner, relatively few females give birth there, and genetic profiling has been ongoing for decades (Feldheim et al., 2004; Gruber et al., 2001; Postaire et al., 2022). The results presented here

indicate that long-term studies focused on identifying kin among blacktip sharks in Terra Ceia Bay may demonstrate a second example of natal philopatry by coastal sharks.

While the observation of three genetically distinct units in the Atlantic and Gulf suggests male and female blacktip sharks reproduce in the region of their birth (i.e., regional philopatry), this behavior cannot explain the fine-scale adaptive structure observed within Gulf units. Adaptive variation could sort among neighboring estuaries if alleles adapted to local conditions conferred phenotypes with greater fitness and matrilines carrying these alleles re-used the same estuaries as parturition sites in subsequent generations (i.e., natal philopatry). Under this scenario, YOY with phenotypes locally adapted to their parturition site would have a higher probability of surviving and reproducing. Differential selection pressures among parturition sites would drive selection for locally adapted phenotypes and overcome gene flow of maladapted alleles from neighboring estuaries via patrilines and/or female straying. Given the heterogeneity among Gulf estuaries in conditions like temperature and salinity and the high rates of mortality experienced by YOY blacktip sharks (Heupel & Simpfendorfer, 2002), directional selection and natal philopatry could facilitate the sorting of adaptive alleles, generating the patterns of adaptive structure observed in this study (Kawecki & Ebert, 2004).

The genetic structure found among parturition sites within management units highlights the importance of policies that focus on the preservation of adaptive variation (Funk et al., 2012). Estuaries in which offspring are born and/or reside as juveniles are considered essential because they are fundamental to lifecycles (Fluharty, 2000), but if neighboring habitats are environmentally heterogeneous and sources of novel adaptive variants, it may be necessary to individually evaluate their contribution to future persistence (Stiebens et al., 2013). These considerations are particularly important for species displaying fine-scale philopatry because the

loss of certain habitats could lead to irreversible declines in recruitment and adaptive potential (Hess et al., 2013; Hueter et al., 2005). Furthermore, as environmental conditions continue to shift with climate change, the capability of organisms to adapt and persist will depend on existing genetic variation and levels of gene flow among habitats.

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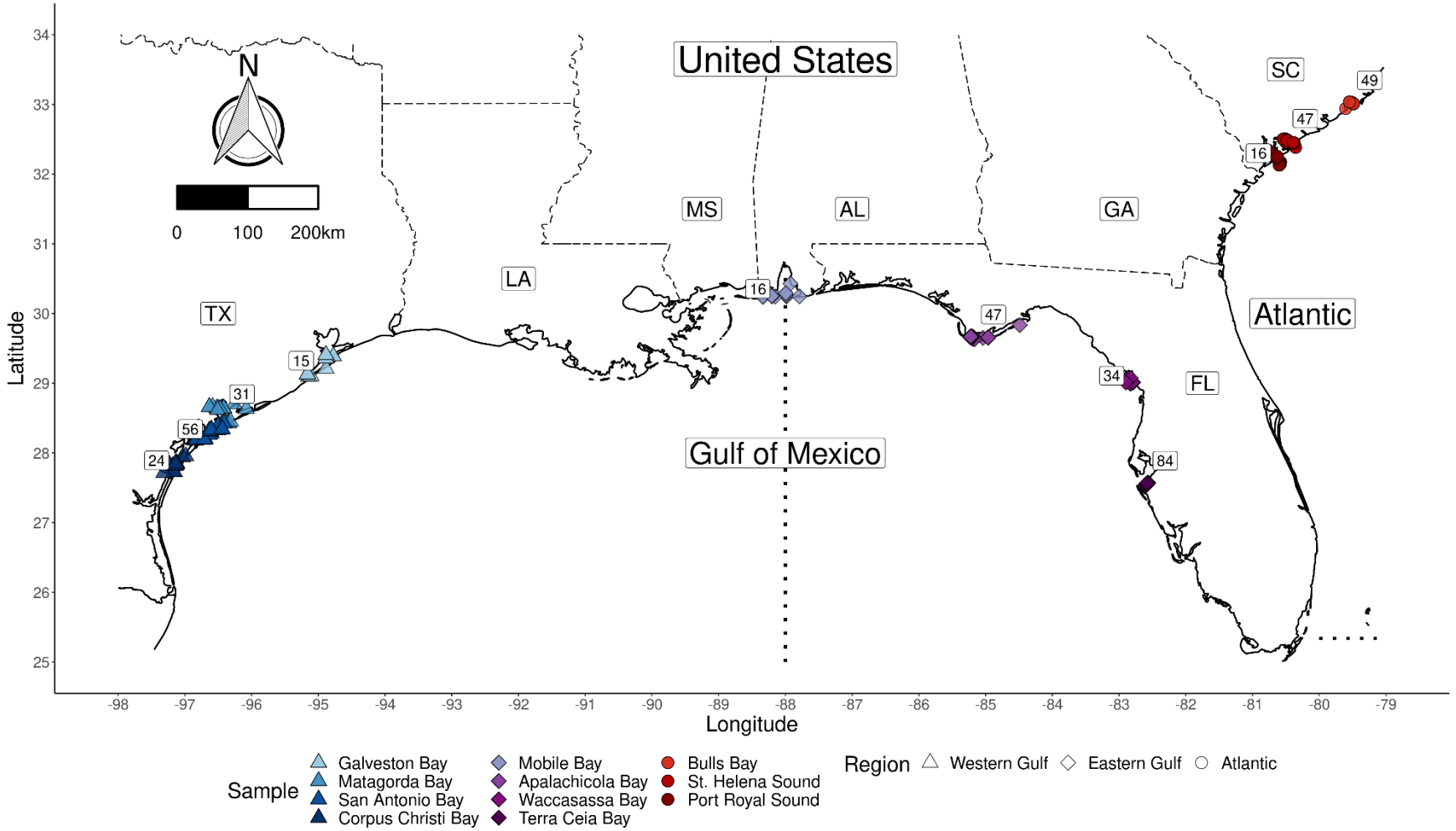

Figure 4.1. Blacktip shark (*Carcharhinus limbatus*) sampling locations in the U.S. Atlantic and Gulf of Mexico. Regions are divided by dotted lines, following designations by NOAA Fisheries. Mobile Bay straddles the 88th meridian which separates the eastern and western Gulf stock subregions and was considered part of the eastern Gulf.

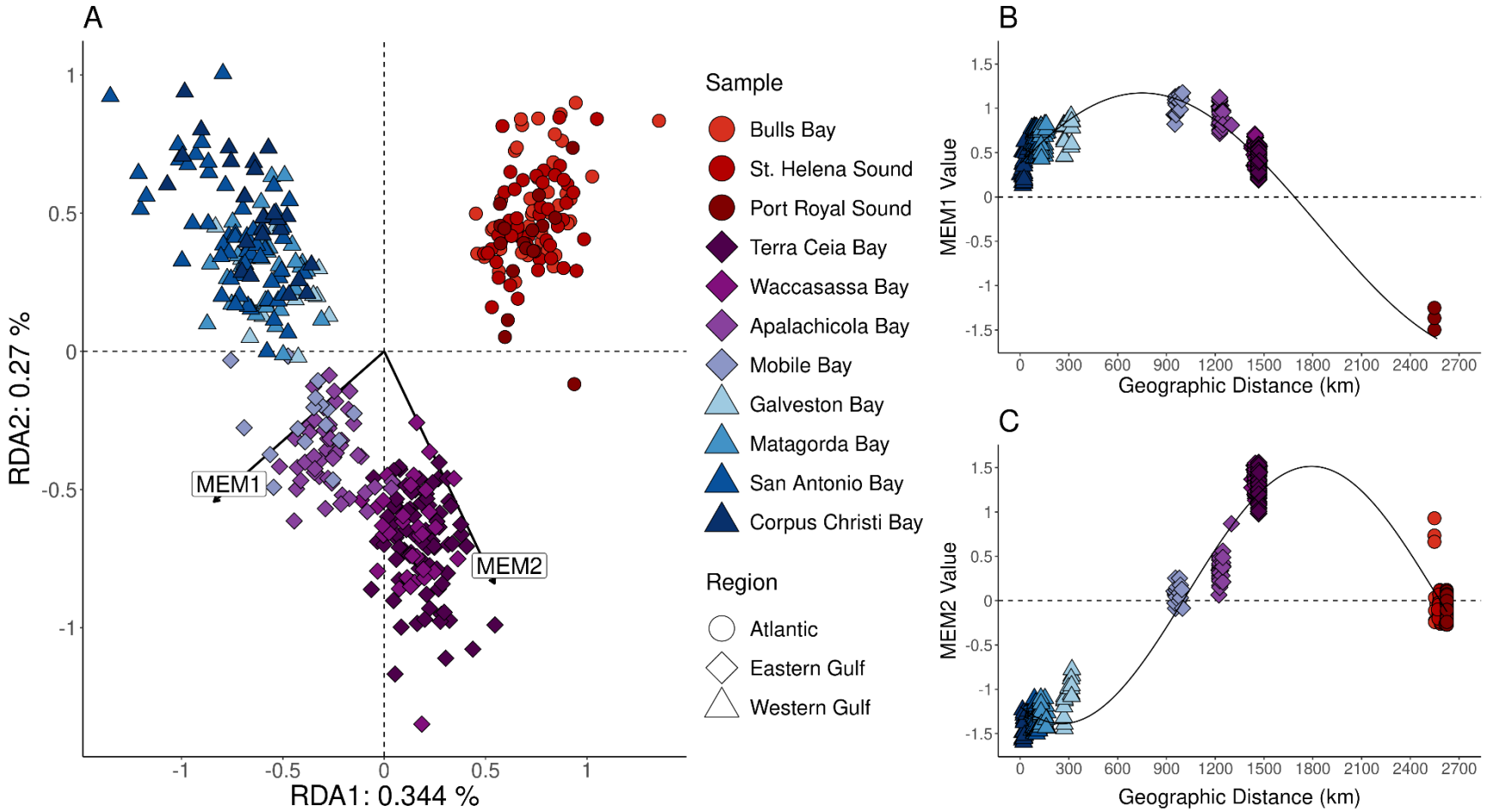

Figure 4.2. A) Biplot showing ordination space loadings determined by MEM1 and MEM2 from the full model of the spatial redundancy analysis. Values for MEM1 (B) and MEM2 (C) with fitted sine waves plotted against geographic distance.

