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Although socially monogamous, both male and female Purple Martins, *Progne subis subis*, seek extra-pair copulations (EPCs) resulting in multiple-sired broods. Studies have attempted to explain this behavior, yet the evolutionary mechanisms driving this mating strategy are not yet known. I tested several predictions of the genetic relatedness hypothesis proposed as evolutionary mechanisms that may drive EPC behavior. I determined paternity and genetic relatedness using microsatellite genotypes derived from Purple Martins in a colony at Severna Park, Maryland.

I predicted that all extra-pair offspring would be sired by adult males after their second year of age. I found that extra-pair paternity was not confined to older males. I predicted that older males sing to attract related subadult males. There was no evidence that adult males were recruiting related subadults to achieve indirect genetic benefits. I predicted that females with multiple-sired broods paired to related males seek EPCs leading to extra-pair fertilizations (EPFs) in an attempt to avoid inbreeding. I found no evidence that avoiding related males was involved in EPC-seeking behavior of females. Finally, I predicted that exclusively monogamous females are less related to their social mate than polyandrous females are to their social mate. My findings do not support the hypothesis that any aspect of genetic relatedness is involved in the EPC behavior of female Purple Martins.

EXTRA-PAIR COPULATION-SEEKING BEHAVIOR
IN PURPLE MARTINS, (*PROGNE SUBIS SUBIS*):
THE RELATEDNESS HYPOTHESIS

by
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APPROVAL PAGE

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CHAPTER I

INTRODUCTION

Avian mating behaviors may have evolved in response to obtainable mates and ecological resource availability, i.e. suitable nesting space, safety, and food availability (Gill, 1994). Mate selection is an important means of driving the evolution of these mating systems (Koko et al., 2003). Mating systems, defined by type of sexual union within a pair-bond, include genetic monogamy, polygamy (polygyny and polyandry) and promiscuity (Oring, 1982; Gill, 1994).

Monogamy

In birds, monogamy refers to a male and a female that mate exclusively within their pair-bond with the purpose of raising young either seasonally or for life (Gill, 1994). Both sexes tend to share in nest building and maintenance as well as bi-parental contribution to the care of offspring (Lack, 1968). Few species adopt this mating strategy because both sexes experience reduced reproductive success, as there are a limited number of eggs in which to fertilize (Gill, 1994).

Polygamy – Polygyny and Polyandry

Polygyny is a class of polygamy in which a male will pair with 2 or more females, usually without aiding in the care of offspring while females maintain monogamy throughout the breeding season (Gill, 1994). This is an uncommon mating strategy in birds with only 26% of North American passerines exhibiting the behavior and tends to only occur in few individuals within the species (Ford, 1996). For example, Johnson and Best (1980) documented a single Gray Catbird (*Dumetella carolinensis*) tending to two nests simultaneously. It was hypothesized that this behavior was dependent on resource availability as the male aided in provisioning for all of his offspring (Ford et al., 1996).

Typical polyandrous females maintain a nest or nests of multiple males. This strategy is most noted within the orders Gruiformes (coots, cranes and rails) and Charadriiformes (shorebirds) (Gill, 1994). Oring et al. (1986) explained this type of mating strategy in the Spotted Sandpiper (*Actitis macularia*). Females took on reverse roles as they attracted mates and defended territories as males brooded eggs (Oring, 1986;1997). Thus reproductive success in females is dependent upon the number of mates they attract (Oring, 1986;1997). In the case of Purple Martins, females may have multiple mates but maintain a single nest (Wagner et al., 1996a).

Promiscuity – Leks

Lek species are those species that have adopted promiscuous mating systems in which aggregate males exhibit elaborate courtship displays to attract females (Gill, 1994). Females freely visit leks and mate with chosen males but are obligated to provision

offspring independently (Höglund and Alatalo, 1995; Pizo et al., 2001). The Swallow-Tailed Hummingbird (*Eupetomena macroura*) is one of 29 hummingbird lek species that seemingly adopted this mating strategy because of easy access to, and abundance of, food sources (Pizo et al., 2001).

Extra-Pair Copulation-Seeking Behavior – A Mixed Mating Strategy

One type of mating system that incorporates some degree of previously described mating systems is extra-pair copulation-seeking behavior resulting in a mixed mating strategy. Referred to as social monogamy, both parents provision offspring but actively seek copulations outside of their pair-bond that may result in extra-pair fertilizations (Lack, 1968).

Extra-pair copulation-seeking behavior is a common reproductive strategy particularly in socially monogamous birds (Birkhead and Møller, 1992; Charmantier et al., 2004). Of the more than 9,000 species of birds, nearly 90% are considered socially monogamous rather than genetically monogamous (Lack, 1968). Like many socially monogamous species, bi-parental care of nestlings is characteristic in Purple Martins (Doughty & Fergus, 2002).

One common theoretical approach in determining ultimate mechanisms involving mixed mating strategies is to examine behaviors in the context of sexual selection (Trivers, 1976). Males and females may pursue different mating strategies as they have markedly different investment in their gametes (Trivers, 1976). Males, producing unlimited quantities of sperm, may maximize the number of fertilizations by fertilizing as

many eggs as possible. Females producing limited numbers of eggs may seek fertilizations from highest quality males (Jennions and Petrie, 2000). Furthermore, sub-optimal males who are cuckolded by dominant males should attempt to nest in proximity to high quality males to increase their inclusive fitness (Wagner et al., 1996A).

Many avian species seek extra-pair copulations, particularly if they are colonial, leading to extra-pair fertilizations resulting in multiple-sired broods (Lack, 1968; Jennions and Petrie, 2000). Males may mate outside of pair-bonds increasing the likelihood of spreading their genes (Jennions and Petrie, 2000). Female motivation may be more selective, based on preference of a specific primary or secondary trait (Jennions and Petrie, 2000). Thus, females paired to sub-optimal males should seek extra-pair copulations from superior quality males (Jennions and Petrie, 2000). Regardless of mechanism, resulting benefits are genetic, increasing fitness for both sexes (Jennions and Petrie, 2000).

Study Animal

Socially monogamous Eastern Purple Martins (*Progne subis subis*) are the largest avian species in the swallow family, Hirundinidae (Doughty and Fergus, 2002). Like most dimorphic passerines, both sexes have delayed plumage maturation not reaching prime pigmentation until after their second year (Pyle, 1997) referred to as older, adults or ASY throughout this paper. Males and females less than two years will be referred to as younger, subadults, second year or SY.

Eastern Purple Martins winter in the neo-tropics east of the Andes from northern

South America to southern Bolivia and southeast Brazil (Doughty and Fergus, 2002).

Purple Martins have a delayed breeding season (Figure 1) lasting about 60 days from onset of pair-bonding until their fledglings become independent (Pyle, 1997).

Purple Martins are exclusive cavity nesters, but are dependent on human “landlords” to provide artificial housing. Multiple-compartmented condominium houses or hollowed out gourds atop secured poles provide breeding territories for this colonial species (Doughty and Fergus, 2002). Typically, colony formation consists of older adult males arriving initially to the nesting site and establishing territories within gourds or compartments of a condominium complex (Wagner et al., 1996). Older females arrive secondly and engage in pair-bonding with older males (Morton et al., 1990). After breeding, and while females brood their clutches, adult males sing during pre-dawn hours (“dawnsong”) attracting migrating subadult males and females to the colony (Morton, 1987). Males, and females who have not begun to lay eggs, may actively seek extra-pair copulations.

Previous Hypothesis of Extra-Pair Copulation-Seeking Behavior in Purple Martins

Female Purple Martins may seek extra-pair copulations to obtain “good genes” (Møller et al., 1994). This hypothesis is an extension of the “hot shot” model (Beehler et al., 1988). Females solicit extra-pair copulations from available high quality males, receiving superlative genes (Beehler et al., 1988). Superior males may exhibit phenotypic characters favorable to females (Hamilton and Zuk, 1982). To date, however, there is no evidence that female Purple Martins seek males to increase the likelihood of

receiving a superior genetic contribution to their offspring. The only test of this hypothesis involved the concept that ASY males who achieve extra-pair fertilizations had specific genetic profiles in accordance with parasite immunity (Davidar and Morton, 1992). It was found that *Haemoproteus prong*, a blood parasite ubiquitous in Purple Martin colonies, (Davidar and Morton, 1992) was not a factor in the pattern of EPFs (Wagner et al., 1997). In fact, while maintaining a high degree of paternity, adult males had greater parasite loads than subadult males (Wagner et al., 1997). There is also no evidence that adult male fertility entices females to pursue extra-pair copulations as a mechanism to ensure fertilization if she is pair-bonded to a subadult (Wagner et al., 1996b).

Females may experience forced extra-pair copulations when the number of males in the colony is disproportionate to females (Westneat et al., 2003). Unmated males known as “floaters” may travel from colony to colony seeking unmated or widowed females (Westneat et al., 2003). However, in my study colony, each male was assigned to a female social mate, indicative of a lack of “floaters” about the colony (Wagner et al., 1996a). Also, there were no instances where paternity could not be assigned further dismissing the possibility that lingering males sired any offspring.

