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MATING SYSTEM IN A CAPTIVE POPULATION OF A DASYATID RAY, THE SOUTHERN STINGRAY (*Hypanus americanus* Hildebrand and Schroeder 1928), AT THE NATIONAL AQUARIUM, BALTIMORE, MARYLAND

by

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THESIS APPROVAL PAGE

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ABSTRACT

The southern stingray, *Hypanus americanus*, is a common coastal ray, ranging from New Jersey to Brazil, including the Gulf of Mexico and Caribbean Sea. It is an important resource for ecotourism in the Bahamas and Cayman Islands. It is a hardy species that survives many years as well as commonly reproduces in captivity. Despite the fact that many aspects of its life history have been described from captive as well as wild conspecifics, the mating system is not well understood. Behavioral polyandry has been well documented in both groups. While genetic polyandry or multiple paternity appears to be common in many species of elasmobranchs, it has not been reported in the southern stingray. The goal of this investigation is to document multiple paternity as an aspect of the mating system in this species based upon a population of reproductively active southern stingrays that were maintained at the National Aquarium. Amplicon deep-sequencing using next generation sequencing (NGS) technology was used to determine multiple paternity. The two-step PCR approach combined Illumina's dual indexing strategy and species-specific primers. Results confirmed multiple paternity, and that two to three potential sires were involved. Since it was documented in this group of captive rays, it is likely to occur in wild conspecifics given the similar polyandrous mating events. Although the southern stingray is not a species of concern for extinction, it is an important consideration for population management. This investigation provided the first documented case of multiple paternity in a dasyatid ray, and only the second case in a viviparous batoid.

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INTRODUCTION

1.1 CHONDRICHTHYAN FISHES

Cartilaginous fishes are in the class Chondrichthyes and consist of sharks, batoids (rays) and chimaeras. They are diverse with approximately 1,250 living recognized species and are distinguished from the bony fishes, class Osteichthyes, by having a true upper and lower jaw, an internal cartilaginous skeleton, and four to seven internal and/or external gill openings. Although cartilaginous fishes are less diverse than bony fishes, they are more diverse than all the other groups of marine vertebrates which include: jawless fishes, marine reptiles, birds, and mammals (Compagno *et al*. 2005). They occupy a large range of habitats and niches in every marine environment and even some freshwater environments. They can be found from coral reefs to lagoons and estuaries, coastal waters to the pelagic ocean, and waters of the continental shelf to depths of over 4,000m. Some species are resident part of or the entire year in polar waters. Most chondrichthyans are found on the continental and insular shelves and slopes and in the open ocean, with some endemic to small areas, or confined to narrow depth ranges. The greatest diversity occurs in the Indian Ocean and western Pacific Ocean. Some large-sized coastal and oceanic species are wide-ranging and make extensive migrations across ocean basins. Most favor temperate to tropical seas, but approximately 5% of species live in freshwater (Bigelow & Schroder 1948; McEachran 1982; Compagno 1999, Musick 2005).

Other notable characteristics in Chondrichthyes include: uniformly covered body with small dermal denticles or placoid scales, teeth in replicate rows, lacking lungs or swim bladder, spiral valve, modified pelvic fins called claspers (used for reproduction) in males, internal fertilization, and modes of embryonic nutrition ranging from retained oviparity to placental viviparity (Hamlett 1999). All species are carnivorous and predominantly predatory; however, some are scavengers or filter feeders. Sharks and rays are meso to apex predators, positioned at the middle to highest levels of the aquatic food chain. Their prey spans the food chain to include zooplankton, invertebrates, bony fishes, other chondrichthyans, and large marine mammals or reptiles. In addition to visual, gustatory, auditory, and olfactory senses, chondrichthyan fishes have highly developed mechanoreception and electroreception systems, the lateral line (detects movements and vibrations) and the ampullae of Lorenzini (detects weak electrical fields emitted by living prey or anything moving through the Earth's field). They have been shown to have a high brain weight to body weight ratio (Bigelow & Schroder 1948; McEachran 1982; Compagno 1999; Last *et al.* 2016b). It is easy to underestimate the diversity of living cartilaginous fishes and according to Stevens *et al.* (2005), there is a danger in not recognizing chondrichthyan diversity because it could decline with little notice.

1.1.1 Taxonomy

Chondrichthyan fishes have undergone several taxonomic revisions, based on morphological, as well as, molecular bases primarily at the order level and below (Compagno 1973, 1977; Douady *et al.* 2003; Aschliman *et al*. 2012; Naylor *et al.* 2012).

New species are discovered and described on a regular basis (Last *et al*. 2016b). The class contains more than 1,250 species, consisting of approximately 50 species of ghost sharks, silver sharks, elephant fish, chimaeras or ratfish (order Chimaeriformes), more than 600 species of batoids, flat sharks, or winged sharks and greater than 500 species of non-batoid traditional sharks (Compagno *et al*. 2005; Last *et al.* 2016b).

The class Chondrichthyes is divided into two subclasses, Holocephali and Elasmobranchii (Compagno 1973, 1977). The subclass Holocephali contains a single living order, Chimaeriformes – modern chimaeras. Elasmobranchii, sharks and rays, can be subdivided into three superoders, Squalomorphii, Galeomorphii, and Batoidea, known as squalomorph sharks, galeomorph sharks, and batoids or rays, respectively. Within the superorder Galeomorphii there are four orders: Heterodontiformes (bullhead sharks), Lamniformes (mackerel sharks), Orectolobiformes (carpet sharks), and Carcharhiniformes (ground sharks). Within the superorder Squalomorphii, there are four orders of sharks: Hexanchiformes (cow and frilled sharks), Squaliformes (dogfish sharks), Squantiniformes (angel sharks), and Pristiophoriformes (sawsharks). The superorder Batoidea includes four orders: Rajiformes (skates), Myliobatiformes (rays), Rhinopristiformes (shovel nose rays, guitarfish, and sawfish), and Torpediniformes (electric rays) (Douady *et al*. 2003; Naylor *et al*. 2012, 2016).

There has been great debate over elasmobranch classification and their interrelationships in the last few decades and contemporary taxonomic specialists are largely divided in their views on chondrichthyan classification (Last 2007). Multiple interpretations have been proposed in scientific literature based on morphological characteristics (Compagno 1973, 1977, 1999; Shirai 1996; de Carvalho 1996; McEachran *et al.* 1996; McEachran & Dunn 1998; Naylor *et al*. 2012) and molecular studies (Douady *et al*. 2003; Dunn *et al*. 2003; Velez-Zuazo & Agnarsson 2011; Winchell *et al.* 2004; Naylor *et al.* 2012; Last *et al.* 2016a). Molecular research has both confused and added weight to these debates. At present, there is no agreement regarding higher systematics (Last 2007; Rocco *et al*. 2007; Naylor *et al*. 2016). According to Naylor *et al.* (2012), interest in elasmobranch biodiversity and taxonomy has grown in recent years and is currently at an all-time high due to four influences: (1) the large number of new species that have been described over the past 30 years; (2) the recognition that many species of elasmobranchs may be threatened with extinction from fishing pressures and habitat destruction; (3) the growing interest in DNA "barcoding" as a tool to augment taxonomic description; and (4) an emerging recognition of the important role that elasmobranchs play as top predators in marine ecosystems.

1.1.2 Life History Characteristics

Among the chondrichthyans there is considerable variation in life history parameters (e.g. gestation rates differ from 3 months to 2 years; Pratt & Casey 1990; Smart *et al.* 2016). Life history patterns can be defined as the characteristics which affect the survival and reproduction of an individual's lifetime in a population. These traits

include where they are born, how long they remain there, growth rates, maximum size, what and how much they eat, social and sexual relationships, gestation rates, reproduction, when, where, and how often they mate, fecundity, their movements and migrations, and longevity (Hoenig & Gruber 1990). Studies on life history characteristics are essential for accurate predictions on how populations will grow and how they will respond to fishing pressure. However, because of the difficulty in validating age, most sharks and rays have not been reliably aged. Coupled with the lack of information on reproductive habits, such statistics are not known for many chondrichthyan species (Cailliet *et al.* 2005, Goldman *et al*. 2012).

The biology of chondrichthyan fishes is the least understood of all major marine vertebrate groups. Chondrichthyans display strongly K-selected life history strategies (Hoenig & Gruber 1990; Cortes 2004). The majority of cartilaginous fishes exhibit: choosing constant and/or predictable habitats, narrow niche, "large" body size, slow growth, late age at maturity, low fecundity and productivity (small, infrequent litters), long gestation periods, high natural survivorship for all age classes, and long life. Hoenig and Gruber (1990) suggest elaborate social structure and mating systems, lack of parental care of young, and storage of energy should be added to the list. These animals only need to produce a few young capable of reaching maturity to maintain population levels under natural conditions. However, these traits combined with the tendency of many species to aggregate by age, sex, and reproductive stage has major implications for their sustainability (Cailliet *et al.* 2005, Dulvy *et al.* 2008).

All traditional fisheries management strategies are based on teleost reproductive strategies and life history characteristics (Cailliet *et al.* 2005). As a result, a different approach for chondrichthyan management than that currently being used for teleosts is required for sustainable chondrichthyan fisheries (Stevens *et al*. 2005). Life history studies tell us immediate management values as input to assessment models and can provide massive amounts of information (Hoenig & Gruber 1990).

1.1.3 Reproduction

Chondrichthyan reproduction and reproductive behavior are not new areas of research to scientists, but have suffered due to the difficulty of obtaining direct observations. Few species have been recorded mating in the wild; however, scientists have recently learned some elasmobranch species exhibit involved pre-copulatory and copulatory behaviors (Pratt & Carrier 2001, 2005). Mating behavior is often implied from examinations of freshly caught animals, laboratory studies of reproductive structures and functions, and observations of captive or free-swimming animals. Much of our knowledge up until the past couple of decades came from observations of captive animals (Henningsen *et al*. 2004b). Understanding all aspects of elasmobranch reproduction and behavior is becoming increasingly significant in managing their exploitation and the consequences (Pratt & Carrier 2001).

Several elasmobranch populations have declined from overexploitation, due to directed or incidental fisheries as by catch worldwide (Musick 1999; Dulvy *et al*. 2008). Reproductive behavior, habits, nursery grounds, breeding areas, and segregation of females are characteristics that need to be thoroughly understood for the successful

conservation and management of these animals. All elasmobranchs share similar reproductive organ systems which include: claspers, siphon sacs, ovaries with large follicles, and oviducal glands (Pratt & Carrier 2005). For a successful mating event to occur, the male is required to bite and hold the female, while inserting the clasper(s) into the female's cloaca. Repeated copulations might be needed for fertilization to occur. During mating season, females and some males bear the marks of courtship and mating events. Scarring patterns are species specific and depend on where the male bites the female. The most common patterns observed are abrasions and tooth rakes on pectoral fins and around the cloaca (Pratt & Carrier 2005). Males of some species use aggressive behavior to maintain a sexual hierarchy (Gordon 1993). Many elasmobranchs may also display a social hierarchy that can precede or follow mating and carry over into non-reproductive periods, especially in species which remain together over multiple mating seasons like the sand tiger shark, *Carcharias taurus*, and the nurse shark, *Ginglymostoma cirratum* (Gilmore *et al.* 1983; Carrier *et al*. 1994).

Of all the areas relating to Chondrichthyan reproductive biology, reproductive modes or methods of embryonic nutrition are among the best understood (Hamlett & Koob 1999) and can be divided into two general modes, lecithotrophy and matrotrophy. In lecithotrophy, the embryo receives nutrition solely from the yolk. In matrotrophic species, embryos receive nutrition from the mother (Musick & Ellis 2005). It is within these two general modes that the diversity of reproductive modes occurs (Wourms 1981; Hamlett & Koob 1999).

Oviparity refers to lecithotrophic development which occurs in pouch-like eggs which are deposited externally on the seafloor or supporting structures. Incubation periods can last months to more than two years, largely dependent on the temperature of their environment. Oviparity can be further divided into single and multiple oviparity. Single oviparity, also known as extended oviparity, is found in Heterodontiformes (bullhead sharks), Rajidae (skates), Orectolobiformes (carpet sharks), and most Scyliorhinidae (catsharks). A single egg is deposited from each oviduct, usually in pairs. Tens and even hundreds of eggs can be deposited over the span of a mating season. Multiple oviparity, also known as retained oviparity, is characterized by the retention of a small number of eggs in the oviduct during most of the development before depositing on the seafloor to hatch. This only occurs in a small number of Scyliorhinid species (Hamlett & Koob 1999; Musick & Ellis 2005; Last *et al.* 2016b).

Aplacental yolk-sac viviparity, formerly known as ovoviviparity, involves retention of fertilized eggs throughout development within the uterus with no additional nutritional input from the mother. This is the most common reproductive strategy, found in Squaliformes, Hexanchiformes, some Orectolobiformes, Carchariniformes, and batoid groups (Compagno 1990; Hamlett & Koob 1999; Carrier *et al.* 2004; Musick & Ellis 2005; Last *et al.* 2016b). Another form of viviparity is aplacental viviparity with trophonemata. In this mode, the developing embryo receives supplementary nutrition from protein and fat-enriched uterine milk (histotroph) secreted by long villi on the uterine lining called trophonemata, after the yolk is utilized. This mode reaches its pinnacle in the batoid order Myliobatiformes. The embryo consumes the histotroph

which enables it to increase in size more rapidly than other reproductive modes. Gestation rates are typically shorter, only lasting from a few months to up to a year (Hamlett & Koob 1999; Musick & Ellis 2005; Last *et al.* 2016b).