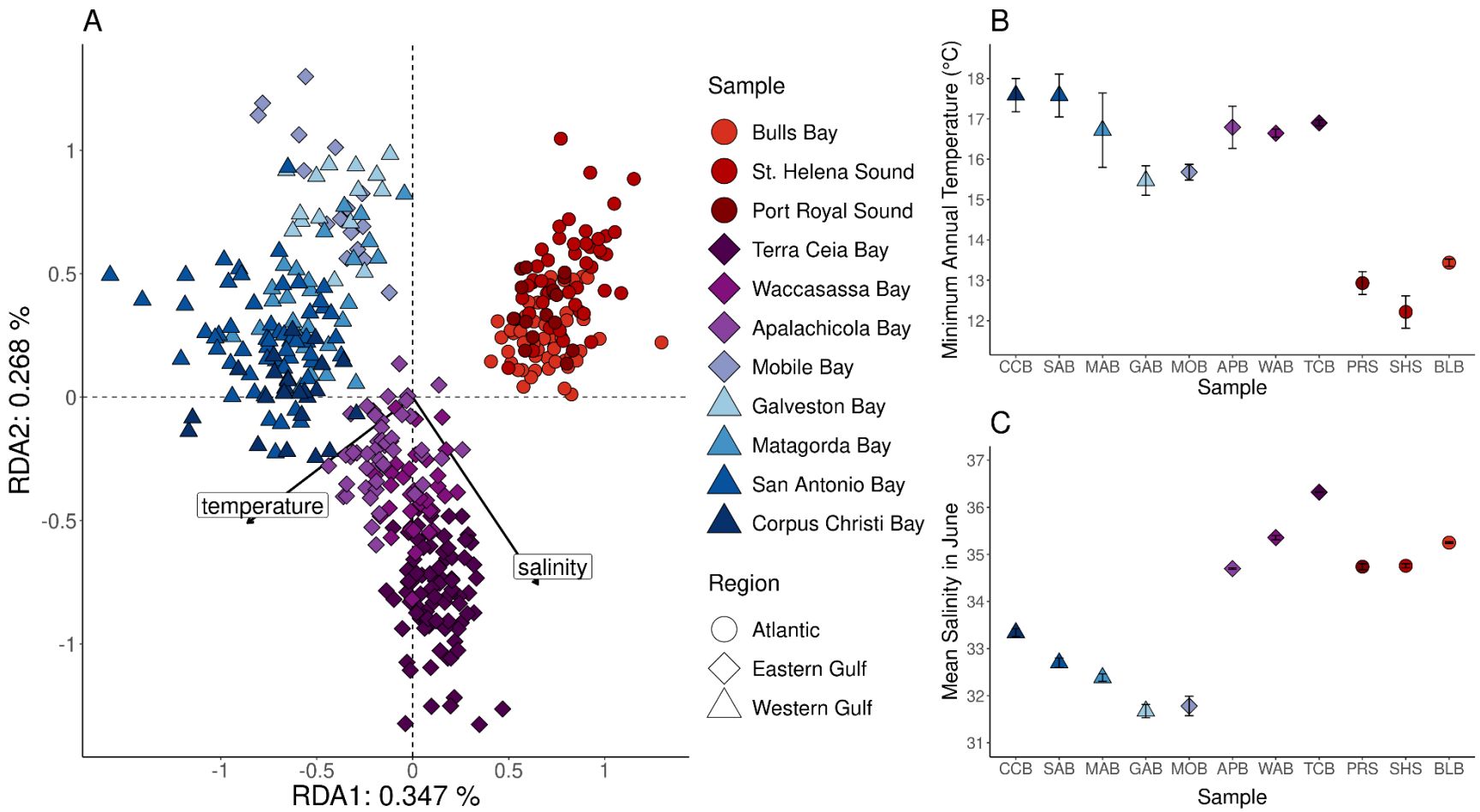

Figure 4.3. A) Biplot showing ordination space loadings determined by minimum annual temperature and mean salinity in June from the full model of the environmental redundancy analysis. Mean values (± one standard deviation) for minimum annual temperature (B) and mean salinity in June (C) for coastal areas encompassing geographic samples.

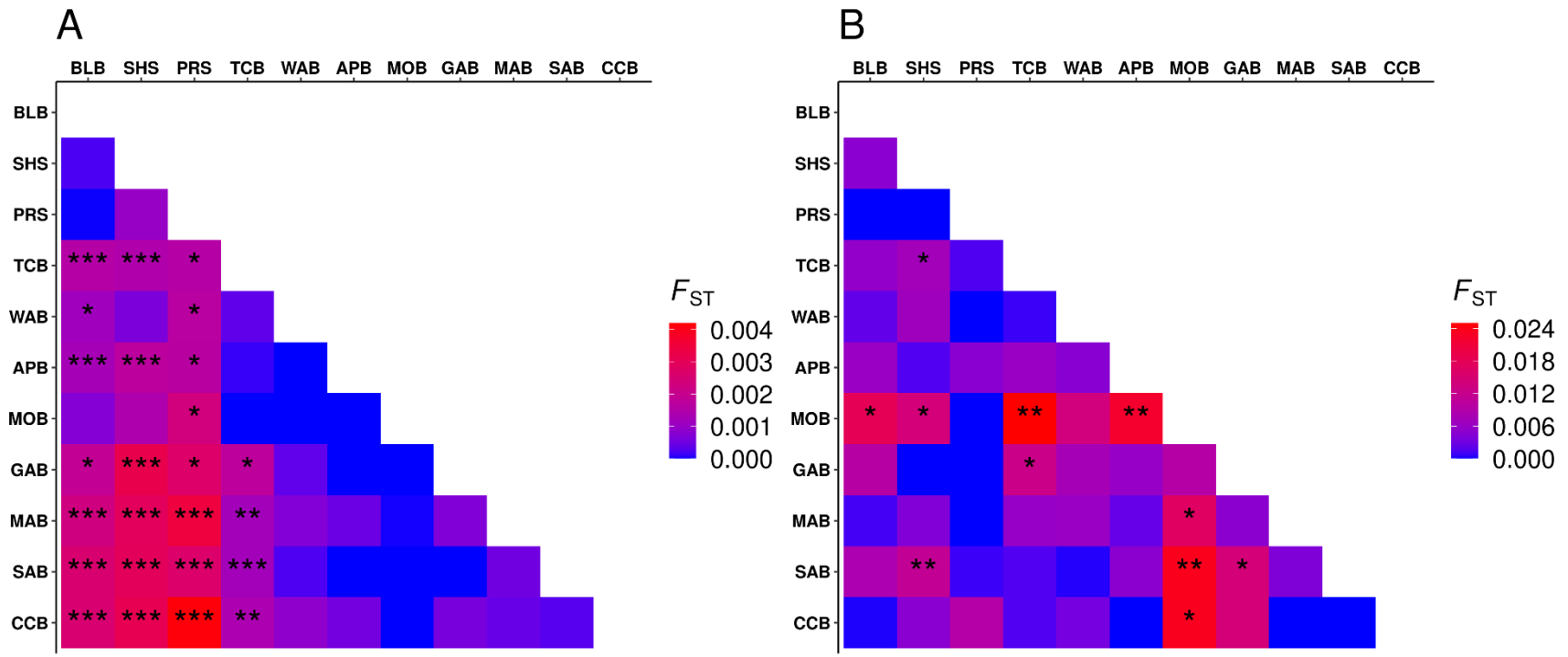

Figure 4.4. Heatmaps illustrating locus-by-locus pairwise F_{ST} values between samples and associated *p*-values, corrected for multiple comparisons, at neutral (A) and putatively adaptive loci (B). *p*-values are only shown for comparisons in which the lower 2.5% of bootstrapped F_{ST} values were greater than zero: $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***). Samples by region: Atlantic (BLB, SHS, PRS), eastern Gulf (TCB, WAB, APB, MOB), and western Gulf (GAB, MAB, SAB, CCB).

Table 4.1. Environmental variables determined by model selection to explain a significant proportion of genomic variation.

Variable	Minimum Annual Sea Surface Temperature	Mean Sea Surface Salinity in June
Dataset	Bio-ORACLE	MARSPEC
Unit	°C	unitless
Measurement Type	Aqua-MODIS satellite	*In situ*
Spatial Resolution	5 arcminute	1 arcdegree
Source	Feldman & McClain, 2010	Antonov et al., 2010

Table 4.2. Hierarchical and single-level locus-by-locus AMOVA using neutral and putatively adaptive datasets. Underlined *p*-values denote statistically significant heterogeneity; * denotes lower 2.5% of bootstrapped *F*-statistics were greater than zero.

Dataset	Samples	Source of Variation	Variance Components	Percent Variation	*F*-statistic	*p*-value
Neutral	All	Among groups (i.e., Atlantic and Gulf)	0.5283	0.1577	0.0016	<0.0001*
		Among samples within groups	0.1342	0.0401	0.0004	0.0101*
	Atlantic	Among samples	0.1060	0.0320	0.0003	0.2075
		Among individuals within samples	330.7439	99.9680	-	-
	Gulf	Among samples	0.1375	0.0410	0.0004	0.0128*
		Among individuals within samples	335.5742	99.9591	-	-
Putatively Adaptive	All	Among groups (i.e., Atlantic and Gulf	0.0041	0.0682	0.0007	0.2228
		Among samples within groups	0.0307	0.5157	0.0052	<0.0001*
	Atlantic	Among samples	0.0125	0.2126	0.0021	0.2310
		Among individuals within samples	5.8751	99.7874	-	-
	Gulf	Among samples	0.0356	0.5971	0.0060	<0.0001*
		Among individuals within samples	5.9312	99.4029	-	-

Table 4.3. Locus-by-locus pairwise F_{ST} values (lower) between samples and associated *p*-values (upper) at neutral (A) and putatively adaptive loci (B). Underlined and bold denote statistically significant comparisons before and after correction for multiple comparisons, respectively. * denotes the lower 2.5% of bootstrapped F_{ST} values were greater than zero. Symbols denote regions: U.S. Atlantic Ocean (ǂ), eastern Gulf of Mexico (¡), western Gulf of Mexico (§).

A)	BLBǂ	SHSǂ	PRSǂ	TCB¡	WAB¡	APB¡	MOB¡	GAB§	MAB§	SAB§	CCB§
BLBǂ	-	0.2968	0.5448	**0.0001***	**0.0034***	**0.0001***	0.1769	**0.0037***	**0.0001***	**0.0001***	**0.0001***
SHSǂ	0.0002	-	0.0785	**0.0001***	0.0704	**0.0001***	0.0250	**0.0001***	**0.0001***	**0.0001***	**0.0001***
PRSǂ	0.0000	0.0010	-	**0.0101***	**0.0105***	**0.0051***	**0.0181***	**0.0056***	**0.0001***	**0.0001***	**0.0001***
TCB¡	**0.0016***	**0.0015***	**0.0016***	-	0.1964	0.3720	0.6876	**0.0069***	**0.0015***	**0.0001***	**0.0020***
WAB¡	**0.0011***	0.0006	**0.0017***	0.0004	-	0.6479	0.8030	0.3306	0.0775	0.2697	0.0587
APB¡	**0.0013***	**0.0017***	**0.0017***	0.0001	0.0000	-	0.9895	0.4497	0.1225	0.6930	0.1213
MOB¡	0.0007	0.0014	**0.0023***	0.0000	0.0000	0.0000	-	0.7559	0.5724	0.9319	0.8596
GAB§	**0.0019***	**0.0032***	**0.0027***	**0.0018***	0.0004	0.0000	0.0000	-	0.2219	0.5095	0.3027
MAB§	**0.0023***	**0.0029***	**0.0034***	**0.0012***	0.0007	0.0005	0.0000	0.0007	-	0.1004	0.2799
SAB§	**0.0026***	**0.0029***	**0.0027***	**0.0012***	0.0002	0.0000	0.0000	0.0000	0.0005	-	0.2869
CCB§	**0.0026***	**0.0031***	**0.0041***	**0.0014***	0.0009	0.0005	0.0000	0.0006	0.0004	0.0003	-

B)	BLBǂ	SHSǂ	PRSǂ	TCB¡	WAB¡	APB¡	MOB¡	GAB§	MAB§	SAB§	CCB§
BLBǂ	-	0.0386	0.7396	**0.0104**	0.2463	0.0173	**0.0011***	0.0437	0.3537	**0.0022**	0.5124
SHSǂ	0.0049	-	0.6438	**0.0022***	0.0250	0.2761	**0.0061***	0.6386	0.0913	**0.0001***	0.1326
PRSǂ	0.0000	0.0000	-	0.3436	0.7727	0.2041	0.7010	0.8508	0.8239	0.4421	0.1591
TCB¡	**0.0054**	**0.0071***	0.0014	-	0.3450	**0.0064**	**0.0001***	**0.0097***	0.0249	0.2021	0.3130
WAB¡	0.0022	0.0067	0.0000	0.0007	-	0.0837	0.0147	0.1168	0.0619	0.4477	0.2670
APB¡	0.0062	0.0015	0.0047	**0.0061**	0.0047	-	**0.0001***	0.1344	0.2268	0.0331	0.6390
MOB¡	**0.0180***	**0.0142***	0.0000	**0.0250***	0.0139	**0.0222***	-	0.1247	**0.0037***	**0.0001***	**0.0018***
GAB§	0.0096	0.0000	0.0000	**0.0126***	0.0075	0.0059	0.0093	-	0.1753	**0.0092***	0.0436
MAB§	0.0010	0.0041	0.0000	0.0058	0.0062	0.0023	**0.0167***	0.0049	-	0.0949	0.5969
SAB§	**0.0083**	**0.0112***	0.0008	0.0015	0.0003	0.0049	**0.0236***	**0.0144***	0.0040	-	0.6980
CCB§	0.0002	0.0047	0.0093	0.0015	0.0033	0.0000	**0.0234***	0.0144	0.0000	0.0000	-

Table 4.4. Lower, point, and upper estimates of contemporary effective number of breeders (N_b) by sample and region calculated using NEESTIMATOR. Lower and upper values are based on 95% confidence intervals determined using the jackknife approach. n denotes the number of individuals genotyped.