As more males join a colony, females that lay eggs later would have more genetic mates from which to choose. However, there is no statistical correlation between egg-laying dates and paternity (Wagner et al., 1996a). In previous studies, older males achieved 100% of paternity, whereas late-arriving subadult males only attained 50% (Wagner et al., 1996a).

A Female may actively seek extra-pair copulations, particularly if she is paired with a young, SY male (Morton et al., 1990). DNA fingerprinting analysis was used to substantiate male age class correlation to paternity (Morton et al., 1990). This was confirmed in a previous study where 43% of offspring assigned to subadult males had actually been sired by older, ASY males (Wagner et al., 1996a). Only 4% of older social males had broods that were multiple-sired (Wagner et al., 1996a).

Current Hypothesis of Extra-Pair Copulation-Seeking Behavior in Purple Martins

Hidden Leks and Female Preference

One of the mixed mating strategies adopted by Purple Martins is the hidden lek (Wagner et al., 1998; Tarof et al., 2004). Tarof et al. (2004) described hidden leks as being similar to traditional leks but less obvious because of territory size and bi-parental care. The hidden lek hypothesis predicts that extra-pair copulation-seeking females actively pursue clusters of socially monogamous males, which is analogous to females seeking promiscuous copulations at a traditional lek (Tarof et al., 2004).

Hidden lek evolution is based on two evolutionary models: the hotshot model (Beehler and Foster, 1988) and the female preference model (Bradbury, 1981). The “good genes” hypothesis is an extension of the hotshot model in that females choose to mate with older males that have a higher genetic quality than their younger counterparts (Trivers, 1972; Davidar and Morton, 1992; Wagner et al., 1997). Hotshot males may be preferred over other males as they exhibit some phenotypic trait that specifies their genetic superiority (Höglund and Alatalo, 1995). As females congregate around hotshot

males, other males are attracted to the hidden lek (Tarof, 2004). Although late-arriving males will probably be cuckolded, they will have the opportunity to be chosen as a mate and achieve partial paternity (Wagner et al., 1996a). The female preference model predicts that females favor males in collective groups over solitary males (Bradbury, 1981; Tarof, 2004).

Genetic Relatedness Hypothesis

As described in the hidden lek hypothesis, females base their mate choice on male cues, particular traits or characteristics (Trivers, 1972; Davidar and Morton, 1992; Wagner et al., 1997). In this study I investigated relatedness as a cue driving the evolution of extra-pair copulation-seeking behavior in Purple Martins. The genetic relatedness hypothesis predicts that females choose genetically dissimilar males to sire their offspring reducing the negative effects of inbreeding by increasing offspring viability, heterozygosity and overall fitness (Bloomqvst et al., 2002). Thuman and Griffith (2005) tested this hypothesis on the basis of sperm competition in a polyandrous shorebird species, *Philomachus pugnax*. The results gave evidence that females cryptically chose sperm from genetically dissimilar males increasing genetic diversity of offspring.

The genetic relatedness hypothesis also predicts that adult males allow related subadult males to nest in close proximity in order to receive indirect genetic benefits. Although the mechanism of kin recognition between males is not understood in Purple

Martins, it is possible that subtle song differences among adult males' "dawnsongs" are recognizable by related males (Morton 1985, Sharp et al., 2005).

The genetic relatedness hypothesis is difficult to test directly because it is not possible to measure the long term genetic diversity and fitness that a female may achieve by seeking extra-pair fertilizations. For example, to accurately test the prediction that extra-pair fertilizations lead to increased genetic diversity, one would have to demonstrate that a female who obtained EPFs both increased the genetic diversity of her brood relative to the genetic diversity of her brood if she did not obtain extra-pair fertilizations. It would have to be shown that the long-term fitness is higher as a result of extra-pair fertilizations. However, there are several predictions that can be tested if extra-pair fertilization-seeking behavior is driven by the benefits of increased genetic diversity in Purple Martins. I tested four of these predictions as described below.

Four Predictions of the Genetic Relatedness Hypotheses:

1) All extra-pair paternity is confined to older, ASY males.

In a previous study of this same colony of Purple Martins, Wagner et al. (1995) found that females pair-bonded to younger, SY males, frequently sought extra-pair copulations almost exclusively from older males. This study was based on minisatellite and RAPD paternity identification methods. My study, in contrast, is based on single locus markers (microsatellite repeats). Confirmation of this hypothesis would validate the consistency between the different methods used in the previous study and my study. If the results are inconsistent, it will be necessary to evaluate the power of each approach

for paternity assignment. Because microsatellites are single locus markers and are potentially much more informative for both paternity assignment and measurements of genetic variability, the evaluation of the consistency between the different types of markers may be highly informative for future studies of parentage and genetic diversity in this species.

2) Pioneering, older, ASY males are attracting related, younger, SY males to the colony.

By attracting related males to the colony, the pioneering, older male martins would have indirect means of gene dispersal as pair-bond establishment and fertilization occurs.

3) Females avoid genetically similar mates and actively seek extra-pair copulations leading to extra-pair fertilizations with less related males.

Females may greatly decrease negative effects of inbreeding by increasing offspring viability, heterozygosity and overall genetic diversity. In contrast, exclusively monogamous females should be less related to their social mates. The support for this hypothesis has been mixed. For example, Blomqvist et al. (2002) examined genetic parentage of three species of shorebirds, Western Sandpipers (*Calidris mauri*), Common Sandpipers (*Actitis hypoleuca*) and Kentish Plovers (*Charadrius alexandrinus*). The study found, in all 3 species, that females with multiple-sired broods were closely related to their social mates. By contrast, Lane et al. investigated relatedness in North American

red squirrels (*Tamiasciurus hudsonicus*). Although females mate multiply, there was no evidence that relatedness influenced parentage (Lane et al., 2006).

4) Females that are socially and genetically monogamous are less related to their social mates than females that are polyandrous.

Monogamy refers to an exclusive pair-bond with a single member of the opposite sex lasting throughout a breeding season or during the course of a lifetime (Gill, 1994). In Purple Martins, females may be exclusively monogamous even though her social mate may seek EPCs outside of their pair-bond (Gill, 1994). Under the genetic relatedness hypothesis, those females socially bonded with less related males have no cause to seek extra-pair copulations and should elect their social mates as their sole genetic mates. Although broods are not multiple-sired, genetic diversity should be the product of mating with less related or unrelated males. In contrast those females that have any degree of relatedness to their social mates should seek extra-pair copulations. Therefore the number of extra-pair offspring should be greater than within-pair offspring.

Methods Used for Testing the Predictions of the Genetic Relatedness Hypothesis

All of these predictions rely on the ability to determine paternity as well as overall genetic relatedness between particular individuals. The most accurate means of determining paternity and quantifying relatedness is to use DNA- based markers that are highly variable and unlikely to be influenced by natural selection. Previous studies of the Severna, Maryland Purple Martin colony determined paternity using VNTR

(minisatellite) DNA fingerprinting and RAPDs (Wagner et al., 1996b). These genetic markers are either multi-locus (VNTRs) or dominantly inherited (RAPDs). Although they are excellent for paternity assignment, they are not ideal for estimating genetic relatedness. One of the major goals of my study was to identify highly polymorphic single locus DNA markers (microsatellites) that are inherited as codominant loci, and are thus highly reliable for both paternity assignment and quantification of genetic relatedness (Queller and Goodnight, 1989; Blouin et al., 1996). My study reports both the results of microsatellite identification in the genome of Purple Martins and tests predictions of the genetic relatedness hypotheses using these highly variable DNA markers.

CHAPTER II

METHODS

Field Methods

I studied a colony (19 nests) of Purple Martins from Severna Park, Maryland consisting of three condominiums each with twenty-four compartments atop 4.3-meter poles (Morton 1987; Morton et al., 1990). The closest colony was nearly 5 km away and was not observed to have influenced the population density of the Severna Park colony (Wagner et al., 1996b). During the breeding season of 1993, prior to my involvement in this study, blood samples from all adults and nestlings were taken by Dr. Richard Wagner of the Konrad Lorenz Institute for Ethology and Dr. Eugene Morton of the Smithsonian Institution (Wagner et al., 1996a). Procedures for collections and animal handling were described in Wagner et al. (1996a). Based on observations that most eggs had hatched, birds were trapped in a single night using custom-made traps that covered cavity entrances (Morton and Paterson, 1983). Blood samples were taken via jugular venipuncture, separated by centrifugation and stored in PBS buffer (Wagner et al., 1996a). Adult birds were banded in accordance with the bylaws set forth by the National Geological Survey Breeding Bird Laboratory (Wagner et al., 1996a). Color leg bands were applied to all adults for quick visual identification and behavioral observations made throughout the breeding season, i.e. social mates, EPCs, etc. (Wagner et al., 1996a).

Age Class Assignment

Based on field data, male age class was recorded as second year (SY) or after the second year (ASY). Ages were recorded either based on degree of plumage pigmentation or actual leg banding information (Wagner et al., 1996a).

Laboratory Methods

DNA Microsatellite Library Development

I used a Dynabead® enrichment, hybridization capture approach to isolate microsatellite loci by cloning small fragments of genomic DNA and hybridizing fragments with two different oligonucleotide probes of tandem repeats (Mix #2 and Mix #3) (Table 1) (Glenn and Schable, 2005). I sequenced DNA fragments, scanned them for simple-sequence repeats (microsatellites), and designed oligonucleotide primers for those with repeats to amplify in multiple individuals using the polymerase chain reaction (PCR). Loci that amplified consistently and were polymorphic in a sample of 10 adult individuals were used in the remainder of this study.