Oophagy is an additional form of aplacental viviparity where after the initial yolk-sac nutrition, developing embryos consume the unfertilized eggs to support their development. This usually results in large (>100cm TL) neonates and occurs in a few species of the lamnoid sharks and a small family of carcharhiniform sharks, the Pseudotriakidae, the false catsharks, (Hamlett & Koob 1999). However, the mechanism is different in the two groups. In the lamnoids, unfertilized eggs are continuously produced for the embryos to ingest and store in a yolk-stomach. In the pseudotriakids (ground sharks), the developing embryo ingests a multitude of apparently unfertilized ova that is contained within the same egg envelope and stores the food source in the external yolk sac. Adelphopagy, or intrauterine cannibalism, is a form of lamniform oophagy in which the largest developing embryo in each uterus consumes all the smaller embryos first, and then relies on maternal production of unfertilized eggs for the duration of development. This mode has only been documented in the sand tiger shark, *Carcharias taurus* (Musick & Ellis 2005, Gilmore *et al*. 1983).

Placental viviparity occurs in five families of carcharhiniform sharks. In this mode of reproduction, early development is supported by the yolk and the timing of placentation varies (Compagno 1988). Prior to implantation, the egg envelope is greatly reduced in thickness and all metabolic exchange between the uterus and fetus must occur

across the egg envelope. Once the term is reached, the yolk stalk is transformed into an umbilical cord while the yolk sac contributes to the functional placenta (Hamlett & Koob 1999).

Parthenogenesis is the last reproductive mode to note. Parthenogenesis is a form of asexual reproduction in which embryos develop in the absence of fertilization (Dudgeon *et al.* 2017). It is most commonly found in plants and invertebrates but increasing occurrences of parthenogenesis have been seen in vertebrate species (Dudgeon *et al.* 2017). This mode has been reported in in two orders of sharks and two orders of batoids and can also be considered a mating system (Feldheim *et al.* 2016).

1.1.4 Mating Systems

There is a growing interest in mating systems of elasmobranchs and how to manage a species due to widespread overexploitation. Traditionally, the understanding of mating systems in elasmobranchs came from direct observational data of mating in only a few species (Pratt & Carrier 2001). Based on a few genetic and field observational studies, speculation is that elasmobranchs commonly use polyandrous mating strategies and multiple paternity is common (Chapman *et al*. 2004). Emlen & Oring (1977) characterize polyandry as individual females gaining access and mating with multiple males. Polyandry and multiple paternity (a single litter of offspring sired by multiple males) are now recognized as common strategies, and are widespread in the animal kingdom, utilized by birds (Petrie & Kempenaers 1998), mammals (Clapham & Palsbøll 1997; Firman & Simmons 2008), reptiles (Uller & Olsson 2008), insects (Boomsma *et al.* 1999; Bretman & Tregenza 2005), and fishes (Evans & Magurran 2000). Molecular

techniques are often used in studies determining elasmobranch mating systems. Genetic polyandry and multiple paternity has been documented in twenty-one species of sharks and two species of batoids (Marino *et al.* 2015; Russouw *et al.* 2016; Green *et al.* 2017). Based on the litters sampled, frequency of polyandrous females seems to vary both among and within species with some species being primarily genetically monogamous and some predominantly polyandrous (Marino *et al.* 2015). For males, there is a clear benefit to having multiple breeding partners: the more females it inseminates, the more offspring it fathers, the greater his reproductive fitness. The benefit of this strategy to females is less obvious. In elasmobranch mating systems, there have been no direct benefits to the female, although, there is potential for indirect or genetic benefits (Daly-Engel *et al.* 2010) as well as detriments to the female from mating wounds.

Monogamy is classified as neither sex having the opportunity to monopolize additional members of the opposite sex, directly or through resource control. Fitness is increased through shared parental care and is the most often seen mating system in the birds (Emlen & Oring 1977). Some shark species have been observed to follow genetically monogamous mating strategies. For example, over 81% of sampled litters from female bonnethead sharks, *Sphyrna tiburo*, (Chapman *et al.* 2004) and in 89% of sampled litters from female shortspine spurdog, *Squalus mitsukurii*, (Daly-Engel *et al.* 2010) produced offspring sired by a single male. Polygyny is described as individual male's frequently controlling or gaining access to multiple females. It occurs if environmental or behavioral conditions bring about the clumping of females and males have the capability to monopolize them. There are multiple types of polygyny and they

are classified according to the means that males use to control females (Emlen & Oring 1977).

1.2 SUPERORDER BATOIDEA

The superorder Batoidea is a highly modified, highly diverse, and most speciose group of cartilaginous fishes. They are presently comprised of 26 families and 633 valid named species. An additional 50 unrecognized or undescribed species are known to exist (Last *et al.* 2016b). They are characterized by dorsoventrally flattened bodies and typically modified into a disc formed by the complete or partial fusion of pectoral fins with head and trunk. Stingrays and guitarfishes are examples of complete and partial fusion, respectively. The disc shape may be circular, oval, triangular, heart-shaped, or rhombic. Five to six gills slits are located ventrally with the eyes and spiracles found on the dorsal surface. All rays lack an anal fin, and the caudal and dorsal fin are variable or sometimes absent. Like sharks, rays have denticles, but vary in size and distribution. Their body shape provides a good indication of their life history strategy and ecological role. A flattened, soft, and flexible disc enables the ray to burrow into substrate or move around reefs and crevices, while a firmer, large musculature, and angular body shape allows for active swimming (Last *et al*. 2016b). Most species of rays live in demersal habitats where they are either primarily benthic or benthopelagic, however, some members of the group, such as mobulid rays, have powerful and enlarged pectoral fins for swimming in the pelagic ocean, well above the seafloor. They successfully occupy a

variety of niches including shallow estuarine, coastal, and shelf environments, tropical freshwater habitats, all ocean basins, and deep continental slopes up to 3000 meters. Batoids feed mainly on crustaceans, mollusks, and fishes (McEachran & Dunn 1998; Ebert & Bizzarro 2007, as cited in Frisk 2010; McEachran & Aschliman 2004; Last *et al.* 2016b).

Some rays have been studied in greater detail than others due to the ability to catch certain species, geographical locations, large size of some specimens, and storage options. According to Last *et al.* (2016) in "Rays of the World", data varies greatly among Batoidea with some families being heavily studied, for example Pristidae (sawfishes) and Mobulidae (devilrays), but few of the groups have been subjected to recent taxonomic revisions. Using an expanded practice of DNA analysis, scientists have been able to define the structural elements and species compositions of regional fauna.

1.2.1 Taxonomy

Naylor *et al.*(2016), among others (Douady *et el.* 2003; Dunn *et al.* 2003; Winchell *et al.* 2004; McEachran & Aschliman 2004) suggest that rays form a monophyletic group and all major classifications of elasmobranch fishes have batoids as a single well-defined group due to their unique features which sets them apart from sharks. Rays as the sister group to sharks has been supported by recent studies based on DNA sequence data. Advances in molecular methods led to a revolution in the understanding of the taxonomy of ray groups and their classification, yet morphological and molecular analyses are not in total agreement (Last 2007). Debate is ongoing as

studies based on molecular data continue to classify elasmobranchs into two groups, sharks and rays (Naylor *et al.* 2016) and there is presently no agreement at the higher systematic levels. Other studies based on anatomical features suggest rays are derived sharks and closely related to sawsharks (Pristiophoriformes) and angelsharks (Squatiniformes) (Shirai 1996). Interestingly, parasite studies have provided insights into ray taxonomy and distribution. Species of parasites (external or internal) are often confined to a single host species or group of related species. Major initiatives to characterize chondrichthyan parasite fauna have recently shed light on the rich diversity of these animals and their hosts (Last *et al.* 2016b). Within the superorder Batoidea there are four orders: Rajiformes (skates), Myliobatiformes (rays), Rhinopristiformes (shovelnose rays, guitarfish, and sawfish), and Torpediniformes (electric rays) (Naylor *et al.* 2012, Last *et al.* 2016b).

1.2.2 Conservation

Ray fisheries are an important food source for many regions. According to the Food and Agriculture Organization of the United Nations (FAO) fishery data, world production of rays was 26,000 tonnes in 2013. This is 30% of the total production of Chondrichthyan fishes. However, this does not include actual catches, as they do not include discards or undeclared and the majority of landed rays are bycatch. This is especially high in the Indo-Pacific region. Indonesia has the largest shark and ray fishery in the world ranging from 2,000 to greater than 100,000 tonnes caught annually (White *et al.* 2016). Another issue with this is most catches are not reported down to level of species which does not allow for accurate stock management. Often, rays get classified

as sharks (due to the close morphological appearance), so the actual number of ray landings may be seriously underestimated. Groups like the stingrays (Order Myliobatiformes, Family Dasyatidae) and wedgefishes (Order Rhinopristiformes, Family Rhinidae) are particularly at risk in the Indo Pacific. Approximately 43% of ray species are declared Data Deficient by the International Union for Conservation of Nature (IUCN) as population numbers are not maintained and life histories are poorly known (Grubbs *et al.* 2016). Last (2007) stated that rays are amongst the most seriously threatened marine groups on the planet. Currently, the IUCN Red List of Threatened Animals lists 112 out of 557 species of rays threatened and five of the seven most threatened families of elasmobranchs are rays (Dulvy *et al.* 2014).

1.2.3 Family Dasyatidae

The order Myliobatiformes contains eleven families including Dasyatidae (stingrays). Stingrays vary in size from small to massive ranging from 23 cm to at least 220 cm disc width and weighing up to 600 kg. Their disc is circular to rhombic in shape with the head and pectoral-fin skeletons joined. Their eyes are found dorsolaterally on the head with the spiracles close behind eyes. The mouth is located forward on their ventral surface with five pairs of small gill openings located next to mouth. There are teeth small to moderate in size and do not form crushing plates. Their tail is often longer than their disc and whip-like. Most species have one or more serrated caudal venomous barbs. Their skin can be entirely smooth or covered with small denticles or thorns, with those located on the tail being larger (Compagno 1999; Last *et al.* 2016c).

Stingrays are represented by at least 86 living species. The family presently contains 19 genera including *Hypanus*, which contains the subject of this investigation. Additional taxonomic revisions are needed as the status of some of these species has not been fully resolved. Stingray taxonomy is difficult because of the similarity in appearance of many species, and they are often poorly represented in museum collections due to their large size (Last *et al.* 2016c). Most occupy demersal inshore habitats on continental and insular shelves and are found rarely deeper than 400 m; however, one member, the pelagic stingray, *Pteroplatytrygon violacea*, is free-swimming in the open ocean. Some stingrays live in freshwater and occur more than 240 km away from the coast. They display viviparous reproduction with litters of 1 – 13 pups and gestation periods can last more than 12 months (Last *et al.* 2016c). The Dasyatids can be targeted for fisheries, but are typically caught as bycatch in trawls, longlines, or gill nets. In fact, Dasyatids comprise 90% of the total number of rays caught in Indonesia (White *et al.* 2016). Many stingray species live in shallow water coastal and inshore habitats, where they are more susceptible to fishing activities (Kyne 2016). Some species are popular game fish, used for human consumption (shark-fin trade) and leathery products (wallets, shoes, bags) (Kyne 2016; White *et al.* 2016). Multiple species in this family are common in aquariums due to their large size and colorful appearance, while others are regionally important for ecotourism. They serve a role in education and science, as knowledge about food habits, reproductive biology, and interactions with other species is based on these rays in aquariums. (White *et. al.* 2016).

1.2.4 Use of Microsatellites in Elasmobranch Investigations

The use of molecular techniques can be valuable in expanding our understanding of reproductive biology of elasmobranchs where experimental or observational approaches are difficult (Portnoy 2010). Molecular approaches in elasmobranch research has been primarily used for phylogenetic investigations but has recently revolutionized the study of mating systems. For example, monogamy or polygyny were thought were considered the dominant mating system in species with internal fertilization. Studies using high-resolution molecular markers have revealed genetic polyandry are common (Portnoy 2010). Time and cost effective advances in genetic technologies has enabled development of new markers and analyses (Dudgeon *et al.* 2012).

Microsatellites, also known as simple sequence repeats (SSR), variable number tandem repeats (VNTR), and short tandem repeats (STR), are a commonly utilized DNA marker. Microsatellites are tandem repeats of nuclear DNA composed of a motif of 1 to 6 nucleotides repeated *n* times located between less repetitive flanking regions. They are found at high frequency in the nuclear genome of most taxa and can be visualized between flanking regions that are generally conserved across individuals of the same species. Individual microsatellites and their flanking regions can be amplified using polymerase chain reaction (PCR) and appropriate primers, short stretches of DNA which are designed to be complementary and bind to the flanking regions.

They are the most widely used marker for inferring familial relatedness and polyandry because they are highly polymorphic (Selkoe & Toonen 2006; Portnoy 2010; Portnoy & Heist 2012) and have opened up additional areas of research in, identification, reproduction, and movement and philopatry (Dudgeon *et al.* 2012).