Sample	n	Lower N_b Estimate	N_b Point Estimate	Upper N_b Estimate	Region	n	Lower N_b Estimate	N_b Point Estimate	Upper N_b Estimate
BLB	49	1,901	14,653	inf	Atlantic	112	4,482	15,167	inf
SHS	47	1,378	18,229	inf					
PRS	16	2,505	inf	inf					
TCB	84	410	689	1,982	Eastern Gulf	181	1,833	3,148	10,622
WAB	34	1,074	inf	inf					
APB	47	1,021	inf	inf					
MOB	16	299	inf	inf					
GAB	15	inf	inf	inf	Western Gulf	126	5,338	44,094	inf
MAB	31	5,261	inf	inf					
SAB	56	2,383	inf	inf					
CCB	24	784	inf	inf					

CHAPTER V: A GENOMIC ASSESSMENT OF BLACKTIP SHARK (*CARCHARHINUS LIMBATUS*) STOCK STRUCTURE AND MOVEMENT ACROSS NATIONAL BOUNDARIES IN THE WESTERN NORTH ATLANTIC OCEAN

This chapter has been prepared for submission to *Evolutionary Applications*.

Abstract

The implementation of sustainable management plans for chondrichthyans is critical because approximately one-third of these species are threatened with extinction and many are highly susceptible to fishing pressure. However, management is complicated by stocks that straddle and/or mix among the jurisdictions of multiple nations where the effectiveness of plans may vary considerably. The blacktip shark (*Carcharhinus limbatus*) is heavily exploited in the western North Atlantic Ocean and has been documented traveling through the waters of the United States, Mexico, Cuba, and The Bahamas, where shark fisheries management differs greatly. Therefore, the genetic structure and movement of blacktip sharks sampled in the waters of these nations were assessed using a reduced representation genomic approach. At least five genetically distinct units were identified using 772 young-of-the-year and small juveniles genotyped at 5,155 SNP-containing loci. Three units were found in the U.S. Atlantic and Gulf of Mexico, with a western Gulf stock that may straddle U.S. and Mexican waters. Distinct stocks found in Cuba and The Bahamas were highly genetically differentiated relative to the other stocks. Larger juveniles and adults (n = 455) were then assigned to their genetic stock of origin to determine if they had moved across stock boundaries. One individual captured in Cuba and four others captured in the U.S. Atlantic were assigned to the Gulf, indicating limited mixing. The results have important implications for shark fisheries and suggest that internationally-coordinated plans could facilitate sustainable management.

Introduction

Molecular genetic studies can inform fisheries management through the identification of reproductively independent management units (hereafter stocks) by describing how genetic variation is partitioned among and within geographic samples (Carvalho & Hauser, 1994). Independent stocks are affected more by local demographic processes (e.g., birth and mortality rates) than emigration and immigration, so understanding levels of migration/gene flow between stocks is of great interest (Waples et al., 2008). When gene flow between stocks is limited or absent, genetic drift causes allele frequencies at neutral loci to diverge, generating genetic stock structure that reflects (in part) the magnitude of reproductive isolation (Wright, 1931). While differences caused by genetic drift take many generations to accrue, only a few migrants per generation are required to erase/dampen signals of genetic structure (Mills & Allendorf, 1996; Spieth, 1974). Consequently, stocks that appear genetically homogenous may still be demographically independent (Carvalho & Hauser, 1994). By contrast, a sufficient period of reproductive isolation is required to produce detectable divergence, thus genetically distinct stocks are demographically independent and generally warrant management as separate units (Jamieson, 1973; Ovenden, 1990). In addition, because individuals may move between stocks without mediating gene flow (i.e., vagrants), individuals from genetically distinct stocks can mix seasonally and/or at specific points during the life cycle. Effectively harvesting mixed stocks to maximize sustainable yield from productive stocks while avoiding the depletion of at-risk stocks is a considerable challenge for managers (Waples et al., 2008). Therefore, studies that identify genetically distinct stocks and instances of stock mixing have the potential to reduce overexploitation by helping managers to determine both the optimal locations and periods for harvest.

In the last century, global landings of cartilaginous fishes (chondrichthyans) increased by more than 200% and peaked at approximately 870,000 tons in 2000 (FAO, 2022). Landings have since decreased, and while the trend is to some extent the result of population declines caused by exploitation, it is also due to the implementation of fisheries management plans (Davidson et al., 2016; FAO, 2010). However, the efficacy of management varies widely among the nations that contribute most to global landings of chondrichthyans and is often limited by available catch data (Fischer et al., 2012; Iglésias et al., 2010; Simpfendorfer & Dulvy, 2017). Developing nations identify just 17% of chondrichthyan landings to species or genus level, in contrast to 72% for more developed nations (FAO, 2014). More than 30 nations that are responsible for 64% of global landings have developed National Plans of Action for the conservation and management of chondrichthyans, but only 9% of landings occur in nations that have implemented sustainable plans (Davidson et al., 2016). Chondrichthyans are susceptible to fishing pressure because they grow slowly, mature late, produce few offspring per reproductive effort, and thus have lower intrinsic rates of population growth relative to most bony fishes (Cortés, 2004; Musick, 2005; Smith et al., 1998). As a result, approximately one-third of chondrichthyan species are thought to be threatened (Dulvy et al., 2021). Therefore, the identification and sustainable management of stocks are critical but are complicated by aspects of migratory and reproductive behavior that can cause stocks to straddle and/or mix among exclusive economic zones of neighboring nations (Fowler, 2012).

Aspects of shark behavior and movement that inform fisheries management have been elucidated using genetic approaches (Heist, 2005; Portnoy & Heist, 2012). Multiple studies indicate that females of many shark species remain in or return to their birth region for reproduction, a behavior known as female regional philopatry (Day et al., 2019; Karl et al., 2011;

Tillett et al., 2012). Philopatry has important implications for fisheries management because it can reduce gene flow across geographic distances within a species' dispersal potential (Chapman et al., 2015). For example, genetic structure associated with female philopatry was observed between regions that white sharks (*Carcharodon carcharias*) are capable of traversing (Bonfil et al., 2005; Pardini et al., 2001), suggesting gene flow is restricted due to behavior rather than limits on dispersal potential. Moreover, because many shark species display seasonal migrations and ontogenetic shifts in habitat use (Castro, 1993; Grubbs, 2010; Kohler & Turner, 2019), individuals that exhibit philopatry might mix with individuals from other stocks during particular times of the year and/or life stages. Shark movement has traditionally been studied by tracking individuals using mark-recapture and acoustic/satellite tags (Hammerschlag et al., 2011; Heupel & Hueter, 2001; Kohler & Turner, 2019). However, with sufficient divergence between genetic stocks, putative migrants/vagrants can be assigned to a stock of origin using genetic data (Manel et al., 2005), enabling the detection of sharks that have ventured outside of their natal region.

The blacktip shark (*Carcharhinus limbatus*) is distributed throughout the western North Atlantic Ocean, including the Gulf of Mexico (hereafter Gulf) and Caribbean Sea (Compagno et al., 2005), where it is heavily exploited by commercial, recreational, and artisanal fisheries (Pérez-Jiménez & Mendez-Loeza, 2015; SEDAR, 2020; Tavares, 2009). In waters of the United States, males and females mature sexually after five and six years (respectively), and females live birth one to eight pups (four on average) every two years (Baremore & Passerotti, 2013; Natanson et al., 2019), rendering the species vulnerable to localized depletion. The blacktip shark is highly migratory and has been documented traveling hundreds to thousands of kilometers through the jurisdictions of multiple nations (Kohler & Turner, 2019). Nevertheless, females display regional philopatry and some re-visit the same coastal areas in the spring/early

summer for parturition (Keeney et al., 2005; Swift et al., 2022). Young-of-the-year (YOY) remain within parturition sites until the fall of their first year when decreases in water temperature stimulate them to migrate south and/or offshore (Castro, 1996; Heupel, 2007; Heupel et al., 2004). Furthermore, tagging studies demonstrate that some juveniles return to the vicinity of their parturition site the following spring and repeat this seasonal migration for at least three years (Hueter et al., 2005).

Stock assessment and management plans for coastal species such as the blacktip shark vary considerably among neighboring nations in the western North Atlantic. The U.S. currently manages blacktip sharks as part of the aggregated large coastal shark complex in the Atlantic and as a separate, species-specific stock in the Gulf (SEDAR, 2018, 2020). Although multiple assessments found the species to be overfished and experiencing overfishing in U.S. waters (NMFS, 1998, 2006), more recent assessments determined that blacktip sharks are no longer overfished nor undergoing overfishing (SEDAR, 2012, 2018, 2020). Mexico officially classifies shark landings in two size-based categories (CONAPESCA, 2004), preventing the interpretation of fisheries data by species, and limiting the effectiveness of management (Pérez-Jiménez et al., 2020). As such, blacktip sharks are neither assessed nor managed separately. Similarly, in Cuba, limited data collection and a lack of specific regulations prevent the direct management of shark fisheries (MINAL, 2015). By contrast, shark fishing is prohibited in The Bahamas (Ward-Paige, 2017), thus blacktip sharks are in theory afforded full protection but may be harvested if they leave these waters.

Considering the potential for straddling and mixed stocks in the western North Atlantic, assessments of genetic structure and movement are vital to evaluate whether international coordination will benefit the sustainable management of coastal sharks. Therefore, genetic stock

structure and movement of blacktip sharks sampled in the waters of the U.S., Mexico, Cuba, and The Bahamas were examined using a reduced representation genomic approach. Tissues were collected from YOY and small juveniles thought to be residing within the region of their natal stock to ensure that genetic structure reflected differences among reproductive units. Samples were also collected from larger juveniles and adults to determine if older individuals move between stocks. By examining thousands of nuclear-encoded loci distributed throughout the genome, this approach allowed for the identification of genetically distinct stocks, detection of kin sampled within and between stocks, and assignment of individuals to genetic stocks of origin.

Methods

Fin clips were collected from 1,534 individual blacktip sharks captured in nearshore and offshore locations throughout the western North Atlantic in the exclusive economic zones of the United States, Mexico, Cuba, and The Bahamas. Fin clips were immersed in 20% DMSO-0.25M EDTA NaCl-saturated buffer (DMSO; Seutin et al., 1991), or ethanol before being transferred into DMSO, and stored at room temperature until DNA extraction. Sharks were captured year-round from 2007 to 2019. Sex, length measurements (e.g., fork length), and location of capture (latitude and longitude) were recorded for each individual. If fork length was not recorded, it was estimated based on published relationships between length measurements (Carlson et al., 2006) using a customized R script (v3.6.0; R Development Core Team, 2008).

Because blacktip sharks were captured at all life history stages, individuals that were assumed to have been sampled in their region of birth were used to define genetic stocks. Based on the evidence of regional fidelity by blacktip sharks as old as three (Hueter et al., 2005), individuals sampled within or near bays and estuaries and estimated to be less than four using

length-at-age data (fork length, Carlson et al., 2006) were categorized as putative residents (hereafter residents). For population genetics analyses, residents were grouped into 18 geographic samples nested within six regions. There were three samples in the U.S. Atlantic Ocean (hereafter Atlantic), four in the eastern Gulf, six in the western Gulf, two in the southwestern Gulf, one in Cuba, and two in The Bahamas (Table 5.1; Figure 5.1). The three regions in the U.S. (i.e., Atlantic, eastern, and western Gulf) were defined following Swift et al. (2022), and the U.S.-Mexico border was used to demarcate western and southwestern Gulf regions. Individuals sampled at locations that could not be assigned to a proximal bay or estuary, or estimated to be older than four, were not used to establish genetic stocks because they may have been captured outside of their birth region. Thus, these individuals were designated as putative non-residents (hereafter non-residents).