DNA Sampling

DNA from all samples was extracted and suspended in TE previous to this study. I quantified DNA concentrations using a Nano-Drop® spectrophotometer and uniformly diluted all stock samples to 25ng/μL. From the colony, I was able to assay 20 of 23 males, 21 of 23 females and 69 of 87 offspring.

Restriction Enzyme Digest and Linker Ligation

DNA (5-25 μ g) from a single adult bird (ID number 21-01) was cut into fragments between 200bp and 500bp using 4-cutter restriction enzymes HaeIII and RsaI (Glenn and Schable, 2005) leaving blunt ends. The 95 μ L reaction consisted of 80.5 μ L (280ng μ L / DNA (approximately 22.54 μ g), 1 μ L 100X BSA, 2 μ L HaeIII, 2 μ L RsaI and 9.5 μ L 10X Buffer (1X final concentration). I incubated the sample for three hours at 37°C then proceeded to ligate linkers onto fragment ends.

I used USB Ligate-IT™ to ligate double-stranded linkers (ds SuperSNX linkers) onto fragments' blunt ends. Linkers of known sequences provided primer-binding sites for PCR in later steps. The forward primer, SuperSNX24 (Table 2) consisted of twenty-four bases including a GTTT "pig-tail" at the 5' end that facilitated non-template adenine addition by Taq polymerase during PCR (Glenn and Schable, 2005). The reverse primer, SuperSNX24+4P (Table 2), was complimentary to the forward primer but also included a poly-A tail on the 3' end that allowed for TA cloning in the later stages of microsatellite library development (Glenn and Schable, 2005).

Double stranded SuperSNX linkers were prepared for a ligation reaction by mixing equal volumes of forward and reverse, single-stranded primers consisting of 100 μ L of 10 μ M each. To the mixture, 4 μ L of 5M NaCl was added then heated to 95°C. The mixture was cooled slowly to room temperature to form, dsSuperSNX linkers (Glenn and Schable, 2005).

To ligate linkers, I combined 8.15 μ L (2 μ g) of digested DNA, 5.85 μ L of dsSuperSNX linkers, 4 μ L of 5X Ligate-IT™ reaction buffer and 1 μ L of Ligate-IT™

DNA ligase. The samples were mixed gently and allowed to incubate at room temperature for 5 minutes at which time 1 μ L XmnI restriction enzyme was added, allowed to continue incubating for another 5 minutes then immediately placed on ice. XmnI prevented linker dimerization during the ligation process. To test the efficiency of the ligation I performed PCR on a small aliquot of linker-ligated fragments in a 25 μ L reaction that consisted of 2 μ L of template, 2.5 μ L 10X buffer (2.5ng final concentration), 2.5 μ L BSA (25 μ g/mL final concentration), 1.5 μ L dNTP's (150 μ M final concentration), 1.3 μ L superSNX-24 forward (0.5 μ M final concentration), 2 μ L MgCl₂ (2.0mM final concentration), 13.0 μ L dH₂O and 0.2 μ L Taq polymerase (5 units/ μ L). Thermocycler conditions were 95°C for five minutes, ramp down to 70°C, step down 0.2°C every five seconds until 50°C, remain at 50°C for ten minutes, ramp down 0.5°C every five seconds until 40°C, quickly ramp down and held at 15°C. A 10 μ L aliquot of PCR product was ran on a 1% electrophoresis gel stained with ethidium bromide at 100 volts for one hour then viewed under UV light using a Bio-Rad gel imager (Bio-Rad Laboratories, Inc.).

Dynabead ® Enrichments/Oligonucleotide Probe Hybridization

I performed hybridizations of linker-ligated DNA fragments with biotinylated oligonucleotide probes, labeled with biotin at the 3' end (Table 1). To two samples, one for each oligonucleotide probe, (mixes #2 and #3 respectively), I added 10 μ L of oligo probes, 5 μ L of dH₂O and 25 μ L of 2X hybridization solution (12XSSC, 0.2% SDS). The oligonucleotides in the mixes hybridized with DNA fragments that contained the same repeats in a step-down thermocycler program; 95°C denature for five minutes, ramp down

to 70°C, step down 0.2°C every five seconds until 50°C, remain at 50°C for ten minutes, ramp down 0.5°C every five seconds until 40°C, quickly ramp down and held at 15°C. The hybridized samples were captured using Dynabeads®. Dynabeads® are magnetic beads coated with Streptavidin which has an affinity for biotin. Fragments that successfully hybridized with a biotinylated probe were captured by a magnet (Magnetic Particle Concentrator). I washed 50µL of Dynabeads® twice with 200µL of TE buffer (10mM tris pH8, 2 mM EDTA), twice with 250µL of 1X hybridization solution (6X SSC, 0.1 % SDS) and resuspended in 150µL of TE buffer. I added 50µL of washed Dynabeads® to 50µL of DNA/probe mixtures and shook them at room temperature for 30 minutes. I discarded the supernatant from both samples, which contained non-hybridized fragments. I washed each sample twice in 400µL 2X SCC, 0.1% SDS, twice with 400µL 1X SCC, 0.1 % SDS and twice with 400µL 1X SCC, 0.1% SDS at 45°C. I captured beads using the magnet after each washing step and discarded the supernatant. After a final wash, 200µL of TLE was added to the fragment/bead mixtures and incubated at 95°C for five minutes. I captured the Dynabeads, leaving behind supernatant that contained fragments with microsatellites. The supernatant was quickly removed and placed in a 1.5 mL centrifuge tube.

Enriched DNA Recovery

I performed PCR on the enriched DNA to ensure that supernatant contained enough fragments for further analysis. Both 25µL reactions contained 2µL of eluded DNA fragments, 2.5µL 10X buffer (1X final concentration), 2.5µL BSA (25µg/mL final

concentration), 1.5 μL dNTPs (0.15 mM final concentration), 1.3 μL superSNX-24 forward (0.5 μM final), 2 μL MgCl_2 (2.0mM final concentration), 13 μL dH_2O , 0.2 μL Taq polymerase (5 units/ μL). Thermocycler conditions were: denaturing at 95°C for 2 minutes then 25 cycles of 95°C for 20 seconds, 60°C for 20, 72°C for 1.5 minutes. After cycling, 72°C for 30 minutes then held at 15°C. A 4 μL aliquot of PCR product was run on a 1% electrophoresis gel stained with ethidium bromide and viewed in the Bio-Rad gel imager to check for recovery. The original template, rather than the PCR, was used for a second enrichment and the steps of hybridization were repeated. This further reduced the retention of non-hybridized fragments. PCR product from the second enrichment was used in the cloning step of library development as it contained only amplified microsatellite-containing fragments.

Cloning

I cloned DNA fragments containing microsatellites using a Topo-TA Cloning® kit from Invitrogen Corporation. First, a vector ligation was set up by adding 1 μL of PCR product, 1 μL of salt solution, 3 μL of dH_2O along with 1 μL of TOPO® Vector, incubated for five minutes at room temperature and placed on ice. One vial of One Shot® Competent Topo vector cells was thawed on ice. After thawing, 2 μL of cloning reaction product was added to the vial, mixed gently and incubated on ice for five minutes. I heat-shocked the vial in a 42°C water bath for thirty seconds then immediately placed the vial on ice. I added 250 μL of room temperature S.O.C. medium to the vial, mixed, and incubated at 37°C for one hour. I then spread approximately 50 μL of

bacterial culture on pre-warmed (37°C), agar plates that contained 50µg/ml ampicillin and incubated overnight at 37°C. Ampicillin was necessary to prevent bacteria without plasmids from growing on the agar plates.

I picked individual colonies, placed each in a well of a 96 well microtiter plate containing 100µL of LB broth with 50µg/µL of ampicillin and incubated overnight at 37°C. I inoculated LB/colony samples by adding 10µL of sample from each titer well to 3mls of LB and shook them at 300 RPM overnight at 37°C.

I used the Wizard® Plus Miniprep DNA purification kit by Promega Corporation to isolate plasmid DNA and purify inoculated bacterial colonies ($n=200$). Using reagents supplied and following protocol published by Promega, I first spun approximately 1mL of each sample into pellets via centrifugation, discarded supernatant and resuspended each pellet in 250µL of TE solution. To each sample I added 250µL of cell lysis solution, inverted to mix then added 250µL of neutralization solution and inverted once more to mix. Supernatant containing plasmid DNA was captured via spin column and resuspended in 100µL of nuclease free water. I then quantified the DNA using a Nano-Drop® spectrophotometer and diluted samples to 10ng/µL.

DNA Sequencing

I used a Li-Cor 4200 DNA sequencer and cycle sequencing to determine the DNA sequence of each cloned fragment. I used a Thermo Sequenase® kit (Epicentre Biotechnologies) and the M13 Universal reverse primer labeled with 700IRD (infrared dye). For sequencing reactions I used 8µL of template plasmid DNA with 1.5µL M13

700IRD dye-labeled reverse primer. To the samples, I added 7.2 μ L 3.5X buffer, 1 μ L DNA polymerase and 2.3 μ L distilled water to create 20 μ L reactions. I then performed cycle sequencing using the following thermocycler conditions: 92°C for 2 minutes, 92°C for 30 seconds, 54°C for 30 seconds, 70°C for 1 minute. Steps 2 through 4 were repeated for a total of 30 cycles and held at 4°C.