CHAPTER 2

MATING SYSTEM IN A CAPTIVE POPULATION OF SOUTHERN STINGRAYS *Hypanus americanus*

2.1 INTRODUCTION

2.1.1 The Southern Stingray, *Hypanus americanus*

The southern stingray, *Hypanus americanus* (Hildebrand & Schroeder, 1928), is a relatively large, looking stingray with a broad rhomboid disc (Figure 1). They are uniform grayish or brownish in color with typical counter-shading (dorsal surface colored and their ventral surface is white). Three rows of thornlets, or enlarged denticles, adorn each shoulder and run continuously down their midline; small denticles run down their tail to the venomous caudal spine. They have a whip-like tail, long (length approximately 2.5 times their disc width (DW)) with one or more venomous spines. The dorsal tailfin fold is small, while the ventral tailfin fold is large and runs most of the length of the tail (Last *et al*. 2016c). They are found along the coastal western Atlantic from New England to Brazil, including the Caribbean and the Gulf of Mexico, in coastal marine and estuarine habitats to 55 m depths (Bigelow & Schroeder 1953; McEachran & Fechhelm 1998; Grubbs *et al.* 2016). They live among sand flats, sea grasses, and coral reefs, and feed mainly on small demersal fish, crustaceans, bivalves, and worms (Gilliam & Sullivan 1993; Last *et al.* 2016c; Grubbs *et al.* 2016). Maximum size is reported to be 80cm DW and 150cm DW, for males and females, respectively (McEachran & Fechhelm 1998; McEachran & de Carvalho 2002). The size at maturity ranges from 46 – 51cm DW for males and approximately 70 – 80cm DW for females (Bigelow & Schroeder

1953). Maturity has been found to vary within geographical region and is attained at smaller sizes in the Gulf of Mexico than in the Western Atlantic (Bigelow & Schroeder 1953; Funicelli 1975, as cited in Henningsen & Leaf 2010) (Table 1).

Figure 1. Adult southern stingray, *Hypanus americanus*, in a natural setting. Photo courtesy of George Grall, National Aquarium.

Maturity for males is assessed according to degree of calcification and rotation of claspers, and the presence of spermatozoa in the clasper groove (Conrath 2005; Henningsen 2000; Henningsen & Leaf 2010). Typical indicators of female maturity in batoids are follicular development, nidamental gland width, and uterus width, as well as, pregnancy (Conrath 2005; Henningsen & Leaf 2010). Results from Henningsen and Leaf (2010) showed elevated growth rates which may be caused by the effect of captive conditions. Sexual dimorphism in DW at age of maturity, as well as maximum size, is common in batoids, especially myliobatoids, and in general the females attain a greater size while growing at a slower rate than males (Cowley 1997). The magnitude of difference in maximum size

varies among batoids, often with little difference in skates (Sulikowski *et al*. 2003). The maximum weight reported by Henningsen & Leaf (2010) was 17.5kg and 87.7kg for males and females, respectively. It was also reported that sexual dimorphism in DW is observed in neonates (Henningsen 2000; Henningsen and Leaf 2010).

Age at maturity is unknown in wild southerns but has been reported for captive specimens to be 3.5 years and 5.5 years, for males and females, respectively. Longevity (years) in wild specimens is unknown but has been estimated as 26.0 years for captive female conspecifics (unpublished data from our individuals at aquarium). Recently, however, a potential sire (sample 590021) for this study reached an estimated age of 31 years old before dying from leukemia or lymphoma (Unpublished data). The von Bertalanffy growth coefficient (k) for a population of captive born rays was reported to be 0.44 yr^{-1} for males, and 0.11 yr^{-1} for females (Henningsen & Leaf 2010).

Although growth in captive specimens may differ from that of wild conspecifics, studies of growth in laboratory and aquarium specimens have provided valuable life history information where none is otherwise available (Cailliet & Goldman 2004; Mohan *et al*. 2004). Henningsen & Leaf (2010) suggest southern stingrays are similar to other myliobatoid rays, and the growth in wild conspecifics may be slower. Recently, however, growth dynamics were found to be similar in a population of captive southern stingrays, and the aggregation of southern stingrays at Stingray City, Cayman Islands (Vaudo *et al.* 2017). Sex specific differences in maximum size based on theoretical growth modes examined have biological implications because of the ecological

Table 1. Life history parameters of the southern stingray *Hypanus americanus*.

Parameters	Captive Specimens	Wild Specimens	Source
Gestation (months)	4.4-7.5	7.0-8	Henningsen 2000, Ramirez-Mosqueda *et al.* 2012
Fecundity (mean)	2-10 (4.2)	2-7 (2.5)	Henningsen 2000, Ramirez-Mosqueda *et al.* 2012, Grubbs *et al.* 2016
Length at birth (cm DW)	20-34	17-19	Bigelow & Schroeder 1953, Henningsen 2000, Ramirez-Mosqueda *et al.* 2012
Reproductive cycle	Biannual	Annual	Henningsen 2000, Grubbs *et al.* 2016, Ramirez-Mosqueda *et al.* 2012
Maximum size male (cm DW)	76	80	Henningsen & Leaf 2010, McEachran & de Carvalho 2002
Maximum size female (cm DW)	125	150	Henningsen & Leaf 2010, McEachran & de Carvalo 2002
Maximum weight male (kg)	17.5	unknown	Henningsen & Leaf 2010
Maximum weight female (kg)	87.7	unknown	Henningsen & Leaf 2010
Longevity (years)	31	unknown	unpublished data from our collection at NAI, Grubbs *et al.* 2016
Natural Mortality	N/A	unknown	Grubbs *et al.* 2016
Size at maturity male (cm DW)	48-52	51	Henningsen & Leaf 2010, McEachran & de Carvalho 2002
Size at maturity female (cm DW)	75-80	75-80	Henningsen & Leaf 2010, McEachran & de Carvalho 2002
Age at maturity male (years)	3-4	unknown	Henningsen & Leaf 2010
Age at maturity female (years)	5-6	unknown	Henningsen & Leaf 2010

importance of early growth (Osse *et al*. 1997, as cited in Henningsen & Leaf 2010) and correlation of life history traits (age of first reproduction) with growth (Beverton 1992, as cited in Henningsen & Leaf 2010). Difference in life history characteristics are observed in geographically separated population or subpopulation of elasmobranchs (Henningsen *et al.* 2004a). Average reproductive age, annual rate of population increase and natural mortality are unknown for this species in the wild (Grubbs *et al.* 2016).

2.1.2 Reproductive Parameters

Wild southern stingrays have been shown to have an annual reproductive cycle in Florida and Virginia waters, as well as the southern Gulf of Mexico, in contrast to captive specimens which display a biannual cycle (Grubbs *et al.* 2016; Henningsen 2000; Ramirez-Mosqueda *et al.* 2012). Ramirez-Mosqueda *et al.* (2012) noted annual and biannual cycles of reproduction have been documented for other dasyatids in the wild (Snelson *et al.* 1989; Johnson & Snelson 1996; Ebert & Cowley 2008; Yokota & Lessa 2007) and for captive specimens a biannual cycle has been documented (Henningsen, 2000; Mollet *et al*. 2002; Janse & Schrama 2010).

The southern stingray reproduces via aplacental viviparity with trophonemata (Hamlett *et al.* 1996). In captivity, these rays exhibit a gestation of 4.4 – 7.5 months. Litters typically produce 2 – 10 pups with a positive linear relationship with maternal DW. At birth, pups are 200 – 340 mm DW with a 1:1 sex ratio (Henningsen 2000). Gestation for wild specimens lasted for a period of 7 – 8 months with fecundity documented as 2 – 7. Disc width of wild pups ranged from 170 – 190 mm (Ramirez-Mosqueda *et al.* 2012; McEachran & de Carvalho 2002) (Table 1).

The difference between gestation periods in wild and captive specimens is most likely associated with environmental conditions; water temperature has been noted to have a profound effect on development (Henningsen *et al.* 2004a). Captive spotted eagle rays (*Aetobatus narinari*) were documented to have longer gestation times at lower temperature (11 – 12.5 months at 19.8 – 29.4 °C) than at higher temperatures (6 – 6.2 months at 28.1 – 30.1°C) (Mohan *et al.* 2004). In the captive southern stingrays used in this study, water temperature was maintained at 24 - 25°C. The southern stingray has the highest fecundity reported among captive dasyatids and among the highest of reported for wild dasyatids (Ramirez-Mosqueda *et al.* 2012). The average fecundity is low in wild specimens (2.5 embryos) which is typical of other dasyatids (Table 1). A reason for this could be because females often abort embryos due to the stress associated with capture. This reason proves difficult to estimate the fecundity of myliobatoid rays in the wild (Smith *et al.* 2007). Other differences between wild and captive conspecifics have been well documented for elasmobranchs (Henningsen *et al.* 2004a, b).

2.1.3 Mating Behavior

Little is known of the southern stingrays' reproductive natural history. Six species of batoids have been observed copulating in the wild (Brockman 1975; McCourt & Kerstitch 1980; Tricas 1980; Young 1993; Nordell 1994; Yano *et al.* 1999; Carrier *et al.* 2004; Ritter & Vargas 2015). Since these behaviors are not often seen in the wild, natural sexual behavior and mating systems is left unknown (Chapman *et al.* 2003). Even though the southern stingray is a common ray, and despite an earlier account by Brockman (1975) and DeLoach (1999), Chapman et al. (2003) were the first to

photographically document a complete mating sequence in detail in the wild and suggest this mating behavior is likely typical in this species. Contrary to Brockman's (1975) report of dorsal to dorsal copulation, all other observations involved venter to venter copulation (Henningsen 2000; Chapman *et al.* 2003). Two separate events were captured in Grand Cayman Island. The first documented mating event showed a sequence of polyandrous mating events between two males and a large female. Both males achieved successful mating without forcibly restraining the female. This suggests these mating events are female driven, the females choose to mate with multiple males as opposed to being forced. Regardless of whether polyandrous mating is by choice or through forcible restraint by males, semen from more than one male is present in the female's reproductive tract at the same time. Among the array of sexual conflicts exhibited in the southern stingray mating system, it is possible that post-copulatory paternity selection is occurring. This could be sperm competition, flushing, and/or sperm selection (Chapman *et al.* 2003). The second mating event occurred between one female and one male resulting in a single copulation. Both mating events occurred with a large female and smaller male in approximately 2 meters of water over a sandy seafloor (Chapman et al. 2003).

In two additional documented events by Chapman *et al.* 2003, observed in Bimini, Bahamas, gravid females were captured on seagrass flats and transported to a plastic mesh pen on a neighboring flat. In both instances, due to capturing and handling stress, females gave birth when they were placed in the pen. No males were observed in the area at that time. A viscous yellow fluid was seen flowing from the female's cloaca,

post-partum. Up to six male stingrays approached from surrounding flats and circled the pen for up to two hours after parturition. In both instances, a single male circling the pen was caught and placed inside which resulted in the male rapidly initiating copulation.

The sequence of events observed at Grand Cayman and Bimini can generally be characterized as: (1) 'close following': the posterior orientation of the male(s) to the female; (2)'pre-copulatory biting': oral grasping of the female's pectoral disc by the male(s), with anterior rotation of one or both claspers and forward arching of the pelvic region such that the claspers are oriented towards the cloaca; (3) 'insertion/copulation': insertion of a clasper followed by vigorous thrusting of the male's pelvic region, lasting from 10-33 seconds; (4) most commonly followed by a 'resting phase', characterized by a rapid fluttering of the males gills while maintaining clasper insertion and oral grip of the females disc; and (5)'separation': the male's release of his oral grip, clasper withdrawal, and movement away from the female. This mating sequence matches closely with that observed in captivity (Henningsen 2000; Henningsen et al. 2004b.), as well as with the mating behavior Yano *et al.* (1999) characterized for manta rays, *Manta birostris*. Manta rays have a pelagic lifestyle and habitat, compared to the demersal southern stingray. Despite extreme differences between these two species, the mating behavior appears very similar which suggests the mating position of 'ventral to ventral' is widespread amongst the batoids.

In the related Atlantic stingray, *Hypanus sabinus*, mating begins in October and continues until late March to early April when annual ovulation and fertilization occurs, and is synchronous. Since female *H. sabinus* are not known to store sperm over this

extended period, copulations months in advance of ovulation are hypothesized to induce the reproduction cycle of the female (Maruska *et al.* 1996). Chapman *et al.* (2003) proposes that a similar ovulation inducement mechanism may explain post-partum copulations. The rapid appearance of male stingrays around the holding pen containing an immediate post-partum female suggests an olfactory attractant that attracts male rays is released during parturition. This has also been observed in captive southern stingrays (Henningsen 2000; Henningsen et al. 2004b). Olfactory cues have been found in other elasmobranchs including the Atlantic stingray, *Hypanus sabinus* (Kajiura *et al.* 2000), blacktip reef shark, *Carcharinus melanopterus* (Johnson & Nelson 1978), clearnose skate, *Raja eglanteria* (Rasmussen *et al.* 1999). Alternatively, if *H. americanus* females in the wild have two reproductive cycles per year (biannual) as they do in captivity (Henningsen 2000), the interval between parturition and the next mating season in this species may be extremely short. Available evidence in wild southern stingrays indicates an annual reproductive cycle (Grubbs *et al.* 2016; Ramirez-Mosqueda *et al.* 2012), while the similar sympatric congeners, *Hypanus guttatus* and *Hypanus marianae* appear to have a biannual cycle (Yokota & Lessa 2007).