High molecular weight genomic DNA was extracted from fin clips using Mag-Bind® Blood and Tissue DNA Kits (Omega Bio-Tek) or phenol-chloroform extraction (Patrinos et al., 2017). A modified version of double digest restriction-site associated DNA sequencing (ddRAD; Peterson et al., 2012), as described by Swift et al. (2022), was used to prepare genomic libraries. To improve genotyping efficacy, a reduced genomic reference was assembled using an initial library of 27 individuals sequenced on a single Illumina MiSeq lane (paired-end, 300 bp). All other libraries were sequenced across 11 lanes on an Illumina HiSeq 4000 (paired-end, 150 bp). Raw sequences were demultiplexed using *process_radtags* (Catchen et al., 2011) and quality trimmed. MiSeq reads were assembled into a genomic reference as described by Swift et al. (2022). Read mapping and SNP (single nucleotide polymorphism) genotyping were performed for HiSeq reads from each library using the DDOCENT pipeline (v2.8.7; Puritz et al., 2014).

Raw SNPs were rigorously filtered using VCFTOOLS (v0.1.14; Danecek et al., 2011) and R functions following practices described by O'Leary et al. (2018) and phased into constituent haplotypes (i.e., SNP-containing loci, hereafter loci) using *rad_haplotyper* (Willis et al., 2017). Retained loci had a minimum mean depth of 17 and were called in at least 90% of individuals, 50% of a given library, and 80% of individuals in a given region. Technical replicates were included within and among multiple libraries and their composite genotypes compared to assess for locus-specific genotyping errors. Loci with genotyping errors in more than one pair of replicates, and one individual from each replicate pair, were removed. In addition, individuals with estimates of F_{IS} less than the first quartile minus 1.5x interquartile range were removed because low F_{IS} may indicate cross-contamination. Finally, to minimize library effects, individuals were grouped by library and BAYESCAN (Fischer et al., 2011; Foll et al., 2010; Foll & Gaggiotti, 2008) executed to identify and remove loci contributing to significant differences among libraries.

Pairwise relatedness was assessed for all individuals using the estimator described by Wang (2002) as implemented in *related* (Pew et al., 2015). Two litters and the mother of one litter were included to determine thresholds for identifying parent-offspring (POP), full-sibling (FS), and half-sibling (HS) relationships between regions. To prevent genetic stock structure from distorting allele frequencies used to estimate relatedness (Wang, 2011), pairwise relatedness was also assessed within each region individually using the same estimator but implemented in *demerelate* (Kraemer & Gerlach, 2017), allowing thresholds for identifying kin relationships to be set using 1,000 pairs of simulated relationships (e.g., HS and FS). Because different kin relationships can result in similar relatedness values (e.g., POP and FS: ~0.5; HS and avuncular: ~0.25), relationships were further evaluated by assigning individuals to cohorts

based on estimated ages using fork lengths, von Bertalanffy growth curves (Carlson et al., 2006), and dates of capture. Kin were assumed to be siblings if they were assigned to cohorts separated by six years or fewer, consistent with the estimated ages at which blacktip sharks mature sexually (Baremore & Passerotti, 2013; Natanson et al., 2019). The inclusion of non-randomly sampled siblings can bias estimates of allele frequencies used for genetic structure analyses (Waples & Anderson, 2017). Therefore, if sibling pairs were captured on the same day and were from the same cohort, one individual from each pair was removed from the dataset for all subsequent analyses.

Three methods were used to screen for loci putatively under directional selection using the resident dataset with individuals grouped by geographic sample. First, a method that does not make assumptions about population structure or demography but identifies F_{ST} outliers using an inferred distribution of neutral F_{ST} was implemented in *OutFLANK*, using default parameters (Whitlock & Lotterhos, 2015). Second, a Bayesian method that generates a null distribution of neutral F_{ST} using an island model was implemented in BAYESCAN (Fischer et al., 2011; Foll et al., 2010; Foll & Gaggiotti, 2008). This method was executed with prior odds of 1,000, a burn-in of 200,000 iterations, 25 pilot runs of 5,000 iterations, 40,000 sampling iterations, a thinning interval of 50, and significance was evaluated using a *q*-value of 0.05. Finally, the FDIST method (Beaumont & Nichols, 1996) was executed in ARLEQUIN (v3.5.2.2; Excoffier & Lischer, 2010) using an island model with 50,000 simulations, 100 simulated demes, with significance evaluated using α of 0.05 corrected for multiple comparisons (Benjamini & Hochberg, 1995). To account for false positives and ensure that genetic stock structure reflected demographic differences rather than those resulting from selection (Luikart et al., 2003), loci determined to be

F_{ST} outliers by more than one method were removed, and all downstream analyses were conducted using only putatively neutral loci.

To assess for genetic stock structure, hierarchical, locus-by-locus AMOVA was performed in ARLEQUIN using the neutral resident dataset, with *F*-statistics calculated as weighted means of locus-specific values to account for uneven levels of missing data among loci (Weir & Cockerham, 1984). Samples were grouped by region and significance assessed ($\alpha < 0.05$) by permuting individuals among samples 10,000 times and by bootstrapping the data 20,000 times to create 95% confidence intervals. Single-level, locus-by-locus AMOVA was then executed for each region separately and significance assessed as above. *Post-hoc* estimates of locus-by-locus pairwise F_{ST} between regions were subsequently calculated using ARLEQUIN, with 95% confidence intervals produced and significance assessed as above but corrected for multiple comparisons (Benjamini & Hochberg, 1995). To visualize genetic differences among regions, principal component analysis (PCA) was performed using *adegenet* (Jombart, 2008; Jombart & Ahmed, 2011), and two biplots were produced using the first three principal components (PCs). In addition, discriminant analysis of principal components (DAPC) with *K*-means clustering was used to identify and visualize the number of genetic groups (*K*= 1-6) based on the lowest Bayesian Information Criterion (BIC; Jombart et al., 2010). To avoid overfitting the data, cross-validation with training and test sets (90% and 10% of individuals, respectively) was used to determine the optimal number of PCs to retain based on mean assignment success across 30 replicates. Subsequently, DAPC was executed using the optimal number of retained PCs and individuals were determined to belong to a genetic group if they had membership probabilities > 95%.

The contemporary effective number of breeders (N_b) was estimated for each genetic stock using the neutral resident dataset and the linkage disequilibrium method (Hill, 1981) implemented in NeEstimator (v2.1; Do et al., 2014), with 0.02 used as the lowest allele frequency. In addition to point estimates, 95% confidence intervals were estimated using the parametric approach.

To assess for individual movements between stocks, non-residents were assigned to a putative genetic stock of origin using *assignPOP* (Chen et al., 2018). After removing neutral loci with variation in < 1% of individuals, 50 Monte-Carlo iterations were used to cross-validate stock baselines by splitting residents into training (75%) and test (25%) groups and determining the percentage of test individuals that were assigned to the appropriate training group. Assignment probabilities for non-residents to each stock were then calculated. Non-residents were assigned to a genetic stock of origin if they had assignment probabilities > 90% – and if captured in a region outside of the assigned stock – they were inferred to have moved between stocks. All figures were generated in R using *ggplot2* (Wickham, 2016).

Results

A total of 143 individuals were removed due to missing data and low read depth; 68 technical replicates were also removed along with 89 individuals that had excessively low F_{IS}. Further, 567 loci at which systematic genotyping errors were detected between replicates, 111 monomorphic loci, and 78 loci determined to be driving library effects were removed. The filtered dataset contained 1,234 blacktip sharks with 779 and 455 individuals considered residents and non-residents, respectively (Table 5.1).

No kin were identified between regions. Because the initial relatedness assessment found no kin within the southwestern Gulf or Cuba, and the sample sizes were small (i.e., 25 and 12

individuals, respectively), within-region relatedness was not assessed for these regions. For the other regions, the thresholds for identifying FS and HS (respectively) were largely consistent: Atlantic (0.41 and 0.20), eastern Gulf (0.41 and 0.20), western Gulf (0.42 and 0.20), and The Bahamas (0.43 and 0.21). Non-randomly sampled siblings were detected in the eastern (one full-sibling pair) and western Gulf (one full-sibling pair and a group of six full- and half-siblings). Randomly sampled kin were detected in the Atlantic, eastern Gulf, and The Bahamas, but not in the western Gulf. One half-sibling pair consisting of an adult male and adult female were sampled in the Atlantic. In the eastern Gulf, 25 kin pairs were detected within (21) and between (four) geographic samples (i.e., estuaries), including three FS and 22 HS pairs. In The Bahamas, 38 kin pairs were detected within (32) and between (six) samples (i.e., islands), including two POP, three FS, 25 HS, and eight putatively avuncular pairs (i.e., individuals with relatedness ~0.25 from cohorts separated by more than six years).

After the removal of non-randomly sampled kin, the resident dataset consisted of 772 individuals genotyped at 5,161 loci (2.57 alleles and 1.98 SNPs per locus on average). A total of 26 loci were determined to be putatively under directional selection by three methods: BAYESCAN was the most conservative (five loci), followed by *OutFLANK* (ten loci) and FDIST (18 loci). However, only six loci were identified by two or more methods, and these were removed to produce a putatively neutral dataset (5,155 loci).

Using the neutral resident dataset, hierarchical locus-by-locus AMOVA demonstrated significant heterogeneity among regions (i.e., Atlantic, eastern Gulf, western Gulf, southwestern Gulf, Cuba, and The Bahamas; $F_{CT} = 0.0076$; $p < 0.0001$) and among samples within regions ($F_{SC} = 0.0003$; $p < 0.05$; Table 5.2). Single-level AMOVA was then executed for each region except Cuba because it consisted of only one geographic sample. While homogeneity was

observed in the Atlantic (F_{ST} = 0.0000; p = 0.7629), western Gulf (F_{ST} = 0.0004; p = 0.0815), southwestern Gulf (F_{ST} = 0.0000; p = 0.8324), and The Bahamas (F_{ST} = 0.0004; p = 0.3158), heterogeneity was observed in the eastern Gulf (F_{ST} = 0.0004; p = 0.0034). However, when single-level AMOVA was executed for the eastern Gulf without randomly sampled siblings, significant heterogeneity was no longer observed (F_{ST} = 0.0003; p = 0.0737). *Post-hoc* estimates of locus-by-locus F_{ST} between regions demonstrated significant differences ($p < 0.001$; after correction) for all comparisons except eastern Gulf-southwestern Gulf (p = 0.0853) and western Gulf-southwestern Gulf (p = 0.4139; Table 5.3). Although the estimate of F_{ST} between the eastern and southwestern Gulf was not statistically significant, the magnitude was greater (F_{ST} = 0.00065) than the estimate between the eastern and western Gulf (F_{ST} = 0.00045), which was significant. This is in contrast to the F_{ST} estimate between the western and southwestern Gulf (F_{ST} = 0.00014), which was also not statistically significant but relatively small. Because it appeared likely that the non-significant difference observed between the eastern and southwestern Gulf was the result of the small sample size for the latter, five genetic stocks were considered in downstream analyses: Atlantic, eastern Gulf, western-southwestern Gulf, Cuba, and The Bahamas.

The biplot based on the first two PCs illustrated three groups corresponding to Atlantic-Gulf, Cuba, and The Bahamas, with subtle differences observed among Atlantic and Gulf regions (Figure 5.2A). These differences were easier to visualize when using the second and third PCs to plot Atlantic and Gulf individuals only (Figure 5.2B). Cross-validation indicated 200 retained PCs (out of 600) were optimal (100% assignment success) and *K*-means clustering resulted in two genetic groups corresponding with Atlantic-Gulf and Cuba-Bahamas; however, one individual from Cuba grouped with Atlantic-Gulf (Figure 5.3A). A second and third DAPC using

K-means clustering were then executed. The second included Atlantic and Gulf individuals only with a maximum of four groups allowed (one for each region). Cross-validation indicated 100 retained PCs (out of 600; 84% assignment success) were optimal, and although Atlantic and Gulf individuals mostly clustered into two distinct groups, some individuals from each Gulf region clustered with the Atlantic group (and vice versa; Figure 5.3B). The third DAPC included Cuba and Bahamas individuals only, with a maximum of three groups allowed (one for each geographic sample/island). Cross-validation indicated 5 retained PCs (out of 60; 98% assignment success) to be optimal and clustering split Cuba and The Bahamas into two groups, with a few Bahamas individuals grouping with Cuba (Figure 5.3C).