I aliquotted 2 μ L of each reagent (G,A,T,C), one in each well for 4 total wells per sample, into a labeled 96 well titer plate. To each well, I added 4 μ L of template and 3 μ L of stop solution/loading dye. I denatured the samples along with 50-750bp size marker at 95°C for 3 minutes and then placed them on ice. I then loaded 1.0 μ L of each sample onto a 41cm, 6.5% polyacrylamide Long Ranger® gel and performed electrophoresis for 6 hrs. I analyzed sequences using BioEdit 7.0.5 (Hall, 1998) and edited only sequences that contained microsatellite repeats by removing vector, M13 700 IRD reverse and linker sequences. For 29 microsatellite-containing sequences that long repeats (6 or more), I designed forward and reverse primers through Primer3 (Rosen and Skaletsky, 2000). Forward primers had an addition sequence at the 5' end that corresponded to a universal primer sequence (Table 3).

Polymorphic Loci Identification and Genotyping

I tested 29 loci for amplification (Table 4) with sequence-specific primer pair in PCR using 10 random adult birds. I identified sequences as polymorphic when half were heterozygous.

I performed PCR for each putative polymorphic microsatellite locus ($n=29$) in

10 μ L reactions that consisted of 0.5 μ L template DNA (12.5ng), 1 μ L 10X buffer (1X final concentration), 1 μ L dNTPs (0.1mM final concentration), 0.5 μ L of either M13 700 or M13 800 forward primer (1.0 pmol final concentration) (Table 3), 0.5 μ L of locus-specific forward primer (5 μ M final concentration), 5 μ L of locus-specific reverse primer (5 μ M final concentration), 2.68 μ L MgCl₂ (approximately 0.15 mM final concentration), 3.12 μ L ddH₂O, 0.2 μ L Taq polymerase (5units/ μ L). Thermocycler conditions were as follows: 95°C for 5 minutes, 95°C for 45 seconds, 68°C for 5 minutes 72°C for 1 minute, increments thereafter consisted of denaturing at 95°C for 45 seconds, then step down in increments of 2°C for 5 minutes and 72°C elongation until 50°C, then 24 cycles consisting of 95°C for 45 seconds, 50°C for 2 minutes, 72°C for 1 minute, 72°C for 5 minutes, then held at 4°C. I diluted samples 1:4 by using 1 μ L template to 3 μ L stop solution/loading dye. I denatured the samples and size standards at 95°, placed them on ice and loaded 1microliter into a 24 cm 6.5% polyacrylamide Long Ranger® gel. After two hours of electrophoresis, I scored polymorphic loci using Gene ImagIR™ software. I then used primers for each locus (Table 5) that produced sharp bands to genotype the entire Purple Martin population.

I performed population level genotyping in 10 μ L reactions as described above. Genotypes were scored and profiles compiled for each bird across all 3 polymorphic loci using Gene ImagIR™ software.

Statistical Analysis

Genetic Parameters

I used Cervus 3.0 to quantify allele frequencies, allele sizes (F), observed heterozygosity (H_{obs}) and expected heterozygosity (H_{exp}) at each locus based on adult genotypes (Table 6) (Marshall, 1998; Kalinowski et al., 2007). I also tested for Hardy-Weinberg Equilibrium (HW) using chi-square goodness of fit tests (Marshall, 1998; Kalinowski et al., 2007).

Paternity Assignment

I assigned paternity using LOD scores, the natural logarithm of the likelihood ratio of the probability that a male is the actual sire to the probability of any random male being the true sire (Appendix C) (Marshall, 1998; Kalinowski et al., 2007). Assignment was based on allele frequencies of adults given the genotypes of known mother ($n=21$), her offspring and candidate father ($n=20$) trios (Marshall, 1998; Kalinowski et al., 2007). A positive LOD score indicated that the putative sire was more likely to be the father compared to any random male. A LOD score of zero signified that the candidate father was equally as likely to be the father as any random male in the population. Negative LOD scores indicated the male assigned paternity is less likely to be the sire than any random male.

Before paternity could be assigned, Cervus calculated critical LOD scores based on adult genotypes and 10,000 simulated offspring (Marshall, 1998; Kalinowski et al., 2007). Critical LOD scores of a strict limit of 95% and relaxed limit of 70% were

calculated. Higher relaxed LOD scores were tried, but paternity could not be assigned to all offspring, thus the relaxed level was lowered to 70%.

EPC and EPF Evaluation

I calculated rates of EPC-seeking behavior among males and females as a simple percentage. Ratios for females were number of mixed broods to total number of females. Ratios for males were total number of males that were extra-pair sires to total number of males.

Prediction 1: All extra-pair paternity is confined to older, ASY males.

After assigning age classes based on field data, I calculated offspring sired by both adult and subadult males as the ratio of offspring assigned to each respective age class to total offspring in the colony. I calculated extra-pair paternity achieved as the ratio of the sum of extra-pair offspring per that age group to total number of extra-pair offspring. I used Relatedness 5.0.4 (Queller and Goodnight, 1989; 1999; 2006) to calculate an index of relatedness between pairs of adults for each locus and averaged across all loci. Standard errors and confidence intervals were calculated by jackknifing (Queller and Goodnight, 1989; 1999; 2006). This method resampled data, chosen at random, to create a new matrix to correct for possible bias (Tukey, 1958).

Prediction 2: Pioneering, older ASY males are attracting related, younger SY males to the colony.

I first compiled pair-wise relatedness values for, but not between, ASY and SY males. This consisted of all pair-wise relatedness values among ASY males only combined with all pair-wise relatedness values among SY only males into a single category ($n=59$). I then tested this group for differences between pair-wise relatedness of 1) the whole population which consisted of pair-wise relatedness values among females as well as between ASY and SY males ($n=110$, 259 pair-wise comparisons), 2) females ($n=21$, 194 pair-wise comparisons), 3) ASY males ($n=15$, 139 pair-wise comparisons and 4) SY males ($n=4$, 6 pair-wise comparisons). I tested for normality in the distribution of relatedness estimates within each group using the Anderson-Darling test. I performed Student *t*-tests for all groups as the data were approximately normally distributed and had similar variances. Group “Whole Population” and group “Females” were not normally distributed. Group “Females” was normalized via Box-Cox transformation ($\lambda=0.31$), however normalization was not achieved for group “Whole Population”. Mann Whitney U tests were implemented for these 2 groups as well as a test between ASY/SY Males and group “Females”.

Prediction 3: Females avoid genetically similar mates and actively seek extra-pair copulations leading to extra-pair fertilizations with less related males.

I extracted and categorized relatedness estimates for; pairs of females with mixed brood and their social mates compared to the mean relatedness of the same females and

their genetic mate(s). The Anderson-Darling test established normal distribution thus a paired *t*-test was performed.

Prediction 4: Females that are socially and genetically monogamous are less related to their social mates than females that are genetically polyandrous.

I first calculated the number of social offspring and extra-pair offspring for each of the 19 females. I extracted pair-wise relatedness values between monogamous females and their social mates ($n=4$) versus genetically polyandrous females and their social mates ($n=15$). I then compared relatedness values between the two groups. Both groups were normally distributed per Anderson-Darling tests and a Student *t*-test was performed.

CHAPTER III

RESULTS

Microsatellite Isolation

I identified 191 DNA sequences that potentially contained microsatellites from the enriched subgenomic DNA library. I found 48 sequences that contained tandem repeats. Thus the total percentage of microsatellites identified from the subgenomic DNA library was 25% of the cloned DNA fragments. Of these, 19 had either single nucleotide repeats or the repeat was too close to either of the fragment's ends and made primer design impossible. The remaining 29 loci ranged from 162-460bp and contained repeats that were: di-nucleotide ($n=7$), tri-nucleotide ($n=2$), tetra-nucleotide ($n=15$), penta-nucleotide ($n=2$), hexa-nucleotide ($n=1$), deca-nucleotide ($n=1$), and a 21 base-long, icosikaihena-nucleotide repeat ($n=1$) and one locus that had both a hexanucleotide and a trinucleotide repeat (Table 4). Of the 29 microsatellite sequences, 9 were monomorphic, and 10 amplified inconsistently. Of the remaining 10 loci, 3 (*PUMA19*, *PUMA49* and *PUMA98*) were tetra-nucleotide repeats (Table 5), produced sharp bands and consistently amplified by PCR. Genetic variation at all three of these loci was high, ranging from $H_{exp} = 0.77$ to 0.83 in a sample of 41 adult Purple Martins chosen randomly from the 1993 colony.

Locus *PUMA19* deviated from Hardy-Weinberg Equilibrium expectations ($\chi^2 =$

28.99, $p=0.00006$, 3d.f.). Neither *PUMA49* ($\chi^2=1.254$, $p=0.7329$, 3.d.f.) nor *PUMA98* ($\chi^2=4.662$, $p=0.1983$, 3d.f.) deviated from Hardy-Weinberg Equilibrium expectations. *PUMA19*'s deviation may have been due to null alleles (Ckisi et al., 2003). Null alleles, probably due to mutations at primer binding sites, did not amplify during PCR and resulted in excessive homozygotes (Ckisi et al., 2003; Kalinowski et al., 2006).