2.1.4 Conservation Status of the Southern Stingray

According to Grubbs *et al.* (2016) the southern stingray population appears healthy in the United States with no apparent threats and the population is assessed at Least Concern. It is frequently caught as incidental bycatch along the east coast by trawls, long lines, and nets, but most are released and mortality is probably low (Graham *et al*. 2009; Grubbs *et al*. 2016). Little information is available, however, for the rest of the

southern stingrays' range on the impacts of fishing and population trends, and thus is classified as Data Deficient globally. The population of *H. americanus* in the southern Gulf of Mexico is of concern because it is the most landed elasmobranch by the fisheries and its reproductive parameters suggest that this species has low biological productivity and probably limited resilience to fishing pressure (Ramirez-Mosqueda *et al*. 2012). It is also harvested in parts of South America where fisheries exist in Venezuela, Colombia, and Brazil and an increase in artisanal fisheries pressure in regions of Brazil may warrant concern (Grubbs *et al.* 2016). In fact, in one Rio de Janeiro municipality it is considered vulnerable (Buckup *et al.* 2000, as cited in Grubbs *et al.* 2016). Protection of breeding and nursery areas in parts of South America may be necessary for long-term survival of the species (Grubbs *et al.* 2016). Currently, no conservation actions are required in the United States, but the impact of the harvesting in South America should be monitored and population studies should be conducted (Grubbs *et al.* 2016).

The southern stingray is an important resource for ecotourism operations within the Caribbean (Corcoran 2006; Grubbs *et al.* 2016; Semeniuk *et al.* 2007; Semeniuk & Rothley 2008). Tours allowing tourists to swim and feed these species in shallow waters have increased in popularity. For example, the extremely popular Stingray City Sandbar in the Cayman Islands causes large aggregations of stingrays to inhabit a sandbar where tourists spend most of the day hand feeding them. The impacts these operations may have on behavioral and ecological factors are unknown, however, Corcoran *et al.* (2013) suggests there can be cascading effects on the surrounding marine ecosystem impacting both stingray predator and prey dynamics. The large aggregations of stingrays may

unnaturally influence the community structure not only by the ray's role as predator, but also as prey. The great hammerhead shark, *Sphyrna mokarran*, are known predators of these rays and are frequently observed in the vicinity of the Stingray City Sandbar. Corcoran *et al.* (2013) and Vaudo *et al.* (2017) were also able to show drastic alterations in movement patterns, diel activity, and habitat use of the stingrays fed at this site compared to the wild rays that do not travel to this site.

The hypothesis of the current investigation is that multiple paternity resulting from polyandrous mating events occurs within litters of a captive population of southern stingrays. The null then, is that litters are sired by a single paternal contributor. This study is the first to determine if multiple paternity in the southern stingray occurs in captivity by using genetic testing. It is hypothesized that multiple paternity does occur due to the observation of polyandrous mating events.

2.2 MATERIALS AND METHODS

2.2.1 Experimental Subjects

Experimental subjects consisted of male (n= 11) and female (n= 10) southern stingrays maintained together in a large multispecies exhibit at the National Aquarium, Baltimore, Maryland (Table 2). The "Wings in the Water" exhibit, a 1,003,025 L recirculating aquarium system, housed the population of wild caught and captive born animals used in the study. All adult rays received a 12 mm passive integrated transponder (PIT) tags injected intramuscularly for individual identification (American Veterinary Identification Devices, Inc., Norcross, California). Water temperature was maintained at 24.0 – 25.0 ^{o}C. Rays were hand-fed a variety of fish and invertebrates.

2.2.2 Tissue Sample Collection

Tissue samples which consisted of fin clips (approximately 15x15mm) or organ samples were taken from 21 individuals or offspring of adult females from the Wings in the Water exhibit. Specifically, 5-10mm samples were collected from 12 pups representing four separate litters born throughout 2005 (Table 3). These samples were kept in a DNA buffer solution suitable for long term storage. Of the eight female southern stingrays in the exhibit, only four contributed to the litters sampled (Table 4). The parturient female was identified by presence of a post-partum concavity in the uterine disc (Henningsen 2000; Conrath 2005). Prior to parturition, the female had a notably convex uterine region. Behavior of the adult male rays was used to substantiate the identification as they immediately or within hours chased and copulated with the female (Henningsen 2004b). Tissue samples collected from two of the mothers were acquired from the National Aquarium Clinical Lab, which collected these samples after the rays died. A liver, kidney, and thyroid tissue sample was used for "Mom 1" mother 592004, who was euthanized using Euthasol (pentobarbital sodium and phenytoin sodium) euthanasia solution after being compromised from an illness. Multiple tissue samples were tested due to issues with the isolation of DNA. The tissue sample used for "Mom 2" mother 595008 was gill tissue. This female was found dead on exhibit. A tissue sample could not be collected from the fourth contributing female "Mom 3"

Table 2. Experimental Subjects. Sample identification with acquisition and de-accession dates.

Sample ID	ID	Sex	Date Acquired	Location Acquired	Location and Date of Deaccession
1	Pup 1	F	3/16/2005	Born to mother 595009	Sent to different aquarium
2	Pup 2	M	3/16/2005	Born to mother 595009	Sent to different aquarium
3	Pup 3	F	3/16/2005	Born to mother 595009	Sent to different aquarium
4	Pup 4	M	3/16/2005	Born to mother 595009	Sent to different aquarium
5	Mother 595009	F	1/19/1995	Born at NA - sister to mom 2	8/22/2012 to Georgia Aquarium
6	Pup 6	M	10/24/2005	Born to mother 592004-mom1	Sent to different aquarium
7	Pup 7	M	10/24/2005	Born to mother 592004- mom1	Sent to different aquarium
8	Pup 8	F	10/24/2005	Born to mother 592004-mom1	Sent to different aquarium
9	Pup 9	F	6/7/2005	Born to mother 595008-mom 2	Sent to different aquarium
10	Pup 10	F	6/7/2005	Born to mother 595008-mom 2	Sent to different aquarium
11	Pup 11	M	6/7/2005	Born to mother 595008-mom 2	Sent to different aquarium
14	Potential sire 590015	M	6/1/1990	Wild - Florida Keys	1/5/2009 to World Aquarium and Conservation for Oceans
15	Potential sire 590021	M	1/1/1985	Wild- Florida Keys	9/22/2016 died
16	Potential sire 596026	M	2/2/1996	Born at NA	1/10/2011 died
17	Potential sire 597301	M	1/1/1987	Wild - Florida Keys	10/29/2009 to World Aquarium and Conservation for Oceans
18	Potential sire 598403	M	5/15/1998	Born at NA to 592004 - mom 1	1/5/2009 to World Aquarium and Conservation for Oceans
20	Pup 20	F	7/3/2005	Born to mother 598401-mom 3	Sent to different aquarium
21	Pup 21	M	7/3/2005	Born to mother 598401-mom 3	Sent to different aquarium
Mom 1	Mother 592004	F	10/12/1992	Wild – Shackelford Bank, NC	3/13/2010 died
Mom 2	Mother 595008	F	1/19/1995	Born at NA - sister to sample 5	4/4/2012 died
Mom 3	Mother 598401	F	1/21/1998	Born at NA to 590020	8/22/2012 to Georgia Aquarium

mother 598401, prior to its transport to another public aquarium. Following a request to this specific aquarium, a fin clip for the last contributing mother was acquired in 2014. Samples were collected from five of the seven adult males maintained in the exhibit (Table 4). One of the remaining samples needed was not obtained before the ray was donated to another aquarium, where it is not possible to collect a sample. The additional two potential sires were discovered to be in the exhibit after the analysis of this investigation was completed. All adult samples were stored in a DNA buffer solution or frozen at -20°C.

Table 3. Weight and disc width (DW) data from each litter sampled. The mother is identified by her unique six-digit accession number assigned by the National Aquarium tracking system (TRACKS®), followed by the sample ID used for this investigation. The pups are identified by the sample ID used for this investigation.

Mother	Pup	Date of birth	Clip location	Sex and measurements
595009 (Sample 5)	1	3/16/05	L pelvic fin	Female: 233mm
	2	3/16/05	L pelvic fin	Male: 217 mm Clasper Length: Not Recorded
	3	3/16/05	R pelvic fin	Female: 225 mm
	4	3/16/05	L pelvic fin	Male: 218 mm Clasper Length: Not Recorded

Mother	Pup	Date of birth	Clip location	Sex and measurements
595008 (Sample Mom 2)	9	6/7/2005	L pelvic fin	Female: 329 mm, 1,230 g
	10	6/7/2005	R pelvic fin	Male: 342mm, 1,472 g Clasper Length: 42 mm
	11	6/7/2005	R pelvic fin	Female: 334 mm, 1,367 g

Mother	Pup	Date of birth	Clip location	Sex and measurements
598401 (Sample Mom 3)	20	7/3/2005	R pelvic fin	Female: 271 mm, 718 g
	21	7/3/2005	L pelvic fin	Male: 281 mm, 758 g Clasper Length: 28 mm

Mother	Pup	Date of birth	Clip location	Sex and measurements
592004 (Sample Mom 1)	6	10/24/2005	Not Recorded	Male: 264mm, 635g Clasper Length: Not Recorded
	7	10/24/2005	Not Recorded	Male: 247mm, 481g Clasper Length: Not Recorded
	8	10/24/2005	Not Recorded	Female: 253mm, 625g

Table 4. All identifiers for the dams and potential sires for this study. A tissue sample was not obtained for potential sire samples: 19 and 7. The accession number is a unique six-digit identifier given to the animal from the National Aquarium (NA) tracking system (TRACKS®) when the animal was brought into the collection. De-accessed is defined as when the animal is traded to a different aquarium or dies.

Accession Number	PIT Tag no.	Sample ID	Sex	Date Acquired	Location Acquired	Date and Location De-assessed
592004	1320350	Mom 1	F	10/12/1992	Wild- Shackelford Bank, NC	3/13/2010 Died
595008	1803854	Mom 2	F	1/19/1995	Born at NA to female 590001	4/4/2012 Died
598401	121156625A	Mom 3	F	1/21/1998	Born at NA to female 590020	8/22/2012 to Georgia Aquarium
595009	121569610A	5	F	1/19/1995	Born at NA to female 590001	8/22/2012 to Georgia Aquarium
590021	53634100	15	M	1/1/1985	Wild- Florida Keys, FL	9/22/2016 Died
597301	2033125	17	M	1/1/1987	Wild- Florida Keys, FL	10/29/2009 to World Aquarium and Conservation for Oceans
596026	121235274A	16	M	2/2/1996	Born at NA to female 597303	1/10/2011 Died
598403	121247271A	18	M	5/15/1998	Born at NA to "Mom 1" 592004	1/5/2009 to World Aquarium and Conservation for Oceans
590015	121149283A	14	M	6/1/1990	Wild- Florida Keys, FL	1/5/2009 to World Aquarium and Conservation for Oceans
598404	121146622A	19	M	5/15/1998	Born at NA to "Mom 1" 592004	10/29/2009 to World Aquarium and Conservation for Oceans
590006	2073074	Potential sire 7	M	1/1/1990	Wild- Florida Keys, FL	7/27/2007 Died

2.2.3 Isolating the DNA

Each sample was mixed with 500µL of Queen's lysis buffer, 20µl of proteinase K (10mg/mL) and 40µl of 10% SDS (sodium dodecyl sulfate). The tubes were shaken and incubated at 55°C for 24 hours. After incubation, 400µl of 6M NaCl was added and each tube was vigorously inverted for 15 seconds. After centrifuging at 4,500 rpm for 15 minutes, 500µl of supernatant was transferred to a new labeled tube. To the supernatant, 1000µl of 95% ethanol (EtOH) was added, and then the tubes were inverted several times. If DNA was visible at this time, it was spooled out using a pipette and tip, and then transferred to a new tube. If DNA was not visible at this time, it was centrifuged at full speed for 5 minutes. The supernatant was removed and the pellet was washed with 70% EtOH.

Once DNA was transferred to a new tube, the pellet was washed with 70% EtOH and centrifuged at full speed for 5 minutes. The supernatant was discarded and this process was repeated 3 more times. Most of the supernatant was removed, then the pellets were allowed to air dry. Pellets were resuspended in 500µl of sterile water by vortexing. Aliquots were made by removing 100µl of the sample to use for the polymerase chain reaction (PCR) process. The original sample was kept in the -20°C freezer to limit thawing and refreezing.

2.2.4 Primer Design

The southern stingray microsatellite library was developed and gifted by Dr. Kevin A. Feldheim of Pritzker Lab of the Field Museum, Chicago, IL and Dr. Mahmood Shivji of Nova Southeastern University, Fort Lauderdale, FL; using the procedure

outlined by Glenn & Schable (2005). From this library, nine microsatellite loci (Appendix 1) were chosen and primers were developed from Integrative DNA Technology for these loci for this study (Table 5).

Table 5. Genetic characteristics of nine microsatellites used for the determination of multiple paternity in the southern stingray *Hypanus americanus*. T_m: melting temperature; T_A: annealing temperature.