Though residents were assumed to have been sampled in their region of birth, the individual from Cuba flagged by the first DAPC was moved to the non-resident dataset for subsequent analyses to independently assess if it had moved between stocks. Cross-validation for the second and third DAPC indicated lower assignment successes (84% and 98%, respectively), suggesting less precise clustering. Therefore, Atlantic, Gulf, and Bahamas individuals flagged by these analyses were not moved to the non-resident data.

Because of the small sample size (seven individuals), no finite N_b estimates were obtained for Cuba. Finite N_b lower estimates were obtained for every other genetic stock, as were finite point and upper estimates, except for the western-southwestern Gulf (Table 5.4). The lower estimate of N_b for the western-southwestern Gulf was the largest (79,579), followed by the Atlantic (12,873), eastern Gulf (5,682), and The Bahamas (537). There was no overlap in N_b confidence intervals among stocks.

Cross-validation demonstrated high assignment precision and accuracy for genetic baselines of Cuba and Bahamas stocks (100% for both). Assignment for the Atlantic stock was

lower and less precise (82% on average), but for eastern and western-southwestern Gulf, assignments were much lower (~60% for both). Therefore, Gulf stocks were grouped as one and assignment accuracy reassessed, resulting in much higher accuracy and precision (95% on average; Figure 5.4). After assigning each non-resident a membership probability for each stock, five individuals were determined to have been sampled outside of their natal stock. Four non-residents captured in the Atlantic were assigned to the Gulf, and the individual captured in Cuba that was flagged by DAPC was also assigned to the Gulf.

Discussion

A reduced representation genomic approach was used to assess the genetic stock structure of blacktip sharks sampled throughout the western North Atlantic Ocean while examining individual movements between stocks. The results indicate there are at least three genetically distinct stocks in the United States and that the western Gulf stock likely straddles U.S. and Mexican waters. Genetic stocks are also present in Cuba and The Bahamas. Estimates of N_b for The Bahamas stock were considerably smaller than for all continental stocks, and while too few samples were collected to generate finite N_b estimates for Cuba, the overall data suggest this stock likely has very low N_b too. Five individuals were determined to have moved across stock boundaries; however, the majority of putative non-residents appear to have been sampled in the region of their natal stock. This study has important implications for fisheries management and suggests internationally-coordinated policies may help to maintain sustainable stocks of coastal sharks.

Three genetically distinct units of blacktip sharks were found in U.S. waters corresponding with Atlantic, eastern Gulf, and western Gulf stocks. Estimates of pairwise F_{ST} demonstrated significant divergence between the Atlantic, eastern Gulf, and western Gulf

regions (Table 5.3), and non-overlapping confidence intervals of N_b estimates provided further support for three genetic stocks (Table 5.4: Waples, 2010), consistent with findings of Swift et al. (2022). Principal component analysis and DAPC with *K*-means clustering (not employed by the previous study) provide further evidence of distinct Atlantic and Gulf stocks but do not illustrate differences between eastern and western Gulf stocks as well. Because this study assessed genetic structure using juveniles less than four years old – in contrast to Swift et al. (2022) which included only YOY – the finding of three genetic stocks supports the idea that blacktip sharks born in the Atlantic and Gulf generally reside in their birth region until they are approximately three years old (Hueter et al., 2005).

The results also indicate that the western Gulf stock potentially straddles U.S. and Mexican waters. Overlap was observed between western and southwestern Gulf individuals using PCA, and the estimate of pairwise F_{ST} between these regions was not significant and much lower than all other pairwise comparisons (Table 5.3). The finding of homogeneity among blacktip sharks sampled in U.S. and Mexican waters is in contrast to a previous study that found significant differences between samples from Texas and Mexico using mitochondrial DNA and nuclear-encoded microsatellites (Keeney et al., 2005). Individuals from Mexico in that study were collected from the northern Yucatán Peninsula (Laguna Yalahau), hundreds of kilometers northeast of Campeche (i.e., the closest state sampled in this study; Figure 5.1). Therefore, genetic differences between Yucatán and Texas could denote an area of transition between the Gulf and Caribbean Sea, as noted by the authors (Keeney et al., 2005). The lack of heterogeneity observed between western and southwestern Gulf samples in this study could be due to the relatively small sample size for Mexico (21) as compared to Texas (222). However, tagging data have shown blacktip sharks seasonally migrating from Louisiana and Texas to Mexican waters,

as far southeast as Campeche (Kohler & Turner, 2019). These movements could facilitate gene flow if females born in the U.S. frequently stray to Mexico to give birth and/or U.S. individuals breed with those born in Mexico. While blacktip sharks are known to give birth and mate in proximal locations in the U.S. Atlantic (Castro, 1996), mating grounds in the Gulf are unclear and could be discrete from estuaries used for parturition. Therefore, elucidating where mating occurs may help to interpret patterns of gene flow between the U.S. and Mexico. In addition, if YOY and small juveniles migrate south along the western Gulf coast in the fall – as they do in the eastern Gulf (Hueter et al., 2005) – some of the southwestern Gulf residents may have been born in U.S. waters because the majority (67%) were sampled in November. Thus, further study is necessary because it is unclear if the lack of genetic structure between U.S. and Mexican waters indicates a straddling stock or substantial mixing of YOY and small juveniles due to seasonal migration. To adjust for the potentially confounding effects of seasonal movement/mixing, additional samples from southern Gulf YOY and small juveniles captured during the summer would be necessary. Samples from Tamaulipas to Quintana Roo would also help to evaluate the extent of a straddling stock (if present) and the potential for a genetic break between the southern Gulf and Caribbean Sea.

Samples from Cuba and The Bahamas, the two island nations included in this study, were more differentiated from all other stocks (including each other) as compared to continental stocks. This could be visualized in both PCA and DAPC (Figure 5.2A and 5.3C, respectively) and validation of genetic baselines that indicated complete resolution between Cuba and The Bahamas and all other stocks (Figure 5.4). Further, estimates of pairwise F_{ST} that included Cuba or The Bahamas were at least six times greater than pairwise estimates that did not include either (Table 5.3). For example, estimated F_{ST} between the Atlantic and The Bahamas (F_{ST} = 0.0315)

was more than an order of magnitude greater than the estimate between the Atlantic and southwestern Gulf (F_{ST} = 0.0028), even though The Bahamas is approximately three times closer to the Atlantic coast than the Atlantic is to the southwestern Gulf. A similar disparity was observed by Gledhill et al. (2015) who found that Φ_{ST} between the Atlantic and The Bahamas was almost two-fold greater than Φ_{ST} between the Atlantic and Mexico (Yucatán).

The observations of increased divergence in island stocks relative to those along the continental shelf are consistent with the idea that deep water may act as a barrier to gene flow for coastal sharks (Duncan & Holland, 2006; Karl et al., 2012; Schultz et al., 2008). Blacktip sharks are usually found in waters < 50 m deep but are captured at depths of up to 100 m, albeit infrequently (SEDAR, 2020). The U.S. Atlantic coast is separated from The Bahamas and Cuba by stretches of deep water (> 600 m; Lynch-Stieglitz et al., 1999), which blacktip sharks may avoid to reduce the risk of predation by larger predators such as tiger sharks (*Galeocerdo cuvier*) and great hammerheads (*Sphyrna mokarran*; Kajiura & Tellman, 2016). However, a blacktip shark was documented moving from The Bahamas (Bimini) to Cuba (Kohler & Turner, 2019), likely via the Old Bahama Channel (sill depth: ~500 m). Moreover, tagging data have documented three blacktip sharks – including two juveniles – traveling from St. John in the U.S. Virgin Islands to the U.S. Atlantic coast, a journey of > 2200 km through the jurisdictions of multiple island nations and waters deeper than 2,000 m (Legare et al., 2020). By contrast, tagging data have not documented movements between the Atlantic and Gulf, nor between the eastern and western Gulf (Kohler & Turner, 2019). Therefore, factors in addition to movement and gene flow likely explain the patterns of genetic structure observed here.

Multiple kin pairs and genetic homogeneity were observed between Andros and Bimini, two islands in The Bahamas that are separated by ~100 km of shallow water (~5 m deep). In

Andros, ~60% of the blacktip sharks sampled were small juveniles, and the detection of mothers and offspring and siblings captured within and across cohorts indicate this area is likely used as nursery habitat (Heupel et al., 2007). Conversely, ~70% of blacktip sharks sampled around Bimini were adults, consistent with the idea that these waters are a mating ground for the species (Gledhill et al., 2015). Notably, the peak period of blacktip shark mating in The Bahamas is thought to be September-October (Gledhill et al., 2015), in contrast to the Atlantic and northern Gulf where mating peaks in May (Baremore & Passerotti, 2013; Castro, 1996). Consequently, if individuals move between Atlantic/Gulf and Bahamas stocks, differences in the timing of mating could contribute to the apparent lack of gene flow.

The genetic diversity of blacktip sharks in Cuba and The Bahamas is also likely to be impacted more by genetic drift as compared with continental stocks due to differences in population sizes. Estimates of contemporary N_b for The Bahamas (~500) were an order of magnitude smaller than estimates for all continental stocks (> 5,000; Table 5.4), and though N_b is not directly related to N_c, the measures appear relatively coupled in sharks (Portnoy et al., 2009). The inference that N_c is small in The Bahamas is further supported by the large proportion of randomly sampled kin (~0.2% of all pairwise relationships) which was ten times greater than in any other region (e.g., eastern Gulf: 0.02%). While complementary kinship and N_b results are lacking for Cuba due to the small sample size, the magnitudes of F_{ST} estimates involving Cuba suggest population size is potentially small there as well. Similar patterns of divergence were seen in the blacknose shark (*Carcharhinus acronotus*) sampled across the same geographic area, with estimates of long-term N_e much smaller (> 10 times) in The Bahamas than in the Atlantic and Gulf populations (Portnoy et al., 2014). Disparities in size and genetic diversity of island and mainland populations are well known and thought to be a consequence of founder effects and

limited habitat availability (Frankham, 1996, 1997). However, comparisons between island and mainland populations of marine organisms have received relatively little attention (Dawson, 2016). Therefore, a greater understanding of the factors contributing to decreased abundance and genetic diversity of island stocks is critical for management.

Though the majority of putative non-residents appear to have been sampled in the region of their natal stock, five blacktip sharks were determined to have moved between stocks based on their probability of membership (> 90%) to a genetic stock of origin distinct from the stock in which they were sampled. A male sampled in Cuba that was initially categorized as a putative resident (age < three years; fork length < 930 mm) clustered with Atlantic-Gulf using DAPC (Figure 5.3A) and was assigned to the Gulf stock based on a membership probability of 94.6%. Intriguingly, this was the only shark sampled from the northern coast of Cuba (Figure 5.1). When assignment to the eastern or western Gulf stock was assessed, a much higher membership probability was observed for the western Gulf (80% vs. 14%), suggesting the individual may have reached Cuba by crossing the Yucatán Channel rather than the Straits of Florida. The other individuals were found to have moved from the Gulf to Atlantic. All four were females estimated to be between five and 12 years old; two were sampled in July 2014 and the other two were sampled 16 days apart in September 2014. Notably, the individuals sampled in September were caught less than 1 km away from each other. Sexual segregation is widespread among sharks (Drymon et al., 2020; Klimley, 1987; Sims, 2005) and blacktip sharks often swim in single-sex schools (Kajiura personal communication). The species migrates seasonally along the Atlantic coast in polarized schools contained within larger aggregations (Kajiura & Tellman, 2016), beginning the northward phase in southeastern Florida in March (Castro, 2011). Evidence from a study of bonnethead sharks (*Sphyrna tiburo*) suggests coastal sharks navigate by orienting in

northward or southward directions using geomagnetic cues (Keller et al., 2021). Therefore, it is possible that the inferred movements from the Gulf to South Carolina occurred after a school strayed into southeastern Florida, oriented north, and subsequently joined larger aggregations migrating up the Atlantic coast.