Paternity Assignment

Because a small number of loci were identified ($n=3$), I evaluated paternity using both the strict and relaxed confidence levels. Critical LOD scores were 4.74 at the strict confidence level of 95% and 2.58 at the relaxed confidence level of 70%. Of the total males in the colony in 1993 ($n=21$), I had DNA samples for 20. Two males had social mates, but no offspring. Only 2 of the 69 trio LOD scores were negative suggesting that in all but 2 cases, paternity was more likely to be the assigned male rather than any random male (Marshall 1998; Kalinowski et al., 2007). Out of the 69 offspring, 3 were assigned a sire at 95% strict confidence, 43 were assigned at relaxed confidence of 70% and 23 were assigned sires below the 70% relaxed critical LOD score (Appendix C).

Paternity assignments should ideally be at a minimum level of 95% confidence, which requires more polymorphic loci than I was able to identify in my enriched subgenomic DNA library screen and sequencing of 191 cloned fragments. Based on the levels of heterozygosity observed at the three loci I did assay, I estimate that a minimum number of 10 microsatellite loci would be sufficient to determine paternity at the strict 95% confidence level required to rigorously test the four predictions of the

genetic relatedness hypothesis. This would require sequencing at least 400 more clones, and was not feasible for this study. I thus based all of my analysis on the paternity assignments for all 69 offspring even though most were below the 95% threshold. Consequently all of the results were interpreted with the caveat that paternity assignments were not necessarily strongly supported by the statistical analysis and were viewed as 'suggestive' rather than definitive for testing the predictions.

Using these relaxed criteria for paternity assignment, 17 of the 19 nests analyzed (89.5%) had offspring with multiple sires. Thus, 2 of the 19 nests (10.5%) contained non-mixed broods (genetically monogamous social mates).

EPC and EPF Evaluation

Rates of extra-pair copulations that lead to extra-pair fertilizations among males and females were high for both males and females (Figure 2). Nearly all of the males successfully mated with more than one female and more than 2/3 of the females had multiply sired broods (Figure 2).

Rate of Return

The rate of return for adult birds ($n=32$) and subadult birds ($n=9$), from the previous year (1992) was calculated. Although 21 of 32 adults (65.6%) were returns from 1992, there were no SY returns.

Prediction 1: All extra-pair paternity is confined to older, ASY males.

Of the 69 offspring I tested for paternity, 28 were from extra-pair matings. Of those 28, adult males sired 22 and subadult males sired 6 (Figure 3). Although older, ASY males achieved most of the extra-pair paternity it was not confined exclusively to older males.

Prediction 2: Pioneering older, ASY males are attracting related, younger, SY males to the colony.

I predicted that old, ASY males that pioneered the colony attracted related young, SY males as an indirect means of increasing inclusive fitness. A test of this prediction required a comparison of the genetic relatedness between old, ASY and young, SY males to the average relatedness among Purple Martins in general. Average relatedness was measured by quantifying genetic relatedness between all adult individuals in the colony. However, this average included the relatedness between ASY and SY birds in addition to all other adult birds. I thus compared average relatedness of ASY with SY males only to the average relatedness of whole population, females, ASY males only and SY males only excluding pair-wise comparisons between ASY and SY males.

I found no significant difference between ASY/SY relatedness and any of the four groups: ASY/SY compared to Whole Population, $t=0.13$, $p=0.901$, $d.f.=294$; ASY/SY compared to Females, $t=-.026$, $p=0.797$, $d.f.=307$; ASY/SY compared to ASY males, $t=-0.09$, $p=0.932$, $d.f.=281$; ASY/SY compared to SY males, $t=0.90$, $p=0.402$, $d.f.=6$. Results for the Mann Whitney U test were as follows: ASY/SY compared to Whole

Population, $p=0.6608$ and for ASY/SY compared to Females, $p=0.7373$. There were no significant differences between the means of ASY/SY and the other groups (Figure 4).

The results demonstrate that adult and subadult males do not differ from any sample of individuals within the colony and thus do not support the prediction that ASY males are recruiting related SY males to the colony.

Prediction 3: Females avoid genetically similar mates and actively seek extra-pair copulations leading to extra-pair fertilizations with less related males.

There is no evidence that females with multiple-sired broods are extra-pair mating with less-related males. Of the 15 females with mixed broods, extra-pair male relatedness values varied greatly and were not consistently lower or higher than relatedness to socially-mated males (Figure 5).

I also predicted that females with multiple-sired broods are more closely related to their social mates than to their genetic mate(s), the extra-pair male(s) that sired some or all of their offspring. Microsatellite genotypes were assayed to determine relatedness between females (with mixed brood) with their social mate and females with their genetic mate(s).

Both groups “Females and their Social Mates” and “Females and their Genetic Mate(s)” were normally distributed and a one-tailed, paired Student t -test was performed to determine if females were more closely related to their social mates than their genetic mate(s). To perform the paired Student t -test I averaged the relatedness values of all extra-pair males for each female and paired it with the relatedness value of the social

male for each female.

The mean relatedness between females and their social mates ($n=15$) was 0.033 whereas the mean relatedness between females and their genetic mates ($n=26$) was 0.0903 (Figure 6). Thus the average relatedness was higher between females and genetic males, opposite of my prediction. However, the difference between the two groups was not statistically significant (paired Student $t = -0.88$, $p = 0.393$).

Prediction 4: Females that are socially and genetically monogamous are less related to their social mates than females that are polyandrous.

I first calculated the number of social offspring and extra-pair offspring for each of the 15 females (Figure 7). I then predicted that females that were exclusively monogamous would be less related to their social mates compared to those females that were polyandrous. An average relatedness of monogamous females with their social mates was 0.076 whereas the average relatedness between polyandrous females was 0.046 (Figure 8). There was no significant difference in relatedness between the two groups ($t=0.18$, $p=0.861$, $d.f.=18$).

CHAPTER IV

DISCUSSION

My results do not support the genetic relatedness hypothesis. I found that 1) females paired to young, SY males did not necessarily seek extra-pair fertilizations from older, ASY males and it is thus unlikely that females are seeking extra-pair copulations from older males who have "good genes"; 2) Females were not more closely related to their social mate than they were to their genetic mate(s) and are thus not likely seeking extra-pair copulations to avoid inbreeding; 3) There is no evidence that older, ASY males are recruiting related, younger, SY males to the colony; and 4) Monogamous females are not more closely related their social mates compared to polyandrous females and their social mates.

A substantial component of this study was to identify and characterize highly polymorphic, single locus genetic markers for paternity assignment and to quantify genetic relatedness. I first discuss the results and analysis of these genetic markers and the implications for their use in my study of Purple Martin breeding biology and then discuss the analytic approach and limitations based on the hypotheses I tested. Finally, I discuss the results of my hypothesis tests using these genetic markers and their implications for future studies of paternity and genetic relatedness in Purple Martins.

Microsatellite Isolation, Characterization and Analytic Considerations

In my enriched, subgenomic DNA library 15% (29/191) of cloned fragments contained microsatellites (Table 4). Although the addition of more loci to the library would have increased its usefulness, the 3 loci I did isolate were highly variable, robust and proved to be an accurate means of paternity assignment and relatedness estimation.

The presence of one locus that deviated significantly from Hardy-Weinberg Equilibrium expectations was likely due to the presence of one or more null alleles, a common phenomenon at microsatellite loci that is usually due to a single nucleotide polymorphism within the region flanking the microsatellite at which the primer anneals (Kalinowski and Taper, 2006). When this occurs, some individuals who are heterozygous at a particular locus appear as homozygous because the alternative allele is not amplified (Kalinowski and Taper, 2006). Because there are more homozygotes than are expected under Hardy-Weinberg Equilibrium, the observed number of observed homozygotes is inflated, as was probably the case for *PUMA19*. In population studies, frequencies can be adjusted (Kalinowski and Taper, 2006) to account for the presence of a null allele by changing the number of heterozygotes in the sample data to the number expected under Hardy-Weinberg Equilibrium. However, the assignment of heterozygote genotypes is done randomly using this correction. In my study, paternity assignment required that the precise genotype of each individual be known. Thus, a correction was not possible.

Ideally, in a paternity assignment study, one would omit any locus that deviates from Hardy-Weinberg Equilibrium expectations from the analysis. However, I only had

3 loci available for the analysis, and thus chose to use the locus, ignoring the potential that some homozygous individuals may be heterozygous.

Both the presence of a locus that deviated from Hardy-Weinberg Equilibrium expectations and the low LOD score threshold that I used for paternity assignments lowered the probability of correct paternity assignment. In fact, in comparison to a previous study that used VNTR and RAPD loci to assign paternity for the same samples, my results were substantially different. Wagner et al. (1996a) found that 8% of the nests contained multiple-sired broods and 5% of ASY males had mixed broods. In contrast, my study found that 90% of nests contained multiple-sired broods and 55% of the ASY males had mixed broods. Because the Wagner et al. (1996a) study used a larger number of loci, and I used a much lower confidence level to assign paternity, it is likely that my results are skewed toward misassignment of offspring to males outside the socially-bonded pair. My results should thus be interpreted with this in mind. They do however show that the identification of microsatellites and their use for paternity analysis and relatedness is promising and will be an excellent tool for multiple levels of analysis in Purple Martin evolutionary ecology studies. In particular, VNTR and RAPD analysis does not produce a reliable analysis of relatedness among groups as VNTRs are multi-locus and RAPDs are dominant genetic markers.