Locus	Primer Sequence (5'-3')	Size Range (bp)	Repeat Motiff	T_m	T_A (°C)
DAM 5	F: CCTCCACTCATGGATTACCATTCT	242	$(TAGA)_{16}$	61.21	58
	R: GCTACAATAAGTGTATGGGACACTGG			60.79	
DAM 17	F: GGTTACCGATTTACAGACTGTCTGTCT	195	$(ATCT)_{15}$	60.91	58
	R: CAGTTAGACCGGCAAACAGACA			61.03	
DAM 20	F: GCTACAATAAGTGTATGGGACACTGG	181	$(TATC)_{12}$	60.79	58
	R: AAACCAGAGCATCTGGAGGAAA			60.91	
DAM 26	F: CGATATCATTGTCAACATTTCTGTTC	215	$(TG)_{27}$	59.63	57
	R: ACGAGCCCGCAGAGTTATAAG			59.36	
DAM 32	F: CTGAGACACTGCAGATAGATACCTAGC	245	$(TAGA)_{27}$	59.84	57
	R: TGTCAGGGATTCTCCATATTGTG			59.66	
DAM 34	F: CTCCAACTCACACCTCTTCCTCT	170	$(AC)_{33}$	59.75	57
	R: GTCCACTAGTTGTTCTTGTGATTTCC			60.13	
DAM 39	F: TAGTTCAGCATGGACTACGTG	187	$(GT)_{31}$	54.94	52
	R: CATCTACCTTCTCTGTCTGTATACAC			54.2	
DAM 45	F: CCAATAGGATGGACAACTAATGTG	298	$(TAGA)_{20}$	58.3	56
	R: CTCACTTTCAAAGACTCTTCATCTCA			59.09	
DAM 60	F: GGTTTCATTTGCTGTGGACTG	149	$(TG)_{41}$	59.03	56
	R: GCTGCGAGTCAGGCTCTCT			59.41	
PCR1 Forward overhang	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG				
PCR1 Reverse overhang	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG				

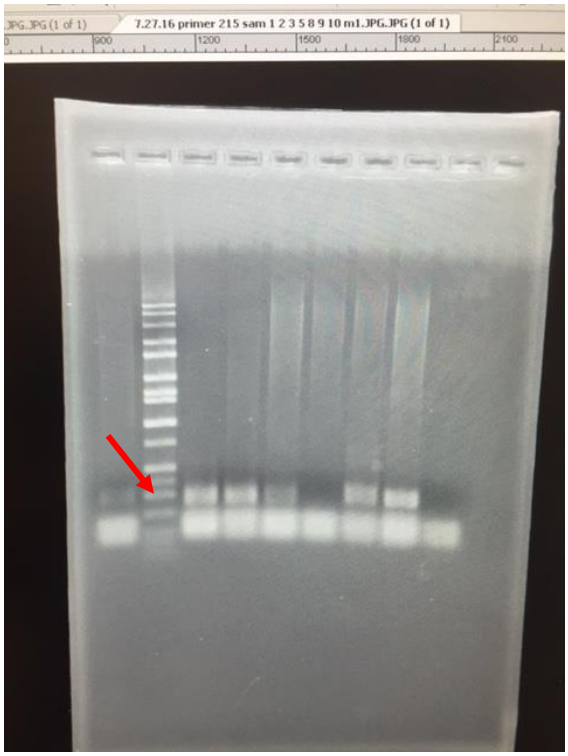
2.2.5 First-Step Amplicon PCR

Reactions for the first-step amplicon PCR were set up in groups depending on the primer being tested which allowed for a master mix to be created. The first-step PCR used primers containing locus-specific sequences as well as a universal 5' tail (Table 5) as specified in the 16S Metagenomic Sequencing Library Preparation (Part # 15044223 Rev. B). Each PCR reaction was carried out in 10µL reaction volumes using the following components: 2µl of molecular grade water, 5µl of PremixTaq (TaKaRa Taq version 2.0, contains Takara Taq, PCR buffer, and dNTPs), 1µl of forward primer, 1µl of reverse primer, 1µl of DNA sample for a total volume of 10µL per PCR tube. Using a thermocycler (Bio-Rad T100™ Thermal Cycler) assembled reactions had an initial denaturation for 5 minutes at 95°C, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing occurred at primer specific temperatures for 3 minutes (Table 5), and extension at 72°C for 60 seconds, before a final extension step of 72°C for 5 minutes. Negative controls lacking template DNA were performed in parallel with each set of PCR reactions.

PCR products were electrophoresed on a Fisher Biotech™ Horizontal Electrophoresis Systems miniwell using Bio-Rad PowerPac 3000 through 1 or 1.5% Fisher BioReagents™ agarose gel in 1xTAE buffer for 30 minutes at 150 volts. Gels were stained with ethidium bromide (10ug/mL) for 15 minutes and then observed and photographed with a Canon camera with UV light (UVB, LLC GelDoc-It®ᵉ Imaging System). The approximate sizes of PCR products were determined by comparison to Hi-Lo™ DNA Marker (Minnesota Molecular cat. No 1010).

If the correct size of products was found, the PCR product was saved (Figure 2). This process was completed for each primer being tested until sufficient products were visualized.

Figure 2. Agarose gel showing a product of the correct size utilizing the primer for locus Dam 26. To accept the reaction worked, the size range of the products needed to be in the 300bp marker line which is demonstrated using a red arrow.



2.2.6 PCR Clean – Up

All primers tested with positive PCR products (as shown in Figure 2) were pooled in individual tubes by sample number, for a total of 21 tubes. A volume of 20 µL of each sample was pipetted to a well on a PCR plate. After vortexing, the AMPure XP beads for

30 seconds, 30µL of beads were pipetted into each well. The entire volume was pipetted up and down 10 times. The sample was then incubated for 5 minutes. The plate was then placed on a magnetic stand for 2 minutes, or until all of the supernatant was clear. While the plate was still on the magnetic stand, a pipette was used to remove and discard the supernatant, carefully avoiding the pellet. The beads were then washed by adding 200µL freshly prepared 80% EtOH to each well. Following an incubation of 30 seconds, the supernatant was carefully removed. This process was repeated one more time. The small amount of excess EtOH left in the well was removed using a P20 pipette. The beads were then air dried for ten minutes. The plate was then removed from the magnetic stand and 52.5µL of 10mM Tris pH 8.5 buffer was added to each well. Using a pipette, the beads were resuspended 10 times. Following an incubation of 2 minutes, the plate was placed back on the magnetic stand for 2 minutes, until the supernatant was clear. 50µL of supernatant was transferred to a new labeled tube so the index PCR could be prepared.

2.2.7 Second-Step Index PCR

The first-step PCR amplicons were then used as templates within the second-stage PCR for further amplification where Nextera XT indexes (barcodes) were included as well as Illumina adaptors for the sequencing process. The Nextera XT index 1 primers used for the sequencer were n724, n727, n729. The index 2 primers were s513, s515, s516, s517, s518, s520, s521, s522. Each index PCR reaction was set up using 5µL DNA, 5µL Nextera XT index primer n7xx, 5µL Nextera XT index primer s5xx, 25µL rTaq, and 10µL PCR grade water. Table 6 provides the sequencer ID. The mixtures were sufficiently mixed then placed on a thermal cycler using the following program: 95°C for

3 minutes, followed by 8 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds; then 72°C for 5 minutes, then held at 4°C. After the PCR was complete, the samples were cleaned using the same PCR cleanup method described previously (16S Metagenomic Sequencing Library Preparation, Part # 15044223 Rev. B) but with 25µL of 10mM Tris pH 8.5 buffer added to each well. Following the remaining protocol, 22µL of each sample was transferred to a new labeled tube to be quantified and diluted to create the DNA library to be sequenced.

Table 6. Nextera XT Index Primers and Illumina MiSeq Identification.

Sample #	Index 1 Primer	Index 2 Primer	Sequencer ID
1	n724	s513	Emily1
2	n724	s515	Emily2
3	n724	s516	Emily3
4	n724	s517	Emily4
5	n724	s518	Emily5
6	n724	s520	Emily6
7	n724	s521	Emily7
8	n724	s522	Emily8
9	n727	s513	Emily9
10	n727	s515	Emily10
11	n727	s516	Emily11
14	n727	s517	Emily14
15	n727	s518	Emily15
16	n727	s520	Emily16
17	n727	s521	Emily17
18	n727	s522	Emily18
20	n729	s513	Emily20
21	n729	s515	Emily21
mom 1	n729	s516	Emilym1
mom 2	n729	s517	Emilym2
mom 3	n729	s518	Emilym3

2.2.8 Illumina MiSeq Library Preparation

Each sample was quantified using a Thermo Scientific NanoDrop 2000 Spectrophotometer using the NanoDrop 2000 program (Table 7). The spectrophotometer was first zeroed using 1µL of Tris, then each sample was quantified using 1µL of sample. Samples were diluted with molecular grade water to the required 4nM concentration for the Illumina MiSeq system. The library was formed by pooling 2µL of each diluted sample, then vortexed sufficiently. The pooled library was then quantified using the Qubit fluorometer with a resulting concentration of 1.56 ng/µL. The library was diluted with molecular grade water to the required 4nM concentration. From the diluted DNA sample, 1 µL was used for sequencing. This was combined with 4µL of samples from different research projects for a total of 5µL going into the Illumina MiSeq to be sequenced.

Table 7. DNA Sample Concentration and Purity Values.

Sample #	Concentration ng/µL	Purity 260/280
1	26.9	1.89
2	35.1	1.79
3	30.5	1.8
4	27.6	1.9
5	30.4	1.73
6	27.3	1.85
7	36.8	1.85
8	23.3	1.89
9	33.4	1.86
10	35.1	1.88
11	38.9	1.85
14	27.1	1.88
15	24.7	1.98
16	27.8	1.87
17	35.1	1.93
18	49.5	1.8
20	24.4	1.92
21	38.3	1.89
Mom 1	17.4	1.83
Mom 2	36.2	1.82
Mom 3	25.6	1.92

2.2.9 DNA Libraries Preparation for Illumina MiSeq Sequencing

The libraries were prepared and loaded onto the sequencer following the protocol from Illumina (Part #15039740 Revision D, October 2013). All prepared libraries were pooled together after quantification with the Qubit fluorometer, then 5 µL of freshly prepared 0.2 N NaOH was added in order to denature the DNA. Samples were briefly vortexed and then centrifuged for 1 minute. After the sample incubated for five minutes, 990 µL of pre-chilled HT1 (Hybridization Buffer) was added and then placed on ice until

ready for the next step. The 4 nM PhiX library was then diluted and denatured by combining 5μL of 4 nM PhiX control and 5 μL of 0.2 N NaOH, samples were vortexed, then centrifuged for 1 minute, then incubated for 5 minutes. The PhiX control is an internal control for the sequencer. A 20 pM PhiX library was prepared by adding 990μL of pre-chilled HT1 to the 10μL of the denatured and diluted PhiX library. The denatured 20 pM PhiX library was then diluted to 12.5 pM by combining 375 μL of the 20 pM PhiX library to 225 μL of pre-chilled HT1 and inverting several times to mix the solution. The denatured DNA sample library and the 12.5 pM PhiX control were then combined by mixing 12 μL of the denatured and diluted PhiX control and 588μL of the denatured and diluted sample library. The 600 μL of sample library and PhiX control was then loaded onto the reagent cartridge which was then loaded into the MiSeq. Prior to sequencing, a sample sheet was uploaded to the MiSeq which detailed which index pairs corresponded to each DNA sample. The MiSeq Reagent Kit v2 2x150 was used for the sequencing process.

2.2.10 Analysis

The technique of parentage analysis used was exclusion. The process of exclusion, based on Mendelian rules of inheritance, uses incompatibilities between parents and offspring to reject particular parent-offspring hypotheses (Jones & Arden 2003). Thus, identification of the paternal allele is simple since the known maternal allele can be eliminated from the offspring's pair at a given locus. In some of the cases, the mother and her offspring have identical alleles, and the paternal allele could be either of

the pair. Multiple paternity is indicated when more than two different paternal alleles are found among the genotype of the offspring.

2.2.11 Results

Genotypes for five of the nine selected microsatellite loci for each mother and her offspring are shown in Table 8. The microsatellite loci Dam 17, Dam 39, and Dam 60 were excluded from further analysis due to consistent problems with amplifying products, while Dam 45 produced no results from the sequencing. Paternal genotypes are illustrated in Table 9. Multiple paternity of offspring, indicated by the presence of three different paternal alleles at any locus, is evident in four of the five loci among broods two and three. In broods one and four, multiple paternity is the most likely scenario since there were two different potential sires discovered, however, it is also possible it could be one of the missing potential sire samples who sired all of the pups. Male 17 fathered 33.3% of the pups across two broods (brood one and four) and male 15 sired 16.6% of the pups found in one brood (brood three). The paternity of the remaining 50% of offspring could not be determined.

Table 8. Alleles at five microsatellite loci in four females and their offspring with the potential sire genotype. Paternal alleles are underlined. Cases where both alleles are underlined represent instances where the paternal allele could be represented by either of the pair. The presence of three or more different paternal alleles is shown in bold (broods two and three). Locus Dam 32 did not sequence for the first and second brood, and pup 11 in brood 3.