The finding that multiple blacktip sharks moved between stocks suggests apparent barriers to coastal shark movement may be traversed more often than tagging studies have indicated. Evidence that a juvenile traveled from the Gulf to Cuba is consistent with tagging data that suggest expanses of deep water between continents and islands are passable, even for smaller individuals (Legare et al., 2020). Also, the idea that this individual reached Cuba via the Yucatán Channel is plausible because the Loop Current could facilitate movement from the eastern Yucatán Platform to the northwest coast of Cuba (Hamilton et al., 2019). Similarly, evidence that four females moved from the Gulf to Atlantic stock indicates deep water and/or constriction of the continental shelf off southeastern Florida do not completely constrain movement. Unidirectional movement from the Gulf to Atlantic has also been documented in blacknose sharks and is thought to be facilitated by the Florida Current which flows eastwards through the Straits of Florida and then to the north (Dimens et al., 2019). Because levels of gene flow sufficient to erase signals of genetic stock structure can be achieved with few migrants per generation (Mills & Allendorf, 1996; Spieth, 1974), the patterns of stock structure observed here among Atlantic, Gulf, and Cuba samples further support the notion that contemporary shark movements do not always equate with gene flow. Thus, distinguishing between vagrants and migrants is important for understanding dynamics within and between stocks (Dimens et al., 2019).

The results of this study have implications for the management of coastal shark stocks throughout the western North Atlantic Ocean. The Gulf stock subregions defined by NOAA Fisheries align with genetically distinct units, but are currently assessed as a single stock with separate landing quotas. There is some evidence of limited mixing between Atlantic and Gulf stocks, and additional mixing between eastern and western units is likely. NOAA Fisheries currently accounts for a potentially straddling stock of blacktip sharks in U.S. and Mexican waters by considering interdictions of Mexican boats in Texas waters and landings of "cazones" (i.e., small sharks) in state waters of Tamaulipas and Veracruz (SEDAR, 2018). However, the results presented here suggest that if a straddling stock exists, it may extend into Campeche, where fishing effort and shark landings are greatest (Castillo-Geniz et al., 1998). While additional research could help to determine the extent of straddling stocks and the location/timing of mixing, policies will not be effective unless they are implemented in the waters of all nations involved. Hence, there is likely a need for internationally-coordinated management.

The observed patterns of stock structure and movement between islands have several other management implications. First, the magnitude of divergence between U.S. stocks and The Bahamas/Cuba suggests the U.S. Caribbean stock – encompassing Puerto Rico and the U.S. Virgin Islands (Deangelis et al., 2008) – likely constitutes a fourth genetic stock in U.S. waters, though samples from these locations are necessary to assess this directly. Also, blacktip sharks in Cuba constitute a distinct genetic stock but detection of a vagrant from the Gulf may indicate mixing. Only 12 blacktip sharks were sampled in Cuba and while the detection of a vagrant among these could be extraordinary, it suggests that understanding the degree of mixing between Cuba and U.S. stocks is vital for management, especially given the lack of shark fisheries

regulations in Cuba (Ruiz-Abierno et al., 2021). Finally, the data indicate that island stocks (e.g., Cuba and The Bahamas) are small in size and receive very little (if any) contemporary gene flow, which is likely related to deep water barriers. By contrast, detection of kin and genetic homogeneity between The Bahamas samples suggests connectivity among islands is more likely if they are separated by shallow water. Consequently, the heterogenous bathymetry of the Caribbean Sea could generate complex patterns of genetic structure among islands due to isolating deep water channels and shallow corridors that facilitate gene flow. Considering the likelihood of low abundance and evolutionary potential, Caribbean stocks that are currently subject to limited management (Hacohen-Domené et al., 2020; Tagliafico et al., 2021) might require a combination of multinational and individually-tailored policies to prevent localized depletion. Therefore, future studies should assess the genetic structure of coastal sharks in the Caribbean because these species help to maintain marine ecosystems and are critical to local economies.

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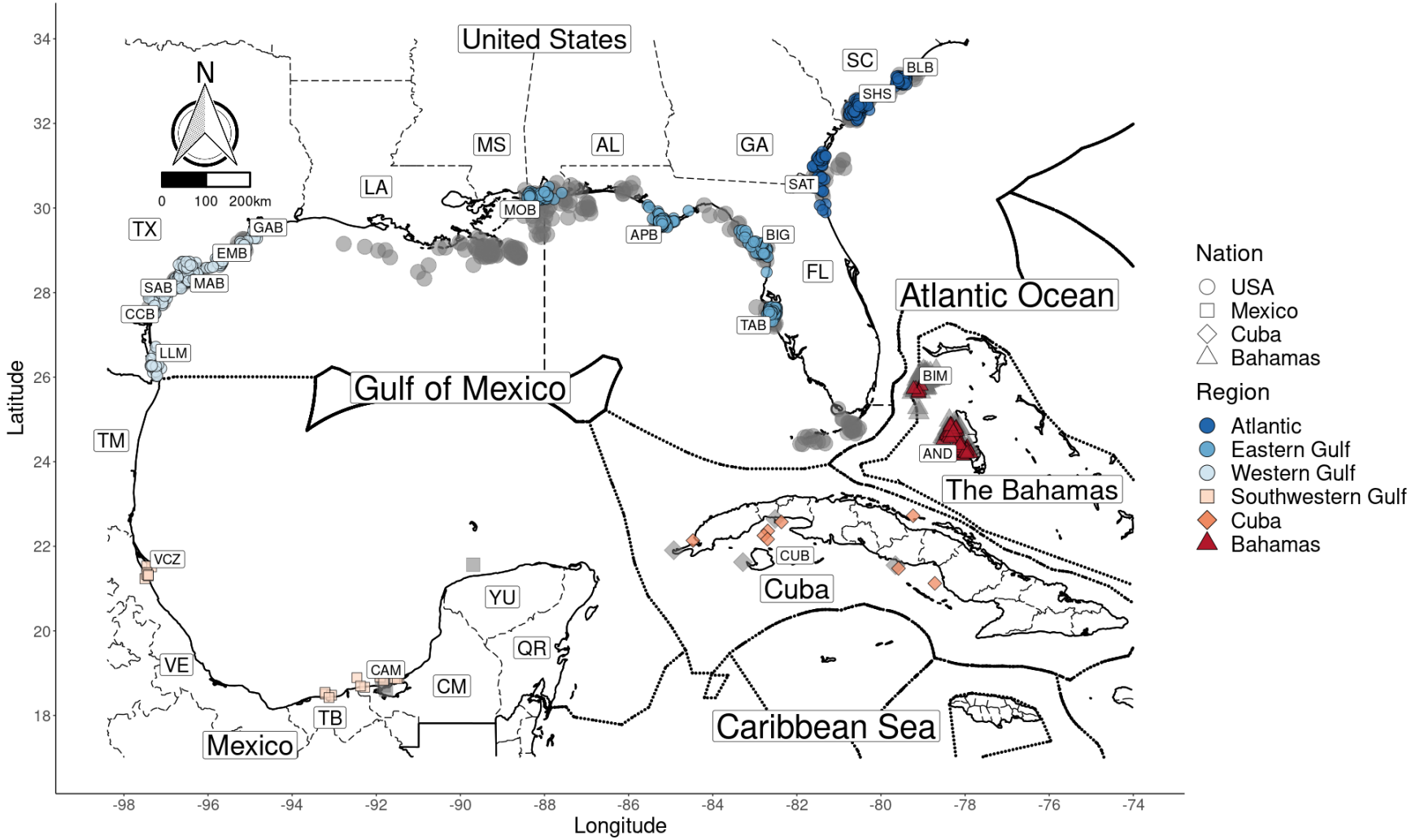
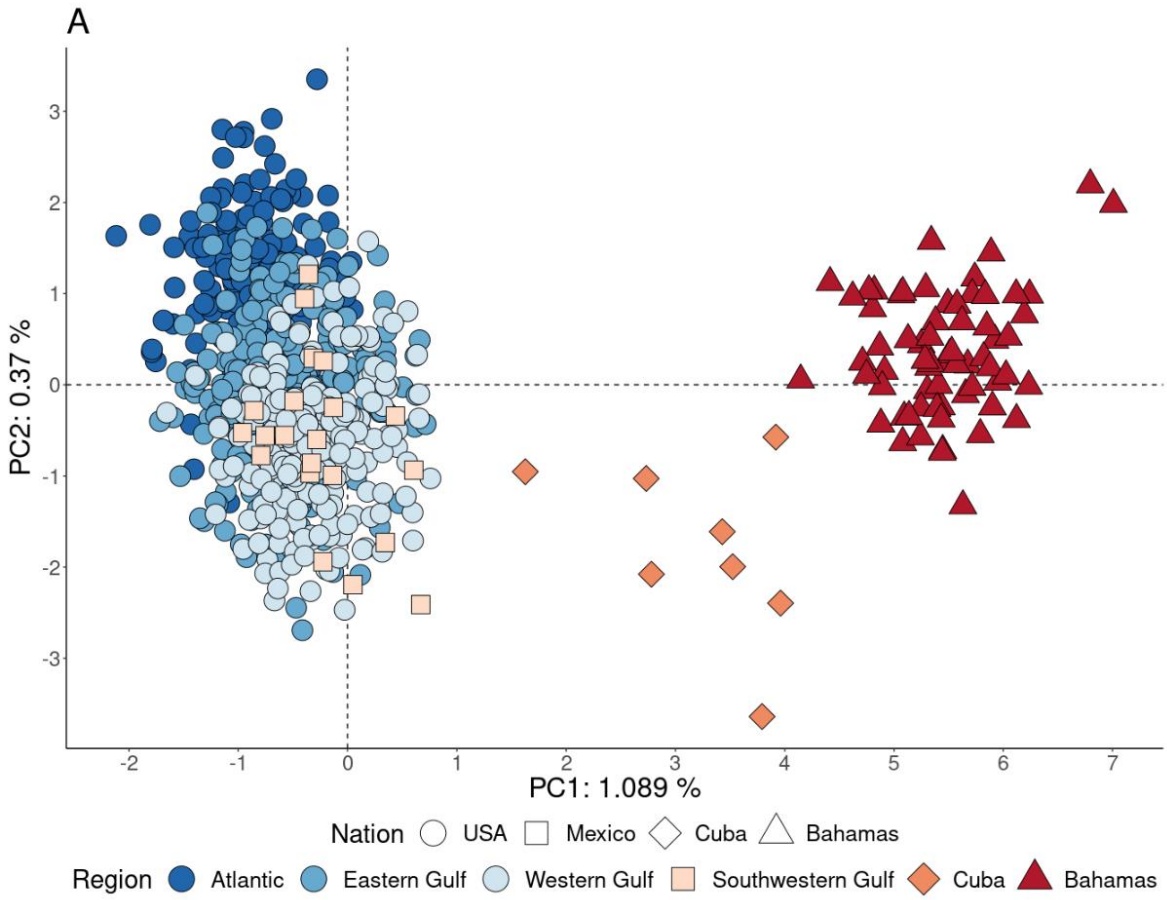


Figure 5.1. Blacktip shark (*Carcharhinus limbatus*) sampling locations in the exclusive economic zones of the United States, Mexico, Cuba, and The Bahamas. Regions and nations where putative residents were sampled are denoted by colors and shapes, respectively. Sampling locations for putative non-residents are colored grey and denoted by nation. Three-letter codes denote geographic samples of

residents: Bulls Bay (BLB), Saint Helena Sound (SHS), South Atlantic (SAT), Tampa Bay (TAB), Big Bend (BIG), Apalachicola Bay (APB), Mobile Bay (MOB), Galveston Bay (GAB), East Matagorda Bay (EMB), Matagorda Bay (MAB), San Antonio Bay (SAB), Corpus Christi Bay (CCB), Lower Laguna Madre (LLM), Veracruz (VCZ), Campeche (CAM), Cuba (CUB), Andros (AND), Bimini (BIM). Two-letter codes denote states in the U.S. and Mexico.