Population size was not a factor in paternity assignment as there is a limited number of gourds and nesting compartments. However, if genetic data were available for several colonies of the same year or the same colony over multiple years' paternity in Purple Martins as a species may have been more predictable.

Prediction 1: All extra-pair paternity is confined to older, ASY males.

According to paternity assignment data, subadults sired some extra-pair offspring, thus this prediction is not supported for this colony in this particular year. Contrary to my results, Wagner et al., (1996a) determined that extra-pair paternity was confined to older males within the same study colony of the same year (1993). These mixed results were probably due to the limitations I encountered during the course of my study discussed further in “Study Limitations” below. However, further analysis with a larger set of single locus microsatellite DNA markers will be required to assess the inconsistencies between the studies. If my current results are supported it will mean that the previous results should be re-evaluated in light of new evidence that demonstrates that extra-pair paternity is not confined to ASY males. Regardless the majority of extra-pair fertilizations in my study were, in fact, from older, ASY males indicating that even though extra-pair mating may not be exclusive to older males, females do show a preference that may be explained by evolutionary mechanisms.

Prediction 2: Pioneering older, ASY males are attracting related, younger SY males to the colony.

I found no evidence to support older, ASY males recruiting related, SY males. This suggests that older males are not attempting to attract younger related males to the colony to increase their inclusive fitness. This prediction has been tested in at least one other study of the túngara frog, *Physalaemus pustulosus*, in which seven polymorphic microsatellite loci were used to determine if there was a relatedness component to mate

selection (Lampert et al., 2006). It was postulated that the pioneering males were calling to related males to join a lek. It was further predicted that females were choosing less-related males based on acoustic cues (Lampert et al., 2006). However the average relatedness calculated among the males was too low to consider them related (Lampert et al., 2006). There was no correlation between female mate choice and relatedness to the male (153 pairs; Lampert et al., 2006).

Prediction 3: Females avoid genetically similar mates and actively seek extra-pair copulations leading to extra-pair fertilizations with less related males.

The prediction that females choose mates based on their relatedness is not supported by my results. Other studies have had similar results. For example, Tarvin et al. (2005) concluded that Fairy-Wrens, *Malurus splendens*, had variable relatedness values within mixed broods. There was no overall genetic similarity between females and their social mates compared to females and their genetic mates (Tarvin et al., 2005). My relatedness values were variable showing that extra-pair sires were no more or less likely to be related to the female than the social mate. On average there was no difference in relatedness between social mates and extra-pair mates.

Prediction 4: Females that are exclusively monogamous are less related to their social mates than females that are genetically polyandrous.

Jennions and Petrie (2000) hypothesized that multiple mating itself is not selected for but females consistently seek higher quality males (“trading up”). Polyandry may be

selected for only if there is a genetic benefit for offspring (Jennions and Petrie, 2000). If relatedness was a factor in brood diversification, Purple Martins would seek genetically dissimilar mates to prevent detrimental effects of inbreeding. However, I found no support for relatedness to explain females' polyandrous behavior.

Study Limitations

My study has limitations. I was bound by the use of three polymorphic loci, one of which failed to meet Hardy-Weinberg Equilibrium expectations. Additional loci may have greatly influenced paternity assignment and relatedness analysis. Unlike the other loci that had 8 or more alleles, there were only 6 alleles associated with *PUMA 19*. Some true alleles probably failed to amplify during PCR (null alleles) resulting in excess homozygotes.

The return rate for adult birds from 1992 to the 1993 colony was 65.6% which could have biased relatedness results among my study groups. However, young, SY birds (4 males and 2 females), were not returns from the previous year thus it is unlikely that any adult was related to the subadults. Wagner et al. (1996b) determined the rate of return for second year birds as less than 10%. Genetic variability at VNTR loci from Wagner et al. (1996b) is among the highest reported for bird species which suggests that relatedness among colony members is not likely.

Currently, Dr. Scott Tarof, a senior postdoctoral researcher at York University in Toronto Canada, is scanning the Purple Martin library for polymorphisms that may have been oversights. He is also looking for loci that may be additions to the library. A more

expansive microsatellite library would lend itself to more extensive future studies.

In addition to improving the quantitative estimates of relatedness and expanding the study to include more individuals in multiple years, one fruitful approach would be to extend the analysis to a closely related species. Purple Martins breed exclusively in man-made gourds or apartment houses which promote coloniality. We do not know if this creates a social lifestyle that promotes the mixed mating strategy (hidden lek) or whether this mating strategy is ancestral and evolved at a time when Purple Martins nested in tree cavities prior to their popularity among humans. Similar studies of related species may shed some light on whether the hidden lek is an ancestral mating strategy or potentially a result of ‘forced’ colonial living. Two such species that may provide insight into the potential ancestral mating strategy are the Gray-Breasted Martin (*Progne chalybea*) and Western Purple Martins (*Progne subis arboricola*) both of which are cavity nesters. If the hidden lek behavior of Purple Martins is not an ancestral mating strategy, it is possible that it may not be adaptive, but is rather a byproduct of a colonial living arrangement. Such a situation may have substantial conservation implications as habitat fragmentation and human intervention influence the direction of mating behavior in this economically important species over long periods of time.

Conclusion

Although Purple Martins from the Severna Park, Maryland colony maintained social monogamy and bi-parental care, polyandry seemed to be the mating strategy for this particular year. Though motive is unknown, females sought extra-pair copulations

leading to extra-pair fertilizations, apparent by brood comprised of mixed offspring. Although males also sought extra-pair copulations, this behavior is not evidence of polygyny as males maintained provisioning of their 'social' offspring. It is possible that mating strategies are plastic in Purple Martins. This colony may have adopted mating behaviors according to environmental stresses and/or change in population structure.

REFERENCES

- Alatalo, R.V., A. Carlson, A. Lundberg and S. Ulfstrand. 1981. The conflict between male polygamy and female monogamy—the case of the Pied Flycatcher *Ficedula hypoleuca*. *American Naturalist*. **117**:738–753
- Atwood, J.L., V.L. Fitz and J.E. Bamesberger. 1991. Temporal patterns of singing activity at leks of the White-bellied Emerald. *Wilson Bulletin*. **103**:373–386.
- Bateson, M. and S. D. Healy. 2005. Comparative evaluation and its implications for mate choice. *Trends in Ecology and Evolution* **20**: 659-664.
- Beck, S.W, L.A.Powell. 2000.Evolution of female mate choice based on male age: are older males better mates? *Evolutionary Ecology Research*. **2**:107-118.
- Beehler B.M., M.S. Foster. 1988. Hotshots, hotspots, and female preference in the organization of lek mating systems. *American Naturalist* **131**: 203-219.
- Berthold, P. and S. B. Terrill. 1991. Recent Advances in Studies of Bird Migration. *Annual Review of Ecology and Systematics* . **22**: 357-78
- Berthold, P. 1993. Bird Migration: A General Survey. Oxford University Press. Oxford, New York, and Tokyo
- Birkhead, T. and AP. Møller. 1992. Sperm competition in birds: evolutionary causes and consequences. Academic Press. London, England.
- Blomqvist, D., M. Andersson, C. Küpper, I.C. Cuthill, J. Kis, R.B. Lanctot, B.K. Sandercock, T. Székely, J. Wallander and B. Kempenaers. 2002. Genetic similarity between mates and extra-pair parentage in three species of shorebirds. *Nature*. **419**:613-615
- Bloom, S.E., M.E. Delany, S.M. Galloway, A.M. Crawford.1993. Constant and variable features of avian chromosomes. Manipulation of the avian genome. CRC Press, Boca Raton, Fl. Pp 39-60,
- Blouin, M.S., M. Parsons, V. Lacaille, S. Lotz.1996. Use of microsatellites to classify individual's relatedness. *Molecular Ecology*. **5**:393-401