	Sample ID	Dam 5	Dam 20	Dam 26	Dam 32	Dam 34	Potential Sire
Brood 1 dam	5	8/21	6*/13	5.2.19/6.2.27		14/33	
Pups	1	21/22+	13/17	6.2.27/5.2.29		14/33	Male 17
	2	8/22+	13/13	5.2.17/5.2.19		14/33	Male 17
	3	8/22+	13/13	5.2.17/5.2.19		14/33	Male 17
	4	8/8	6*/13	5.2.17/5.2.19		33/33	No match
Brood 2 dam	M1	8/16	10/14	5.2.18/5.2.28		14/33	**Potential Sire**
Pups	6	8/**22+**	**10**/**14**	**5.2.18**/**5.2.28**		14/**37**	No match
	7	16/**21**	14/**17**	**5.2.18**/**5.2.18**		**14**/**33**	No match
	8	**8**/**8**	10/**15**	5.2.28/**5.4.31**		14/**42**	No match
Brood 3 dam	M2	8/8	10/15	5.3.21/5.4.31	25*/32+	14/42	**Potential Sire**
Pups	9	8/**16**	**10**/**15**	5.3.21/**5.2.31**	25*/26	**26**/42	Male 15
	10	8/**18**	**15**/**15**	5.3.21/**5.3.24**	25*/25*	14/**39**	Male 15
	11	8/**21**	10/**14**	**5.2.18**/5.3.21		14/**26**	No match
Brood 4 dam	M3	18/21	12/14	5.2.17/5.3.21	23*/25*	14/26	**Potential Sire**
Pups	20	8/18	12/13	5.2.17/5.2.19	19/23*	14/26	No match
	21	21/22+	13/14	5.2.17/5.2.17	23*/23*	26/33	Male 17

Table 9. Alleles at five microsatellite loci for the known potential sires in this study. At least two additional males did not have DNA samples obtained before departure. There was no data for sample 18 at locus Dam 32.

Sample ID	Dam 5	Dam 20	Dam 26	Dam 32	Dam 34
14	8/21	10/12	5.3.21/5.2.28	21*/25*	13/14
15	16/18	10/15	5.3.24/5.2.31	25*/26	26/39
16	8/8	10/14	5.2.18/5.2.28	25*/25*	14/26
17	16/22+	13/17	5.2.17/5.2.28	23*/32+	33/40
18	16/18	6*/17	5.2.17/5.2.19		33/44

2.3 DISCUSSION

2.3.1 Polyandry in Elasmobranchs

Mating systems are now recognized as an important component of population assessment and have become increasingly important for conservation purposes (Byrne & Avise 2012). There is a growing awareness in studying the mating systems of overexploited sharks and rays since many species occupy an important position in their ecosystem (Chapman *et al.* 2004). An increase in molecular tools has allowed for a better understanding of elasmobranch reproductive behavior and all sexual conflicts not easily observed in the wild (Feldheim *et al.* 2002). Questions about their mating systems and life histories can now be addressed, which Rowe and Hutchings (2003) have recognized as a fundamental requirement for any long term, effective conservation. They state that depending on the mating system, exploitation may increase the rate of decline and the time of recovery of a species. An estimated 25% of all sharks and ray species are considered threatened by the IUCN (Dulvy *et al.* 2014). An example of this, cited in

Chevolot (2007), is the disappearance of three skates, the common skate *Dipturus batis* (Brander 1981), the white skate *Rostroraja alba* (Dulvy *et al.* 2000), and the spinytail skate *Bathyraja spinicauda* (Devine *et al.* 2006). Management strategies to protect threatened elasmobranchs include: designation of new marine protected areas, determination of the effects of fishing mortalities, protection of suspected nursery areas and seasons, and captive breeding. These management strategies benefit from detailed knowledge of population structures, genetic diversity, and mating systems (Feldheim *et al.* 2007).

Elasmobranchs exhibit a variety of reproductive modes including various forms of viviparity (live birth), oviparity (egg laying), and parthenogenesis, and display monogamous and polyandrous behaviors. Despite this diversity all species have internal fertilization. The life-history characteristics that make elasmobranchs susceptible to overexploitation, mainly due to overfishing because of their k-selected life history traits (late age at maturity, long life spans, low fecundity, and low natural mortality) result in low reproductive output and limited ability to recover from overexploitations and/or population depletion. Reproductive strategy can have considerable effect on genetic diversity which in turn affects the ability of populations to respond to selection pressures like changes in environmental conditions (Rowe & Hutchings 2003). For this reason, loss of genetic diversity has been associated with increased vulnerability to population depletion and extinction (Dulvy *et al.* 2003).

Determining if multiple paternity is occurring from polyandrous mating events is important because in species with internal fertilization, monogamy or polygyny have

traditionally been considered the dominant mating systems (Portnoy & Heist 2012). Polyandrous behavior is unexpected due to the associated costs excessive mating carries for the female which can ultimately reduce their fitness. These costs may be offset through material or genetic benefits gained by the female through mating (Fedorka & Mousseau 2002).

Based on field observations, group reproductive behavior and polyandrous copulations may be more common in sharks and batoids (Carrier *et al.* 1994, Yano *et al.* 1999, Pratt & Carrier 2001, Chapman *et al.* 2003, Whitney *et al.* 2004). Evidence of behavioral and genetic multiple paternity in elasmobranchs come from the rare observation of females mating with multiple males over the course of a single breeding season (Chapman *et al.* 2003) or from genetic studies where molecular techniques have led to an increase in the number of studies assessing multiple paternity (Feldheim *et al.* 2001a, Chapman *et al.* 2004, Daly-Engel *et al.* 2006, Chevolot *et al.* 2007, DiBatissta *et al.* 2008b, Veríssimo *et al.* 2011, Lage *et al.* 2008, Portnoy *et al.* 2007, Saville *et al.* 2002, Byrne & Avise 2012, Daly-Engel *et al.* 2010, Boomer *et al.* 2013, Hernandez *et al.* 2014). These behavioral observations and genetic studies support the idea that polyandry occurs in elasmobranchs which allows researchers to infer patterns of reproductive behavior without direct observations (Fitzpatrick *et al.* 2012). Multiple paternity resulting from polyandry has been identified in all elasmobranch species where more than one litter has been examined which includes egg layers, placental and non-placental live bearers, and in both shark and batoid species (Byrne & Avise 2012, Boomer *et al.* 2013). However, the prevalence of genetic polyandry and the estimated number of sires

contributing to individual litters differ greatly among species (Portnoy & Heist 2012). Previous research has detected a range of 1-4 sires per brood (Chapman *et al*. 2004; Feldheim *et al*. 2004; Chevolot *et al*. 2007; Portnoy *et al*. 2007; Lage *et al*. 2008; Daly-Engel *et al*. 2010; Heist *et al*. 2011; Veríssimo *et al*. 2011; Griffiths *et al*. 2012, Roussow *et al*. 2016).

The pervasiveness of polyandry in most sharks studied to date has led researchers to believe this behavior provides fitness benefits to females (Feldheim *et al*. 2004; Daly-Engel *et al*. 2006). Multiple paternity is hypothesized to be driven by direct or indirect benefits. For male elasmobranchs the advantages of polyandry is greater reproductive fitness (Bateman 1948, as cited in DiBattista *et al.* 2008a). For female elasmobranchs there have been no direct benefits found, although there is potential for indirect benefits (DiBattista *et al.* 2008a; Daly-Engel *et al.* 2010). Detecting indirect benefits is difficult in long lived species with late maturity because such benefits can only be measured by reproduction of an individual offspring (Portnoy 2010). Multiple mating can be disadvantageous to females due to the risk of injury or exposure to disease during mating events. Often, female sharks and rays sustain injuries, sometimes severe, which may discourage multiple mating by females due to the harm incurred during mating events (Pratt & Carrier 2001). According to a case study completed by Pratt and Carrier (2001) mating entails biting, chasing, and aggression. Males can overwhelm a female during mating events and females are left vulnerable to predation or mortality due to wounds and exhaustion (Pratt & Carrier 2001). In these situations, polyandry may decrease female fitness (Byrne & Avise 2012). But despite the disadvantages posed by polyandry,

multiple mating of females with different males is observed frequently in nature. Many hypotheses have been put forth to explain why polyandry occurs in animals like elasmobranchs, where the female receives no direct benefits from the mating.

2.3.2 Explanations Behind Multiple Mating and Polyandry

Hypotheses fall into two categories of benefits that explain polyandry in the animal kingdom. Direct benefits, which increase the number of offspring a female can produce, include nuptial gifts, increased sperm volume, and parental care on the part of the male (Fedorka & Mousseau 2002; Daly-Engel et al. 2010; Portnoy 2010). When no direct benefit is observed, females are supposed to gain genetic benefits (Yasui 1997). There are several hypotheses proposing the benefits to polyandry are genetic. These hypotheses include (1) the *intrinsic male-quality hypothesis*, that sperm competition or female choice of sperm increases the probability of fertilization by high quality sperm or males (Madsen *et al.* 1992, Birkhead *et al.* 1993, as cited in Zeh & Zeh 2001), (2) the *trading-up hypothesis*, that extra pair copulations compensate for a poor quality mate (Kempenaers *et al.* 1992, Hasselquist *et al.* 1996, as cited in Zeh & Zeh 2001), (3) the *bet-hedging hypothesis* that females actively seeking multiple males could lower the chance of mating with an incompatible, inferior, or infertile male, thus increasing her chances of having higher survivorship for her offspring (Fedorka & Mousseau 2002), (4) the *sexually-selected sperm hypothesis* that multiple mating by females facilitates sperm competition and there is an increased chance her eggs are fertilized by the more competitive sperm, which thereby will increase the chance of her male offspring will produce competitively superior sperm (Keller & Reeve 1995, Evans & Gasparini 2013),

(5) the *offspring diversity hypothesis*, that increased offspring genetic variability enhances females fitness by reducing sibling competition or as a hedge against environmental uncertainty (Ridley 1993, Loman *et al.* 1988, as cited in Zeh & Zeh 2001), (6) the *inbreeding avoidance hypothesis*, that polyandry diminishes the cost of inbreeding in situations in which females cannot avoid mating with close relatives (Stockley *et al.* 1993), and (7) the *genetic incompatibility avoidance hypothesis* proposes that polyandry enables females to exploit post-copulatory mechanisms that minimize the risk and cost of fertilization by genetically incompatible sperm (Zeh & Zeh 1996, 1997, as cited in Zeh & Zeh 2001).

Another explanation of multiple mating by female elasmobranchs may simply be the result of convenience polyandry. According to this hypothesis, females may accept multiple mating partners simply because the costs of resistance exceed the costs of accepting unnecessary copulation. This hypothesis may best explain cases of multiple mating that were found to provide no benefits to offspring but instead function as a means by which females avoid excessive harassment (Chapman *et al.* 2013; DiBattista *et al*. 2008b).

Chapman *et al*. (2004) details another explanation behind multiple paternity in elasmobranchs. They predict that polyandry and multiple paternity are more common in species with low dispersal rates and high levels of philopatry because polyandry may increase genetic diversity of litters and decrease sibling competition for resources (Daly-Engel *et al*. 2007). Examples of this have been shown by the predominance of multiple paternity in two sharks which have a high degree of philopatry and low dispersal rates

(lemon shark *Negaprion brevirostris*, Feldheim *et al.* 2004; nurse shark *Ginglymostoma cirratum*, Saville *et al.* 2002).

2.3.3 Multiple Paternity in this Investigation

This study provides the first demonstration of multiple paternity for a dasyatid ray, and only the second documented case of multiple paternity in a matrotrophic batoid. Multiple paternity was indicated in broods two and three by the presence of three to four paternal alleles in four of the five loci (Table 8). In brood two, there is no potential sire match, and in brood three, male 15 most likely fathered two of the three pups and the third pup did not have a match. Had additional male samples been obtained there is a possibility the paternal allele could have been identified. As shown in brood one, microsatellite genotypes of the female and her offspring indicated that each pup inherited one maternal allele, and two paternal alleles were observed at four of the five loci. Male 17 fathered three out of the four offspring, while the fourth pup had no match from the known paternal genotypes. In brood four, two paternal alleles were observed at all five of the loci. Male 17 fathered pup 21, however, for pup 20 no match was observed. As with brood one, if we procured the additional male samples, there is a chance we could have detected multiple paternity or confirmed monogamous behavior. There was no data from the sequencer for at the locus Dam 32 for the first and second brood, pup 11 in brood 3, and male 18.

Interestingly, the males who sired 50% of the pups were wild caught individuals from the Florida Keys. Male 17 sired 33.3% of the pups across two broods (one and four) and male 15 sired 16.6% of the pups in brood three. This may indicate wild caught

males have a higher reproductive success then the captive bred males in this small population, but this cannot be tested. Male 18 was captive born to Mom 1 of this study while male 14 was also captive born, but not to a dam not used in this investigation. However, without having the DNA samples from the other potential sires, we cannot be certain of this interpretation. Further investigation is required to examine reproductive success in males.

As with any investigation, results lead to more questions. Although this investigation relied upon a limited number of samples from a small number of litters, multiple paternity or genetic polyandry was documented in this captive population of southern stingrays. Due to the similarities in biology and behavior, it is likely that it occurs in wild conspecifics, and possibly in similar species of dasyatids. Information such as obtained in this investigation on the mating system in the southern stingray, can be useful in developing management strategies for the species.

The methods used in this investigation advanced the knowledge of stingray mating systems and the technology used for determining multiple paternity by using next-generation sequencing based 'genotyping-by-sequencing' (GBS) of microsatellite loci. The primary advantage of GBS as a method for microsatellite genotyping is an increase in data showing more allelic diversity. It is a rapid and cost-effective method that can be used for large-scale population genetic studies and provides access to the sequence data, providing an additional advantage over traditional fragment length genotyping by resolving issues of size homoplasy and revealing potentially hidden genetic variation in the amplicons (Vartia *et al.* 2015).