A
PC2: 0.37 %
PC1: 1.089 %
Nation USA Mexico Cuba Bahamas
Region Atlantic Eastern Gulf Western Gulf Southwestern Gulf Cuba Bahamas

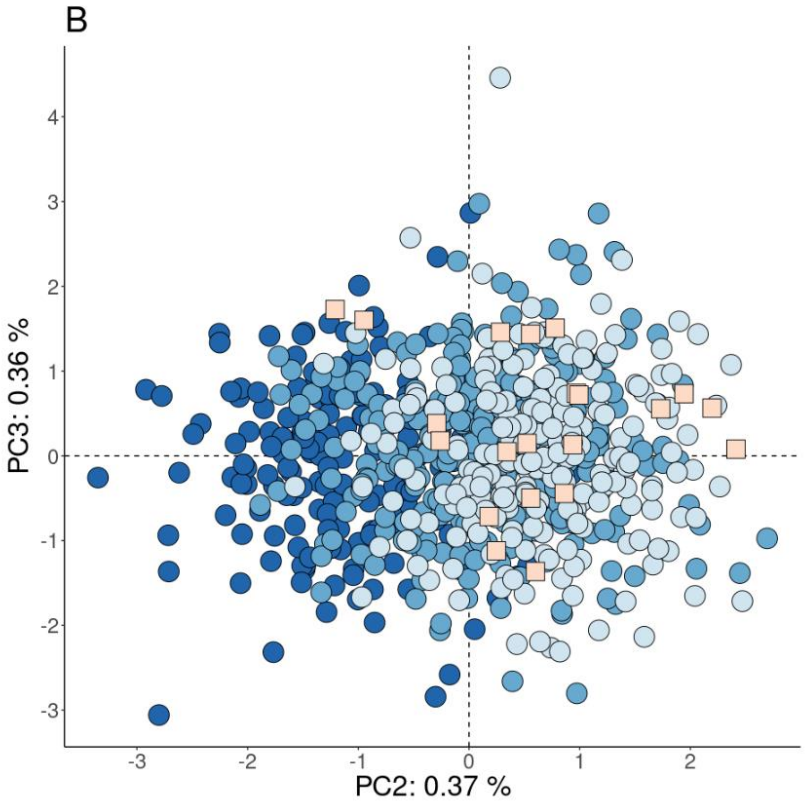

B
PC3: 0.36 %
PC2: 0.37 %

Figure 5.2. Biplots from principal component analysis of the neutral resident dataset with individuals grouped by region. Regions and nations are denoted by colors and shapes, respectively. A) All residents structured using the first two principal components. B) Atlantic, eastern Gulf, western Gulf, and southwestern Gulf residents structured by the second and third principal components.

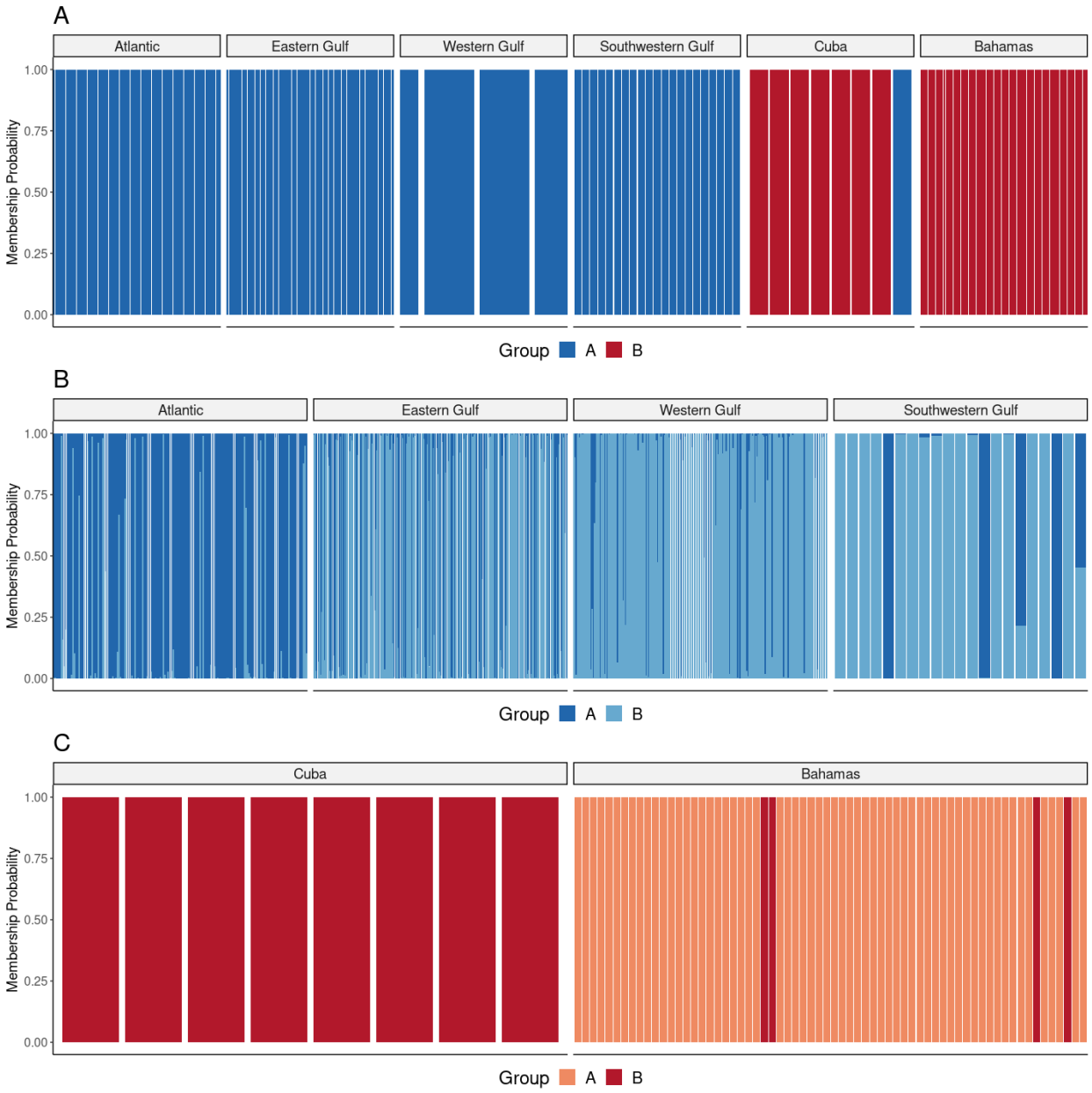


Figure 5.3. Individual membership probabilities from discriminant analysis of principal components with *K*-means clustering for the neutral resident dataset. A) All residents; B) Atlantic and Gulf residents only; C) Cuba and Bahamas residents only.

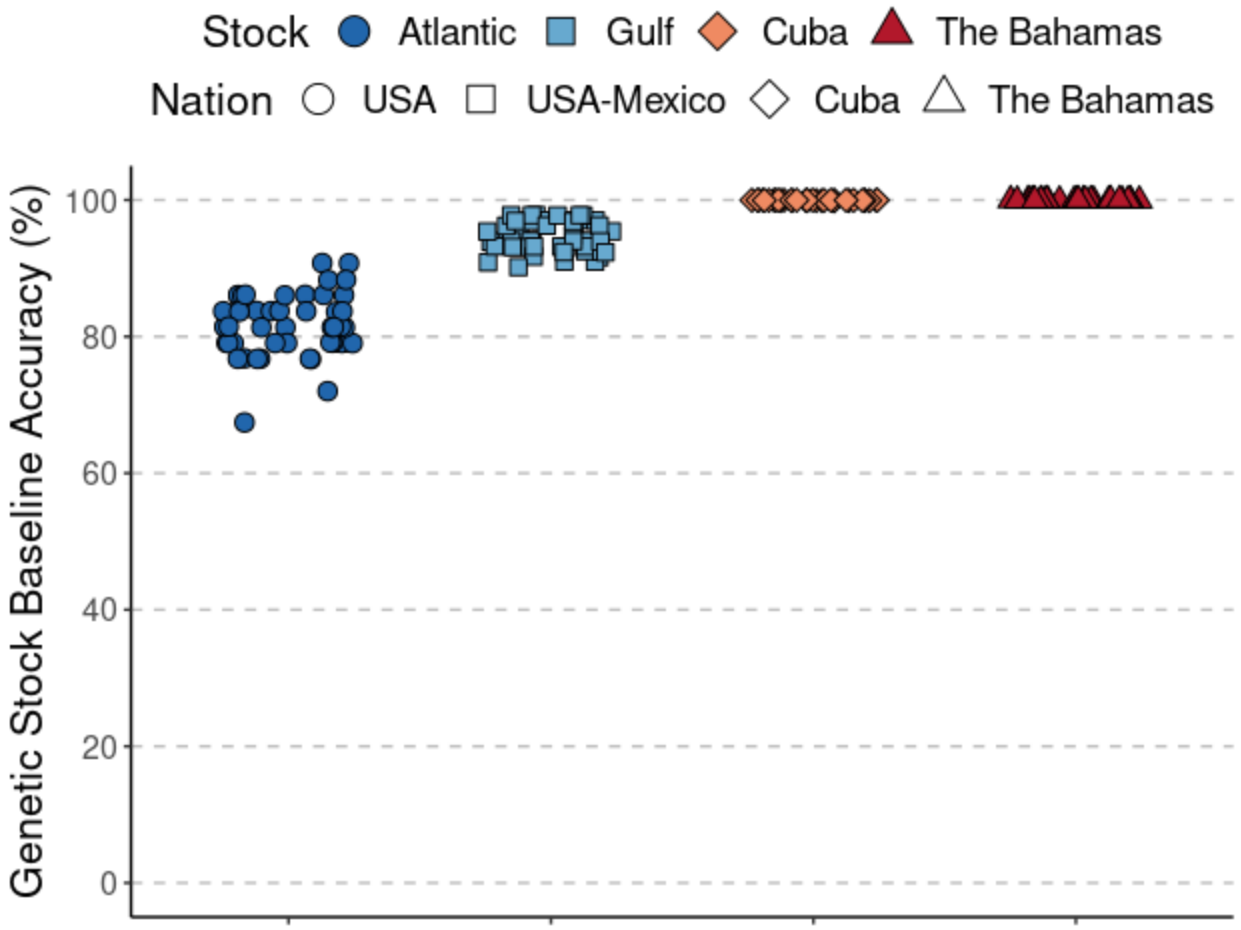


Figure 5.4. Assignment scores from cross-validation of genetic stock baselines used to assign putative non-residents to a genetic stock of origin. Regions and nations are denoted by colors and shapes, respectively.

Table 5.1. Numbers of putative resident and non-resident blacktip sharks (*Carcharhinus limbatus*) sampled in the western North Atlantic Ocean by geographic sample, region, and nation.

Sample	Residents	Region	Residents	Non-residents	Nation	Residents	Non-residents	Total
Bulls Bay	49	Atlantic	172	71	USA	684	343	1027
Saint Helena Sound	73							
South Atlantic	50							
Tampa Bay	127	Eastern Gulf	284	147				
Big Bend	52							
Apalachicola Bay	62							
Mobile Bay	43							
Galveston Bay	17	Western Gulf	228	125				
East Matagorda Bay	13							
Matagorda Bay	62							
San Antonio Bay	83							
Corpus Christi Bay	33							
Lower Laguna Madre	20							
Veracruz	7	Southwestern Gulf	21	4	Mexico	21	4	25
Campeche	14							
Cuba	8	Cuba	8	4	Cuba	8	4	12
Andros	60	Bahamas	66	104	The Bahamas	66	104	170
Bimini	6							
All	779	All	779	455	All	779	455	1234

Table 5.2. Hierarchical (all) and single-level (region) locus-by-locus AMOVA using the neutral resident datasets. Underlined *p*-values denote statistically significant heterogeneity; * denotes lower 2.5% of bootstrapped *F*-statistics were greater than zero.

Samples	Source of Variation	Variance Components	Percent Variation	*F*-statistic	*p*-value
All	Among regions	2.22088	0.75985	0.0076	0.0001*
	Among samples within regions	0.0836	0.0286	0.0003	0.0323*
Atlantic	Among samples	-0.0241	-0.0084	0.0000	0.7629
	Among individuals within samples	287.3920	100.0084	-	-
Eastern Gulf	Among samples	0.1269	0.0437	0.0004	0.0034*
	Among individuals within samples	290.2285	99.9563	-	-
Eastern Gulf (without randomly sampled siblings)	Among samples	0.0765	0.0263	0.0003	0.0737
	Among individuals within samples	290.3855	99.9737	-	-
Western Gulf	Among samples	0.1021	0.0348	0.0004	0.0815
	Among individuals within samples	292.7759	99.9652	-	-
Southwestern Gulf	Among samples	-0.3420	-0.1170	0.0000	0.8324
	Among individuals within samples	292.6117	100.1170	-	-
The Bahamas	Among samples	0.1099	0.0384	0.0004	0.3158
	Among individuals within samples	285.9353	99.9616	-	-

Table 5.3. Estimates of locus-by-locus pairwise F_{ST} values (lower) between regions and associated *p*-values (upper) produced using the neutral resident dataset. Underlined and bold denote statistically significant comparisons before and after correction for multiple comparisons, respectively. * denotes the lower 2.5% of bootstrapped F_{ST} values were greater than zero.