- Boag, P. T., P.J. Weatherhead and S.M. Yezerinac. 1995. Extra-pair paternity and the opportunity for sexual selection in a socially monogamous bird (*Dendroica petechia*). *Behavioral Ecology and Sociobiology*. **37**:179-188
- Box, George E. P.; Cox, D. R. (1964). An analysis of transformations. *Journal of the Royal Statistical Society*. **26**: 211-246.
- Brase, H.B., C.P. Brase. 2006. Understandable statistics concepts and methods. Eighth Edition. Houghton Mifflin Company. Boston, MA.
- Brown, C. R. 1997. Purple Martin (*Progne subis*). The Birds of North America, No. 287 (A. Poole and F. Gill, eds.). The Academy of Natural Sciences, Philadelphia, PA, and The American Ornithologists' Union, Washington, D.C.
- Castelee, T. Van de, P. Galbusera and E. Matthysen. 2001. A comparison of microsatellite-based pairwise relatedness estimators. *Molecular Ecology*. **10**:1539-1549
- Charmantier, A., J. Blondel, P. Perret and M.M. Lambrechts. 2004. Do extra-pair paternities provide genetic benefits for female blue tits *Parus caeruleus*? *Journal of Avian Biology*. **35**:524-532
- Csiki, I., C.Lam, A. Key, E. Coulter, J.D. Clark, R.M. Pace III, K.G. Smith, D.D.Rhoads. 2003. Genetic variation in black bears in Arkansas and Louisiana using microsatellite DNA markers. *Journal of Mammalogy* **84**:691-701
- Davidar, P. and E. S. Morton. 1993. Living with parasites: prevalence and effects of a blood parasite on survivorship in the Purple Martin. *Auk* **110**: 109-116.
- Dawson, R.J.G., H.L. Gibbs, K.A. Hobson, S.M. Yezerinac. 1997. Isolation of microsatellite DNA markers from a passerine bird, *Dendroica petechia* (the Yellow Warbler), and their use in population studies. *Heredity*. **79**:506-515
- DeWoody, J.A. 2005. Molecular approaches to the study of parentage, and fitness: Practical applications for wild animals. Department of Forestry and Natural Resources. *Journal of wildlife management*. **69**:1400-1418
- Doughty, R., and R. Fergus. 2002. The Purple Martin. University of Texas Press. Volume 52.
- Ellegren, H., 2000. Microsatellite mutations in the germline: implications for evolutionary inference. *Trends in Genetics*. **16**:551-558

- Epstein, M.P., Duren, W.L., and Boehnke, M. (2000) Improved inference of relationships for pairs of individuals. *American Journal of Human Genetics*. **67**:1219-1231.
- Ewan, J. and N. Ewan. 1970. John Banister and His Natural History of Virginia 1678-1692. University of Illinois Press. 1st Edition.
- Foerster, K., K. Delhey, A. Johnson, J.T. Lifjeld, B. Kempenaers. 2003. Females increase offspring heterozygosity and fitness through extra-pair matings. *Nature* **425**: 714-717
- Ford, N. L. 1996. Polyterritorial polygyny in North American passerines. *Journal of Field Ornithology*. **67**:10–16.
- Glenn, T.C. and N.A. Schable. 2005. Isolating microsatellite DNA loci. *Methods in Enzymology*. **395**:202-222
- Gill, F.B. 1994. Ornithology. W.H. Freeman and Company. Second edition. p. 403-424
- Hall, T.A. 1999. BioEdit: A User-friendly Biological Sequence Alignment Editor and Analysis Program for Windows 95/98/NT. *Nucleic Acids Symposium*. **41**:95-98.
- Halliburton R. 2004. Introduction to population genetics. Pearson Prentice Hall. p.58-64
- Hamilton, M. B., E.L. Pincus, A. DiFiore and R.C. Fleischer. 1999. Universal linker and ligation procedures for construction of genomic DNA libraries enriched for microsatellites. *Biotechniques*. **27**:500-507.
- Hamilton, W.D. and M. Zuk 1982. Heritable True Fitness and Bright Birds: A Role for Parasites? *Science*. **218**: 384-387
- Hanley, D., W. Minehart III, D.C. Dearborn. 2007. Documentation of a Polygynous Gray Catbird. *The Wilson Journal of Ornithology* **119**:499–502
- Haydock, J., P.G. Parker and K.N. Rabenold. 1996. Extra-pair paternity uncommon in the cooperatively breeding bi-colored wren. *Behavioral Ecology and Sociobiology*. **38**:1-16
- Höglund, J. and R.V. Alatalo.1995. Leks. Princeton University Press, Princeton, New Jersey.
- Jennions, M. and M. Petrie. 2000. Why do females mate multiply? A review of the genetic benefits. *Biological Review*. **75**:21-64.

- Johnson, E.J. and L.B. Best. 1980. Breeding biology of the Gray Catbird in Iowa. *Iowa State Journal of Research* **55**:171–183.
- Jones, A.G., W. R. Arden.2003. Methods of parentage analysis in natural populations. *Molecular Ecology*. **12**:2511-2523.
- Kalinowski, S.T., M.L. Taper.2006. Maximum likelihood estimation of the frequency of null alleles at microsatellite loci. *Conservation Genetics*. **7**:991-995
- Kalinowski, S.T., M.L. Taper and T.C. Marshall. 2007. Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. *Molecular Ecology*. **16**:1099-1106
- Kirkpatrick, M. 1987. Sexual selection by female choice in polygynous animals. *Annual Review of Ecological Systems*. **18**:43-70
- Kokko, H., R. Brooks, M.D. Jennions, J. Morlwy.2003. Evolution of mate choice and mating biases. *The Royal Society of London Review*. B 270, 653–664. DOI 10.1098/rspb.2002.2235
- Kokko, H. and L.J. Morrell. 2005. Mate guarding, male attractiveness, and paternity under social monogamy. *Behavioral Ecology*. **16**:724-731
- Lack, D. 1968. Ecological adaptations for breeding in birds. Methuen and Co., London, England.
- Lampert, K.P., E.B. Ximena, A.S. Rand, G.M. Ulrich and M.J. Ryan. 2006. No evidence for female mate choice based on genetic similarity in the túngara frog *Physalaemus pustulosus*. *Behavioral Ecology and Sociobiology*. **59**:796-804
- Lane, J.E., S. Boutin, M.R. Gunn., J. Slate and D.W. Coltman. 2007. Genetic relatedness of mates does not predict patterns of parentage in North American red squirrels. *Animal Behaviour*. **74**:611-619.
- Mann, H. B., & Whitney, D. R. (1947). "On a test of whether one of two random variables is stochastically larger than the other". *Annals of Mathematical Statistics*. **18**:50-60
- Manning, J.T. 1985. Choosy females and correlates of male age. *Journal of Theoretical Biology*. **16**:349-354
- Marshall, T.C., J. Slate, L.E.B. Kruuk and M. Pemberton. 1998. Statistical confidence for likelihood-based paternity inference in natural populations. *Molecular Ecology*. **7**(5): 639-655

- Meager, T.R. 1986. Analysis of Paternity within natural populations of *Chamaelirium luteum*. Identification of most-likely male parents. *American Naturalist*. **128**:199-215.
- Møller, A.P. and T.R. Birkhead. 1994. The evolution of plumage brightness in birds is related to the evolution of extrapair paternity. *Evolution*. **48**:1089-1100
- Morton, E. S. 1985. Vocal imitation in a captive Purple Martin. *Wilson Bulletin*. **97**:392-395.
- Morton, E. S. 1987. Variation in mate guarding intensity by male Purple Martins. *Behaviour*. **101**:211-224.
- Morton, E. S. 1988. Dawnsong of the Purple Martin. *Atlantic Naturalist*. **38**:38-48.
- Morton, E. S., L. Forman and M. Braun. 1990. Extrapair fertilizations and the evolution of colonial breeding in Purple Martins. *Auk*. **107**:275-283.
- Morton, E. S. and K. Derrickson. 1990. The biological significance of age-specific return schedules in breeding Purple Martins. *Condor*. **92**:1040-1050.
- Oh, K.P and A. Baydyaev. 2006. Adaptive genetic complementarity in mate choice coexists with selection for elaborate sexual traits. Online publication from The Royal Society. doi:10.1098/rspb
- Oring, L.W. 1982. Avian Mating Systems: A comprehensive review of the literature. *Avian Biology*. **6**:1-91
- Oring, L.W. 1986. Avian Polyandry: A critical analysis of current ideas about this perplexing mating system. *Current Ornithology*. **3**:309-351.
- Oring, L. W., E. M. Gray, and J. M. Reed. 1997. Spotted Sandpiper (*Actitis macularia*). *The Birds of North America*. The Academy of Natural Sciences, Philadelphia, PA, and The American Ornithologists' Union, Washington, D.C.
- Pizo, M.A. and W.R. Silva. 2002. The dawn lek of the Swallow-Tailed Hummingbird. *Wilson Bulletin*. **113**:388-397.
- Queller, D.C. and K.F. Goodnight. 1989. Estimating relatedness using molecular markers. *Evolution*. **43**:258-275.
- Queller, D.C., J.E. Strassman and C.R. Hughes. 1993. Microsatellites and kinship. *Trends in Ecology and Evolution*. **8**(8):285-288