CHAPTER 3

PROBLEMS ENCOUNTERED AND SUGGESTIONS FOR FUTURE RESEARCH

3.1 PROBLEMS ENCOUNTERED

3.1.1 Sample Retrieval

This study faced several challenges with acquiring the necessary samples. A sample from a potential sire, labeled as sample ID 19 in table 4 was thought to have been attained prior to transporting the ray to another public aquarium. This aquarium was reached out to, however, it was deemed by the care staff to be potentially harmful to the health of the ray to obtain a tissue sample. A way to mitigate this would be by having keepers remove the stingray's venomous spine by clippers, instead of a fin clip. Stingray barbs are covered in a venomous sheath of skin and once the spine is removed, it will start to grow back (Janse *et al.* 2013). Obtaining a tissue sample this way is relatively non-invasive, particularly if the spines are clipped per routine husbandry practice and would cause the least harm to the animal. This offers a perfect opportunity for collection of tissue samples when animals arrive to the facility or have an exam. If institutions started this process as soon as an elasmobranch enters the collection, building a bank of tissue samples properly stored for future research is easily attainable. After working on this study, this process has been implemented at the National Aquarium for future research.

A similar instance occurred with the fourth mother sampled “Mom 3”, when she was traded to another aquarium in 2012. However, when the receiving aquarium was contacted in 2014, they were able to take a sample (fin clip) and send it (in a vial containing Queen’s Lysis Buffer) to the National Aquarium for this study. Additionally, fin clips were taken in 2005 from a litter of five pups from mother sample “Mom 2” specifically for the potential to research multiple paternity in this species at the National Aquarium. They were to be kept in the -20°C freezer in the National Aquarium’s Clinical Lab. In 2014 when samples were being organized for this study, these samples were sought. However, they were not found to be in the correct spot and could not be located anywhere else. This was a significant loss since it would have increased the number of litters studied and this litter had the highest number of pups to test for multiple paternity.

While completing the analysis, it was discovered there were more potential sires. After looking through old paper logs and cross referencing with our online database, one additional male was discovered to be in the exhibits during the time of fertilization. The National Aquarium did not start using TRACKS® Software until 2006, thus all the paper logs that were kept had to be transcribed into the database which could have led to this error. This additional male brings the total potential sires up to seven individuals, however, based on staff memory there is a possibility of one additional male being in the system during this time making the total potential sires up to eight individuals.

3.1.2 DNA Isolation

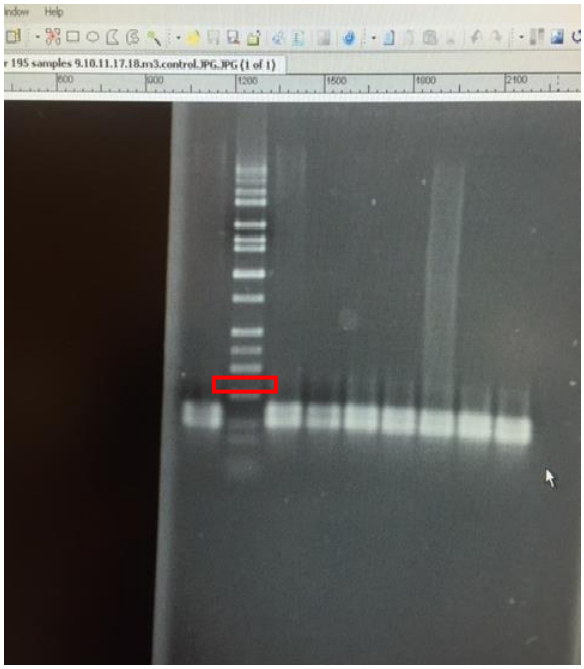
DNA was easily isolated from all samples except for one, sample "Mom 1". The first sample used for attempting isolation was a liver sample obtained from the -20 °C long term storage freezer from the National Aquarium's Clinical Lab. DNA was not visible at any point during the isolation process. The sample was still tested using PCR and electrophoresis. At no time was any product visible. Two other tissue samples from this subject were then obtained for DNA isolation, specifically, thyroid and kidney samples. The same issues occurred with these samples as it did with the liver sample and after PCR and electrophoresis no product was ever visible for any loci. No muscle or fin clips were available to use for DNA isolation. Regardless, the sample was still used for sequencing and after quantification the sample concentration was 17.4 ng/μL, the lowest DNA concentration of all the samples. Improper tissue preservation may be have led to the difficulty in isolating visible DNA from this subject. Regardless of this problem, this sample was still able to be genotyped using the MiSeq.

3.1.3 Issues with PCR and Primers

After several rounds of unsuccessful first-step amplicon PCR, samples 8, 14, 15, 16, 20, 21, and Mom 1 were diluted 1:100 to diminish potential PCR inhibitors. Diluting these specific samples by adding 5 μL of DNA to 495 μL of molecular grade water allowed for PCR to work, except for Mom 1. There were challenges with three primers designed for this study, loci Dam 17, Dam 39, Dam 60. These primers did not work any time they were used for amplification, an example of this is shown in Figure 3. Numerous tactics were tried to determine any error including: trying fresh rTaq,

molecular grade water, and primers, using DNA samples confirmed to work, diluted DNA samples, increasing the % of agarose gel, and checking primer sequences against original sequences. Primer dimers were visible on all gels, however, no bands of the appropriate size were found.

Figure 3. Agarose gel showing PCR reactions that did not produce a product. The primer utilized was for locus Dam 17. A positive product would have been in the high 200bp range, indicated by the red rectangle, while the bands shown here are around 150bp. The most likely cause of bands on this gel is primer dimer.



3.2 SUGGESTIONS FOR FUTURE RESEARCH

3.2.1 Detecting Indirect Benefits

If indirect benefits are found to be lacking, a likely alternative is that females engage in multiple mating simply to reduce the costs of mating (Portnoy *et al.* 2007). Indirect benefits have been shown in a range of taxa (Fedorka & Mousseau 2002; Liu &

Avise 2010) even though their prevalence remains controversial (DiBattista *et al.* 2008a). Indirect genetic benefits of polyandry might be tested by assessing whether offspring from litters sired by a single male differ in fitness from those sired by multiple males (DiBattista *et al.* 2008a). DiBattista *et al.* (2008a) did not find any evidence that polyandry provides indirect benefits in the lemon shark, *Negaprion brevirostris*. They postulate that it is possible genetic diversity is correlated with other unmeasurable fitness components, such as embryo survival *in utero*, survival to adult hood, or lifetime reproductive success. These possibilities are difficult to track in the wild since elasmobranchs are long-lived and highly mobile. Portnoy (2010) suggests testing indirect benefits using large comparative studies across multiple populations with different environmental and demographic characteristics as well as long-term studies will be needed to further examine these questions.

Future work examining the occurrence of convenience polyandry in nature and its relationship to mating systems is also needed (DiBattista *et al.* 2008a). It is a reasonable explanation when females mate multiply with no benefits occurring due to the potential for high costs inflicted as a result of the mating process. Experiments have shown that polyandry can enhance offspring survival in artificial settings but tests in natural populations are rare due to the difficulties of working with elasmobranchs and long-lived species (DiBattista *et al.* 2008a).

3.2.2 Use of Aquariums and Captive Settings

Due to the small sample sizes available in this study, multiple paternity from polyandrous behavior in the captive southern rays was thought to be difficult to

determine. For future investigations to be completed more effectively, if mature southern stingrays are kept in a mixed-sex captive population and breeding events occur regularly, genetic samples from all pups and mature adults should be obtained. Researchers could use these populations to better determine the scope of multiple paternity as well as be available for other molecular techniques from a single sample. Oftentimes following parturition, the pups will be sent to other institutions. Indirect benefits could be investigated if institutions worked collaboratively to keep records of health and breeding success, and developed a way to easily track all animals. For example, this could allow for researchers to seek life history information on the pups from this study and look for comparisons from the pups who were sired by wild-caught males vs. captive-bred males. Testing the genetic mating systems of elasmobranchs in captivity may shed light on what occurs in wild populations (Heist & Feldheim 2004).

Since elasmobranchs, especially dasyatids, are popular in aquaria, it is extremely important to create sustainable breeding programs to become independent from wild collecting (Janse *et al.* 2013). A first step in the process of gathering captive-bred populations is to gather information on current breeding behavior in captivity; such as whether multiple mating occurs and whether poor offspring survival can be linked to particular parents (Townsend *et al.* 2015). Field stations in the southern stingray's natural range, such as the Bimini Biological Field Station in Bimini, Bahamas, also provide opportunities to study these animals in a wild setting. Much like the methods Feldheim et al. (2004) used to investigate lemon sharks, *Negaprion brevirostris*, if researchers come across a pregnant southern stingray, she could be transported to a semi-

captive pen in a sand flat or lagoon until she gives birth. DNA samples of the dam and pups could be attained and used for further analysis of paternity in wild southern stingrays.

3.2.3 Other Possible Studies

The microsatellites used in this investigation could be tried using species that are closely related to the southern stingray, for instance, the Atlantic stingray *Hypanus sabinus*, to see if they work. If so, these specific primers could be used for genetic studies among other dasyatid species, particularly the threatened species. The methods used here present a cost-effective and rapid technique for sequencing an increased amount of loci and using these loci to complete large sample population studies.

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APPENDICES

APPENDIX A: For this investigation, nine microsatellite markers (highlighted in grey) were utilized for determination of multiple paternity in the southern stingray, *Hypanus americanus,* from a microsatellite library developed by Dr. Mahmood Shivji of Nova Southeastern University, Ft. Lauderdale, Florida, and Dr. Kevin Feldheim of Pritzker DNA Lab - Field Museum, Chicago, Illinois. Primers (underlined and highlighted in yellow) were developed from Integrative DNA Technology specifically for this investigation.

Dam 5: $(TAGA)_{16}$

GTTTAAGGCCTAGCTNGCAGAATCACAGTGTTCATGAAGTCAATGCTGCTTTG
CCTCACTTCTGTAGACTCTGTGTCCATCTCCTTAGTAAATACTGATGCAAAAA
TATTTATTTAAGACTCTCCCTCG**CCTCCACTCATGGATTACCATTCT**GATCT
TCCAAAGAAACTATTTTGTCCCTTGCAATCCTTTTGCTCTTAACATATCTGTAG
AAACGCTCAGGATTATCTTTCACCTTATCTGT**TAGATAGATAGATAGATAGA**
TAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATACTTT
ATACATCCCCAAGGGGAAATTCAACATTTTT**CCAGTGTCCCATACACTTATT**
GTAGCAAAACCAATTACATACAGTATTTAACTCAGTATAAATATGATATGCA
TCTAAAAATCAACCCCCCAAAAAAAGCGTTAATAAATAGCTTTTAAAAAGTT
CTTAAATAGTTTACTAAAGTGCATTGAATGGTAACTTAAGCTCAGTCCTAACC
CTGGCACTTAAACATGTCTTGCCCCTGGTGGTTGAATTGTAGAGCCAAATGGC
GTTGGGGAGTAATGATCTCTTCATCCTGTCTGAGGAGCATTGCATTGATAGGA
ACCTGCCGCTGAAGCTGCTTCTCTGTCTCTGGGTGGTGCTATGCAGAGGATGT
TCAGGGTTTTCCATGATTGACCGTAGCCTACTCAGCGCCCTCCGCTCTGCCAC
CGATGTCATACTCTCCAGTTCTGTGCCCACGACAGAGCCCGCCTTCCTTACCA
GTTTATTAAGACGTGAGGTGTCCCTCTTCTTAATGCTGCCTCCCCAGCACGCC
ACCACAAAGAAGAGGGCACTCTCCACAACTGACCGATAGAACAGATTCTGCT
AGCTAGGCCTTAAACA

Dam 17: $(ATCT)_{15}$

GTTTAAGGCCTAGCTAGCAGAATCACATATTGATTCCGCTCCATGGATGAATA
GAGCTGGAATATTAACAAAGAGTGAGAGGTTCTGGGGAGTGCTGATGCAGG
GACAGACAAGGAAATGGACGGTCAGAATCCACTGACAGCAGGGATGCGGGT
TGATATCAGCGACATACCTGTGTTCAGTAGTGGATAATACAATGGACACAGT
TGGAGTGGAAGTGGAAGATTTAGCGTAAGTCCACGTAAGGTGTTGGCTTTCA
TTGTGAACCGTTATTGACCCGGTCTCGGGGGTGAGTGTTAACTAATTTCTCTT
TTTCCGGAGTCTGGTTCACTGGACTAGTTCTCCGAGTCTATGTTTCAGTCTTGG
ACCTTGAAAATGGACTCAGGTCCAGCAGATCATCATCAAAAGAAACAATAAA
CAATTCATTGCGTCCGCTTTTATTTGCTCTAATCAAGTCAGCCTGGAGGAGGA
ACTTCCATTCCTGCGAGCACATGGCAAGAACAACGAACTTCCCTTCGTCTAGT
CTCGTTCCT**GGTTACCGATTTACAGACTGTCTGTCT**ATCTATCTCTCTGGCT
ATCTATCTATCC**ATCTATCTATCTATCTATCTATCTATCTATCTATCTATCT**
ATCTATCTATCTATCTATCTGTCTATCTATCTCTCTGTCTATTGGACTGCCAG
GCTGTCTGTTCTCGACCTGCCTGCT**TGTCTGTTTGCCGGTCTAACTG**TTCAT
CTGCGGCTCTGCCAGATATTGCCACTTTGATCGAGAGGGTTTCACAAGGAGA
GAGACAATGCGAAGCCGCTATGAAAATGGAAATGCTCCTTTTCTCAATCTTA
CCGGATCAAGGTTTACATGATGGATACGCTCTGACATGAGAATGACGTCGTT
TTACCATTCCATGGTCAGTTTATGGGCGTTTACCAAGTCCGACTGAATTTCAT
GACCCATGAGCCACCGAATCCCTCACTTGGTGCACGGGTTTTAACTACATTGA
TGATTTATGGTTTGGTGGAGACAGACAGCAAAGCCTAAGTGAAGGTCCACGT
GAACTGGCTGAGGAAATGAAGCTCAGTGAGTGGACAATGAACTCAAATGGA
AATACCTGTTCTCAGCTATAATGTGGAAAATAGAGATACAATGGAATAATGT
AAAACATAATTATTCAGTAGAGGGCGAAGACTAACATCGAGTGATTCTGCTA
GCTAGGCCTTAAACA