	Atlantic	Eastern Gulf	Western Gulf	Southwestern Gulf	Cuba	The Bahamas
Atlantic	-	**<0.001***	**<0.001***	**<0.001***	**<0.001***	**<0.001***
Eastern Gulf	0.00159	-	**<0.001***	0.085	**<0.001***	**<0.001***
Western Gulf	0.00257	0.00045	-	0.414	**<0.001***	**<0.001***
Southwestern Gulf	0.00283	0.00065	0.00014	-	**<0.001***	**<0.001***
Cuba	0.02694	0.02238	0.01922	0.01840	-	**<0.001***
The Bahamas	0.03130	0.02809	0.02612	0.02629	0.01850	-

Table 5.4. Lower, point, and upper estimates of contemporary effective number of breeders (N_b) for each genetic stock calculated using NEESTIMATOR. Lower and upper values are based on 95% confidence intervals determined using the parametric approach. n denotes the number of individuals genotyped.

Stock	Nation	n	Lower N_b Estimate	N_b Point Estimate	Upper N_b Estimate
Atlantic	USA	172	12,873	17,970	29,709
Eastern Gulf	USA	283	5,682	6124	6,639
Western-Southwestern Gulf	USA-Mexico	243	79,579	inf	inf
Cuba	Cuba	7	inf	inf	inf
Bahamas	The Bahamas	66	537	558	580

CHAPTER VI: CONCLUSIONS

This dissertation focused on studying reproductive strategies of elasmobranchs to address knowledge gaps, identify areas for further study, and inform management. Genetic data were generated for the blacktip shark (*Carcharhinus limbatus*) to assess for polyandry, evidence of mate choice, and examine how philopatry influences population structure at multiple scales. The results build upon previous studies and provide insights into how reproductive strategies can generate, maintain, and disperse adaptive variation. The research provides a foundation for additional studies and has important implications for the conservation of elasmobranchs. To conclude, the major findings of each chapter and potential avenues for future research are discussed.

Identifying Knowledge Gaps

High-throughput sequencing approaches have considerably improved our understanding of vertebrate reproduction (Long, 2020; Van Dyke et al., 2014); however, a lack of genomic data for elasmobranchs has resulted in comparatively few studies of these species. For example, MHC has been shown to influence mate choice via pre- or post-copulatory processes in every group of jawed vertebrates except elasmobranchs (Kamiya et al., 2014). This is a consequence of the challenges in accurately genotyping a sufficient number of individuals at these hypervariable genes without reference genomes or transcriptomes. The prevalence of genetic polyandry suggests females benefit from mating with multiple males, but these benefits are difficult to discern without data for MHC or other genes associated with fitness. Studies of elasmobranch anatomy and copulatory behavior indicate the potential for females to exert mate choice through a variety of mechanisms that could involve MHC. Thus, future research should examine the influence of MHC on elasmobranch mating systems.

Studies of elasmobranchs have demonstrated that some species use nurseries that may increase juvenile survival. However, these studies are biased towards coastal species, with little evidence of nurseries in pelagic or demersal benthic habitats (Heupel et al., 2019). Molecular studies indicate that elasmobranchs display different degrees of philopatry to nurseries used for parturition: some return to nurseries within the region of their birth whereas others may re-use their own nursery (Feldheim et al., 2014; Keeney et al., 2005). Philopatry has important implications for management because it can contribute to patterns of variation within and among populations. Therefore, studies that elucidate the diversity of species using nurseries and the influence of philopatry on population structure can inform management policies. Moreover, studies of elasmobranchs have started to examine the navigational mechanisms that might mediate philopatry (Keller et al., 2021; Newton & Kajiura, 2020). The application of high-throughput techniques to study navigation in salmonids provides a basis for future studies that may help to determine how elasmobranchs locate nurseries.

Mate Choice

Blacktip shark females, each of their offspring, and adults were genotyped at four microsatellites and two MHC genes (*mhc1a* and *b2m*) to assess for polyandry and evidence of MHC-associated mate choice. Multiple sires were detected for each litter and evidence of assortative mating for *mhc1a* was observed for four of six litters. Results from microsatellites and *b2m* suggest that offspring were not the result of inbreeding, indicating assortative mate choice. This chapter provides the first evidence of MHC involvement in elasmobranch mate choice, though the exact mechanism cannot be determined.

While preliminary, this finding is consistent with aspects of blacktip shark biology and provides insight into the potential genetic benefits of polyandry. Blacktip sharks can hybridize

with closely related species (i.e., *Carcharhinus tilstoni*) and show evidence of local adaptation to nurseries (Swift et al., 2022). Assortative mate choice can limit hybridization/outbreeding and the subsequent disruption of co-adapted allele complexes (Palumbi, 1999; Yeates et al., 2009). Thus, mate choice by blacktip sharks for conspecifics with similar MHC alleles may provide benefits via the maintenance of locally-adapted allele complexes. However, the presence of null alleles for *mhc1a* means the results require validation.

Philopatry

Young-of-the-year (YOY) blacktip sharks were genotyped at neutral (4,298) and putatively adaptive (70) SNP-containing loci to examine the influence of philopatry on genetic population structure at multiple spatial scales. Neutral structure demonstrated the presence of three genetically distinct units in the U.S. Atlantic and Gulf of Mexico with limited gene flow occurring between them. In contrast to results from a previous assessment (Keeney et al., 2005), this indicates that both males and females reproduce within the region of their birth (i.e., regional philopatry). The result has important implications for the management of blacktip shark fisheries. While the three genetic units align with regional stocks, blacktip sharks are currently assessed as a single Gulf stock (SEDAR, 2018) with distinct landing quotas in the eastern (37.7 metric tons) and western Gulf regions (347.2 metric tons). Notably, a similar disparity was observed between eastern and western Gulf units in estimates of the effective number of breeders (3,148 vs. 44,094, respectively), supporting the idea of genetically distinct Gulf stocks (Waples, 2010).

Adaptive structure correlated with variation in sea surface salinity and temperature among coastal areas encompassing parturition sites was observed within each Gulf unit. This indicates local adaptation that might be related to differences in salinity tolerance and/or timing of emigration from parturition sites in the fall. However, assessments of putative function are

precluded by the lack of a reference genome. It is also possible that the pattern is related to other conditions that vary with latitude but were not included in the environmental data. Notably, the observed adaptive structure cannot be explained by regional philopatry but may be a consequence of finer-scale female philopatry. In one estuary, five pairs of half-siblings were detected two and four years apart, providing strong evidence that females re-used the habitat for parturition. Fidelity to parturition sites can extend across generations and result in females giving birth in the same habitat in which they were born (i.e., natal philopatry), as documented in the lemon shark (*Negaprion brevirostris*; Feldheim et al., 2014). Natal philopatry could help to generate adaptive structure among neighboring parturition sites if alleles adapted to local conditions confer phenotypes with greater fitness. Considering the high rates of mortality experienced by YOY blacktip sharks and the environmental heterogeneity among estuaries (Heupel & Simpfendorfer, 2002), localized selection may be particularly strong during the YOY/juvenile phase of nursery use (Kawecki & Ebert, 2004). Consequently, selection would counteract gene flow of maladapted alleles from neighboring parturition sites carried by straying females and/or patrilines, and generate the fine-scale adaptive structure observed here. Taken together, the results demonstrate the differential influences of philopatry on genetic structure and highlight the importance of conserving a variety of habitats to facilitate the accumulation of adaptive variation that may increase resilience to environmental change.

Conservation Genomics

The genetic stock structure of juvenile blacktip sharks captured throughout the western North Atlantic Ocean was assessed while examining the movement of larger individuals between stocks. Consistent with results from the previous chapter, at least three genetically distinct stocks were found in U.S. waters, supporting the notion that blacktip sharks remain within their birth

region for several years (Hueter et al., 2005). The results also indicate that the western Gulf stock straddles waters of the U.S. and Mexico as far south as Campeche, but further research is needed to disentangle the effects of seasonal movement from gene flow. In addition, stocks observed for Cuba and The Bahamas were highly genetically distinct relative to the other stocks. Estimates of the effective number of breeders for The Bahamas were significantly smaller than for all continental stocks, and though too few samples were collected to generate finite estimates for Cuba, this stock likely has very few breeders as well. The high degree of genetic divergence between island and continental stocks is likely because of barriers to gene flow and stronger drift for island stocks due to smaller census sizes. While the majority of larger individuals were sampled in their natal stock, five blacktip sharks were determined to have moved across stock boundaries. Four females sampled in the U.S. Atlantic and a male sampled in Cuba were assigned to the Gulf stock, indicating the potential for mixing. However, because few migrants per generation can erase signals of genetic structure (Mills & Allendorf, 1996), these individuals are most likely vagrants.

The results have important implications for the management of coastal sharks in the western North Atlantic. When assessing the Gulf blacktip stock, NOAA Fisheries considers landings by Mexican fishers in the waters of Tamaulipas and Veracruz (SEDAR, 2018); however, if a shared stock exists, it likely extends into Campeche waters where shark landings are greatest (Castillo-Geniz et al., 1998). As a result, management may be less effective unless similar policies are implemented in the waters of both nations. Furthermore, the magnitude of divergence between stocks in the U.S., The Bahamas, and Cuba suggests the U.S. Caribbean Stock – currently assessed as part of the Gulf – likely constitutes a fourth stock in U.S. waters. The detection of a Gulf vagrant in Cuba could be unusual, but the potential for mixing between

these nations is noteworthy given the lack of shark fisheries management in Cuba. Finally, correlation between patterns of bathymetry and genetic structure among islands suggests that coastal sharks in the Caribbean Sea might exhibit complex stock structures. Therefore, stocks for island nations may require specific and multinational management to prevent localized depletion.

Future Directions

Greater availability of genomic and transcriptomic data would benefit molecular studies of elasmobranch reproductive strategies. These data can be used to design primers for genes putatively involved in mate choice/offspring viability and to examine functional properties of loci associated with local adaptation. While such resources would be particularly beneficial if used as species-specific references, the current paucity of elasmobranch genomic data means that "taxonomic gaps" should be addressed by generating high-quality genome assemblies that facilitate research of commonly studied taxa.

There is considerable potential for further research into elasmobranch mate choice. Future studies should first focus on comprehensively assessing MHC diversity to design suitable primers. If this can be accomplished for a variety of elasmobranchs, it might be possible to examine how patterns of mate choice vary among species with different life histories and copulatory behaviors. This would provide insights into the potential genetic benefits of polyandry, enabling the research focus to move beyond convenience polyandry. Furthermore, examining mate choice by species held in captivity will allow for putative mechanisms of choice to be investigated. Approaches using artificial insemination enable researchers to disentangle pre- and post-copulatory processes from differential offspring mortality and provide a compelling direction for further study.

The hypothesis of natal philopatry by blacktip sharks should be examined by assessing relatedness among individuals captured within nurseries over many years. Generating sequence data for a subset of neutral loci that reliably discriminate kin from unrelated individuals would facilitate this assessment by enabling many hundreds of individuals to be genotyped with comparatively little sequencing effort. Such an approach could yield the second direct demonstration of natal philopatry by an elasmobranch by detecting mother-offspring relationships among YOY and juveniles. Similar approaches could be used to examine how movement and habitat use differ between continental and island populations. Relatedness assessments can provide insights into fidelity/philopatry to nurseries – as well as movements to/from these habitats – by enabling the identification of kin and individual recaptures. These data can also be used to estimate the number of females giving birth in specific nurseries (Bravington et al., 2016; Waples & Feutry, 2021). Therefore, long-term projects that detect recaptures and kin among individuals sampled in continental and island nurseries have the potential to describe patterns of movement and changes in abundance – both of which are critical to the development of conservation plans.

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