DAM 20: $(TATC)_{12}$

GTTTAAGGCCTAGCTAGCAGAA*TC*TGTTCTATCGGTCAGTTGTGGAGAGTGCC
CTCTTCTTTGTGGTAAACTATTTAAGGACTTTTTAAAAGCTATTTATTAATGCG
TTTTTGGGGGGGGTGATTTTTAGATGCATATCATGTTTATACTGAGTTAAATA
CTGTATGTAATTGGTTTT**GCTACAATAAGTGTATGGGACACTGG**AAAAATG
TTGAATTTCCCCTTGGGGATGAATAAAGTATATATCTACC**TATCTATCTATCT**
ATCTATCTATCTATCTATCTATCTATCTATCTATCTGTAAGGAGTTTGTATT
TTCTCCTTGTGACCACTTGGG**TTTCCTCCAGATGCTCTGGTTT**TCTCTCACA
GTCCAAAGGCGTGCCAGTTGGAAGGTTAATTGGTCATTGTAAATTGTCCAACT
AATTTGATCAACTCACAAAATGTTGGAAGAAATAAGTGGGTCTATGGAGAGA
AATTAAGTCAATGTTTTGGGTTGAGACTAGTCACATAGTCTTGACGTGATTCT
GCCTTAGCTAGGCATTAAACA

Dam 26: $(TG)_{27}$

GTTTAAGGCCTAGCTNGCAGAATCACACCGACACCATGGCTCTTTAACGCTGT
AATCGATCAAATAAGCATGTTTT**CGATATCATTGTCAACATTTCTGTTC**AAT
TGCAGCAAAAACTATATATATAATGTTAAGTCGTATATATATATTATA**TGTGT**
GTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTG
TTAGTGTTACAACGGAATGTGTCCCCCTGTCCAGATTAGACTTGTTTTTTTTCT
CTGAATTTA**CTTATAACTCTGCGGGCTCGT**CTGCTCACATATATAATGACA
GAAGAAACAGTCTATCTCAATCATTAAATTATTCTGCCATTTCTGTGTTTCCC
ACAACAATTTCACCCAATTCATTCTTCAAGGGCCCAACATTGTTCTTAATTAT
CTTCTTTCTCTTCACATACCTAAAAACGCTTTAGCTATCCTCCTTTATATTCCT
GGCTAGCTTGCGTTCGTGATTCTGCTAGCTAGGCCTTAAACA

Dam 32: $(TAGA)_{27}$

GTTTAAGGCCTAGCTAGCAGAATCACACTCTGACAGAGCTTTAGCAGTTTTAA
AAAGAAGAGTGGGGGGAAATTTGCAGTGTTCTGATGTGCAAAGCTGATGGAG
ACCTATCTACACAGACTCAAGGCTGTAATTGCTGACAAAGGTGCGTCTACTA
AATAATGACTTGAAGGAGCAAATACTTATGCAGTCAATTATTTTGTGTTTCAT
AGTTGTAATTAATTTAGATCACTTTGTGGAGATCTGTTTTCACTTTGACTTTTT
CTGTTTGATCAGTGTCAAAAAATCTAAATTAAATCCATTGTGATTCATTGTTG
TAAAACAATAAAACATGAAAAATTCCAAGGAGGGGTGAACACTTTTTTATT**C**
TGAGACACTGCAGATAGATACCTAGCTAGATAGATAGATAGATAGATAG
ATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGA
TAGATAGATAGATAGATAGATAGATAGATAGATAGATAGGTAGGTAGGT
AGGTAGGTCGGTAGCCAAGCAGATAGAGAGATTGATTGATTGATTGATT
AATAGATAGATAAAATAACTTCAAA**CACAATATGGAGAATCCCTGACATT**
TAATGAAGAGATTCTGCTAGCTAGGCCTTAAACA

Dam 34: $(AC)_{33}$

GTTTAAGGNCTAGCTGGCAGAATCACACCAAACTTAATTTCTTCAGGATTTCT
GTGTATGAGCAAAGATGTCTTGCTACTGTTGTTCTTCAATTGCTATGCCATGG
NACTACAAGCATAAAAAAATGGATATATGGAAACAAAAAAAAGGATACAAC
AGAGACAACACTTCACTCACCAAAACAATACATTTTATTTCTACATTAATGCC
ACCCTAACCCATGTTATCCTCTCCATATTCCCTTAA**CTCCAACTCACACCTCT**
TCCTCTCCCCATCCATACACGCACACGC**ACACACACACACACACACACACA**
CACACACACACACACACACACACACACACACACACACACACACTCACACT
CAATCACTCAGGGGCAGTGGTAAAAT**GGAAATCACAAGAACAACTAGTGG**
ACAACAGAGGGGCTACATCTGATTGAGAAGCGAAAGGAAACTAAGGCACCA
CAACAAGTAGAATGTGGAAGCTTCACTCAAATATCCACATCAGAGTGTGTGA
TCAAGCACATATCATTAGATGTCGAGGGTAGTGATTCTGCTAGCTAGGCCTTA
AACA

Dam 39: $(GT)_{31}$

GTTTAAGGCCTAGCTAGCAGAATCACATGGAAGAGAAGGGTATGGAGGACT
ATGGTCCAAGAGCGGATTGAATAATAATTCAATAATAGGCAGAATAA**TAGTT**
CAGCATGGACTACGTGGGATGAAATGTTTTTCTGTGTTGCAGTATTCTTTAA
TTCAATGACGCTATCAACTTGCTGCACATCCATCTGGA**GTGTGTGTGTGTGT**
GTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTA
TAC**GTGTATACAGACAGAGAAGGTAGATG**TTTTTCTTTGAATGACTACCTC
ATCAGCATCATTTTCCAGTGGTCCAATATCAACTCTCATCTCTCTTTTACTCTT
TATATAACTGAAAAATCATTCAGTATCCTGTTTCATATTATTGGCTAGCTTGC
CCTCATATTTCATCTTTTCCCTTCATACGGTTTTTTTTAGTTGTCTTTTGTTGGA
TTGTAAAAACTTCGTAATCATTCGACTTCCCACTCACTTTTGCTACACTATGTG
ACCTTTGGTTTTTATGCAGTCTTTAACATCCCTTGTCAGCCATGGTTGCCTACA
CTTGCCATTTGTGAACAGATTCTGCTAGCTAGGCCTTAAACA

Dam 45: $(TAGA)_{20}$

GTTTAAGGCCTAGCTGGCAGAATCACATATATGTCACCATTTACAACCCTGAG
ATTCATTTTCTTGCAGGCATATTCTATAAGCAAAAGAGAATCAATGAAAACT
GCA**CCAATAGGATGGACAACTAATGTG**CAAAAGAAAACAAACTGTTACAA
CTACAAAAGCAAAGAAAAGGAAATAATTGTTATTAACCAATCAATAAATAAA
CAAACAAATAAATAAATAAATGGA**TAGATAGATAAATAATTAACTAGATA**
GATAGATAAATAGATAGATGGATGGATGGATAAATAGATAGATAGATAG
ATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGA
TAGATAGATAGATAGATAAGCAACA**TGAGATGAAGAGTCTTTGAAAGTGA**
GTCCATAGGTTATGGGAACAGTTCAGTGATGGGGCATGTGAACATGTGAAGT
TGAGTGGTTATCCCCTTTGGTTCAGGAGCCTGATGGTTAAGAGATAATAACTG
TTCCTAAATCTGTGTGAGTCCTGAGGCTTCTGCACCTTCTTCCTGATGGCAGC
AGTGAGACGAGAGCATGTCCTGGTTGGTAGGGGTCTATGATGAGGGATGCTG
CTTTCCTGGCNAANCACTCCATGTAGATGTGCTCNACAGTGGGGAGGGTTTTA
CC

Dam 60: $(TG)_{41}$

GTTTAAGGCCTANCTNACAGAATCGATTCACTCAGTCATCCAGCCTTCAGAGT
CATCACCGAGTTCACACTGGGGAGAAGCCGTTTAGCTCCTAAGAATGTGGGA
CAGGATTCATTCAGTCATCTCAACTACTGGCACAACAGGCAGTTCACACTAA
AGAGTGGTCGTTCTTATAAATCCATA**GGTTTCATTTGCTGTGGACTG**TATTT
TA**TGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGT**
GTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGAGAGGGAGAGAGAG
AGAGAC**AGAGAGCCTGACTCGCAGC**TTGTTTAAGTCGCTCGAAGCTTGTTT
AGTTTTAACCGAGGACATAGACACTTATAGTCAGACAGAAACGAAGGAGGA
GAAATGGAACAGTCGAGACAAAGGAACTAGTGGCCAAGGGTCACTATTTGTG
ACTTTCCTATGCCCACAAGTCTGGGCTAATTATNGATTCAGCATAAAGNGATT
CTGCTAGCTAGGCCTNAAACA

APPENDIX B: Examples of alleles genotyped at five loci using the Illumina MiSeq for determination of multiple paternity.

Dam 5: ($(TATC)_8$
GCTACAATAAGTGTATGGGACACTGGAAAAATGTTGAATTTCCCCTTGGGGA
TGTATAAAGTATCTATCTATCTATCTATCTATCTATCTATCTAACAGATAAGG
TGAAAGATAATCCTGAGCGTTTCTACAGATATGTTAAGAGCAAAAG
Dam 5: $(TATC)_{21}$
GCTACAATAAGTGTATGGGACACTGGAAAAATGTTGAATTTCCCCTTGGGGA
TGTATAAAGTATCTATCTATCTATCTATCTATCTATCTATCTATCTATCTATCT
ATCTATCTATCTATCTATCTATCTATCTATCTATCTATCTAACAG

Dam 20: $(TATC)_{6*}$
GCTACAATAAGTGTATGGGACACTGGAAAAATGTTGAATTTCCCCTTGGGGA
TGAATAAAGTATATATCTATCTATCTATCTATCTGTAAGGAGTTTGTATT
TTCTCCTTGTGACCACTTGGGTTTCCTCCAGATGCTCTGGTTT
Dam 20: $(TATC)_{13}$
GCTACAATAAGTGTATGGGACACTGGAAAAATGTTGAATTTCCCCTTGGGGA
TGAATAAAGTATATATCTACCTATCTATCTATCTATCTATCTATCTATCTATCT
ATCTATCTATCTATCTATCTGTAAGGAGTTTGTATTTTCTCCTTG

Dam 26: ($(TA)_5(TA)_4(TG)_{30}$
CGATATCATTGTCAACATTTCTGTTCAATTGCAGCAAAAACTATATATATAAT
GTTAAGTCGTATATATATATTATATATATGTGTGTGTGTGTGTGTGTGTGTGT
GTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTTAGTGTTACAACGGAAT
GTGTCCCCCTGTCCAGATTAGACTTGTTTTTTTTCTCTGAATTTACTTATAACT
CTGCGGGCTCGT
Dam 26: $(TA)_6(TA)_2(TG)_{27}$
CGATATCATTGTCAACATTTCTGTTCAATTGCAGCAAAAACTATATATATAAT
GTTAAGTCGTATATATATATATTATATGTGTGTGTGTGTGTGTGTGTGTGTGT
GTGTGTGTGTGTGTGTGTGTGTGTGTGTTAGTGTTACAACGGAAT

Dam 32: $(TAGA)_{25*}$
CTGAGACACTGCAGATAGATACCTAGCTAGATAGATAGGTAGATAGATAGAT
AGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGA
TAGATAGATAGATAGATAGATAGATAGGTAGGTAGGTAGGTAGGTAGG
Dam 32: $(TAGA)_{21*}$
CTGAGACACTGCAGATAGATACCTAGCTAGATAGATAGGTAGATAGATAGAT
AGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGA
TAGATAGATAGATAGATAGATAGGTAGGTAGGTAGGTAGGTAGGTAGG

Dam 34: $(CA)_{26}$
CTCCAACTCACACCTCTTCCTCTCCCCATCCATACACGCACACGCACACACAC
ACACACACACACACACACACACACACACACACACACACACACACACACACTC
AATCACTCAGGGGCAGTGGTAAAATGGAAATCACAAGAACAACTAG

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Publications

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