- Queller, D.C. and K.F. Goodnight. 1999. Computer software for performing likelihood tests of pedigree relationship using genetic markers.
- Piertney, S.B., L. Shore, J. Höglund. 2002. Characterization of microsatellite DNA markers in the White-Bearded Manakin (*Manacus manacus*). *Molecular Ecology*. **2**:504-505)
- Primmer, C.R., T. Raudsepp, B.P. Chowdhary, A.P. MØller, H. Ellegren. 1997. Low frequency of microsatellites in the avian genome. *Genome Research*. **7**:471-482.
- Pyle, P. 1997. Identification guide to North American Passerines: a Compendium of Information on Identifying, Ageing, Sexing "Near Passerine" and passerines in the hand. Slate Creek Press. Bolinas, Ca.
- Rosen, S. and H.J. Skaletsky. 2000. Primer3 on the WWW for general users and for biologist programmers. In: S. Krawetz, S. Misener (eds) *Bioinformatics Methods and Protocols: Methods in Molecular Biology*. Humana Press, Totowa, NJ, pp 365-386
- Sambrook, J. and D.W. RUSSELL (2001) "Molecular Cloning: A Laboratory Manual," Cold Spring Harbor Laboratory Press, 1.105-1.125.
- Snedecor, G.W. and W.G. Cochran. 1989. *Statistical Methods*, Eighth Edition, Iowa State University Press.
- Sharp, S.P., A. McGowan, M.J. Wood and B.J Hatchwell. 2005. Learned recognition cues in a social bird. *Nature*. **434**:1127-1130.
- Slate J, T.C. Marshall and J.M. Pemberton. 2000. A retrospective assessment of the accuracy of the paternity inference program CERVUS. *Molecular Ecology*. **9(6)**:801-808
- Tarvin, K.A., M.S. Webster, E.M. Tuttle, S.Pruett-Jones. 2005. Genetic similarity of social mates predicts the level of extrapair paternity in splendid fairy-wrens. *Animal Behavior*. **70**: 945-955
- Théry, M. and S.L. Vehrencamp. 1995. Light patterns as cues for mate choice on the lekking White-throated Manakin (*Corapipo gutturalis*). *Auk*. **112**:133-145.
- Thuman, K.A. and S.C. Griffith. 2005. Genetic similarity and nonrandom distribution in a genetically similar highly polyandrous shorebird. *Animal Behaviour*. **69**:765-770

- Trivers, R.L. 1972. Parental investment and sexual selection: in Sexual selection and the descent of man. The Darwin Pivot. Aldine-Atherton Press, Chicago, Illinois. Pp. 136–179
- Trivers, R.L. 1976. Sexual selection and resource-accurring abilities in *Anolis garmani*. *Evolution*. **30**:253–269.
- Tukey, J.W. 1958. Bias and confidence in not quite large samples (abstract). *The Annals of Mathematical Statistics*. **29**: 614
- Wagner, R. H., M. D. Schug and E. S. Morton. 1996a. Condition-dependent control of paternity by female Purple Martins: implications for coloniality. *Behavioral Ecology and Sociobiology*. **38**:379-389.
- Wagner, R. H., M. D. Schug and E. S. Morton. 1996b. Confidence of paternity and parental effort by Purple Martins. *Animal Behavior*. **52**:123-132
- Wagner, R. H., P. Davidar, M. Schug, and E. S. Morton,. 1997. Do blood parasites affect paternity and provisioning in Purple Martins? *Condor*. **99**:520-523.
- Wagner, R.H. Hidden leks: sexual selection and the clustering of avian territories. 1998. Female and male extra-pair mating tactics in birds. American Ornithologist Union; 123-146
- Waldman, B., M. Tocher. 1998. Behavioral Ecology, Genetic Diversity and Declining Amphibian Populations. In: Caro, T (ed) Behavioral Ecology and Conservation Biology. Oxford University Press, New York. Pp 394-443
- Webster, S.M., H.C. Chuang-Dobbs, R.T. Holmes. 2001. Microsatellite identification of extrapair sires in a socially monogamous warbler. *Behavioral Ecology*. **4**:439-446
- West S.A., A.S. Griffin. 2002. Cooperation and competition between relatives. *Science* **296**:72-75
- Westneat, D.F., I.R.K. Stewart. 2003. Extra-pair paternity in birds: causes, correlates and conflicts. *Annual Review Ecology, Evolution and Systematics*. **34**:365-96
- Wilcoxon, F. (1945). Individual comparisons by ranking methods. *Biometrics Bulletin*.**1**: 80-83
- Wright, S. 1922. Coefficients of inbreeding and relationship. *American Naturalist* **56**:330-338

Zane, L., L. Bargelloni, T. Patarnello. 2002. Strategies for microsatellite isolation: a review. *Molecular Ecology*. **11**:1-16

APPENDIX A: TABLES

Table 1. Biotinylated oligonucleotide probes labeled at the 3' end. These were used to hybridize with DNA fragments that contain one or more of the nucleotide repeats found in the probe mixture.

Oligonucleotide Mix Number	Nucleotides and Numbers of Repeats
2	(AG) ₁₂ , (TG) ₁₂ , (AAC) ₆ , (AAG) ₈ , (AAT) ₁₂ , (ACT) ₁₂ , (ATC) ₈
3	(AAAC) ₆ , (AAAG) ₆ , (AATC) ₆ , (AATG) ₆ , (ACAG) ₆ , (ACCT) ₆ , ACTG) ₆

Table 2. Forward and reverse linkers that provided primer-binding sites for PCR and allowed for fragments to insert into vectors. Highlighted are the GTTT “pigtail” and poly-A tail used to facilitate TA cloning.

Linker Identification	Linker Sequence
SuperSNX24forward	5 ‘GTTT TAA GGC CTA AGC AGA ATC 3’
SuperSNX24+4P reverse	5’ pGAT TCT GCT AGC TAG GCC TTA AAC AAAA 3’

Table 3. Forward and reverse M13 IRD (infrared dye) primers. Reverse primers were used in sequencing reactions. Forward primers were used in genotyping reactions.

M13 Primer	Sequence
M13 IRD 700 and 800 Forward	5'-CACGA CGTTG TAAAA CGAC-3'
M13 IRD 700 and 800 Reverse	5'-GGATAACAATTTACACAGG-3'

Table 4. 29 Loci, repeat motifs and definition of morphology. NA indicates that the locus either failed to amplify via PCR or produced inconsistent genotypes. Those loci shaded in gray were robust and used to assign paternity and estimate relatedness.

LOCUS	REPEAT MOTIF	MORPHOLOGY
PUMA2	(AAT) ₄	POLYMORPHIC
PUMA3	(CTTT) ₆₁	NA
PUMA9	(CAA) ₄	MONOMORPHIC
PUMA10	(GAAA) ₄₃	NA
PUMA13	(GTTGGTTTCTTCTCTTTCTTT) ₁₉	NA
PUMA14	(GAAA) ₂₇	POLYMORPHIC
PUMA16	(CA) ₄ , (CT) ₄	MONOMORPHIC
PUMA19	(AAAC) ₄	POLYMORPHIC
PUMA25	(GAATCACACA) ₃	MONOMORPHIC
PUMA36	(CTTT) ₃₉	MONOMORPHIC
PUMA49	(CAAA) ₉	POLYMORPHIC
PUMA66	(CCTTCT) ₃₆ (CTT) ₁₆	NA
PUMA74	(GAAGA) ₁₉ , (GAAAA) ₂₄	POLYMORPHIC
PUMA78	(TC) ₄ , (TC) ₄	MONOMORPHIC
PUMA82	(TG) ₇ , (TG) ₅	NA
PUMA87	(CA) ₅	NA
PUMA88	(CA) ₆	MONOMORPHIC
PUMA89	(GTTT) ₆	MONOMORPHIC
PUMA91	(GAAA) ₃₁	POLYMORPHIC
PUMA92	(CAAA) ₅	MONOMORPHIC
PUMA98	(GTTT) ₄ , (GTTT) ₅	POLYMORPHIC
PUMA100	(ACA) ₅	NA
PUMA133	(AAAC) ₅	POLYMORPHIC
PUMA141	(AGAA) ₂₀	NA
PUMA147	(CAAA) ₁₁	MONOMORPHIC
PUMA154	(GT) ₁₆	POLYMORPHIC
PUMA155	(GAAA) ₁₀	NA
PUMA159	(TC) ₆	POLYMORPHIC
PUMA160	(TGGTT) ₂	NA

Table 5. A description of the primers that were designed via Primer3 for the 3 microsatellite loci used in genotyping all individuals from the 1993 Purple Martin colony. Forward primers shown with the 5' tail sequence (highlighted) are the M13-700 IRD or M13- 800 IRD (infrared dye) added to the forward primer.

Locus	Repeat Motif	Primer Sequences
<i>PUMA19</i>	(AAAC) ₄	For- CACGACGTTGTAAAACGACACTATGTCATTACCTTCAAGTGG Rev- CTCTTCTCTGCCTCAGGAAACC
<i>PUMA49</i>	(CAAA) ₉	For- CACGACGTTGTAAAACGACAAAACCACAAACAAACACACAAAA Rev- GAAAGAACTTCAAATTCAGGGAAA
<i>PUMA98</i>	(GTTT) ₄ (GTTT) ₅	For- CACGACGTTGTAAAACGACTCTCCTCTCTCTTTCTCTCTCTCG Rev- TACATATGGATTCATGGATTGACC

Table 6. Genetic variation calculated by CERVUS 3.0 based on 41 adult Purple Martins (21 females and 20 males) at three microsatellite loci. The measure of genetic variation consists of allele size range (bp), number of alleles at each locus (F), observed heterozygosity (H_{obs}), expected heterozygosity (H_{exp}). Deviations from Hardy-Weinberg Equilibrium expectations (HW) was also calculated and recorded numerically as (***) if significant and NS if the locus did not deviate from Hardy-Weinberg Equilibrium significantly.

Locus	F	Allele Size ranges (bp)	H_{obs}	H_{exp}	HW
<i>PUMA19</i>	6	216 - 225	0.69	0.766	***
<i>PUMA49</i>	8	218 - 242	0.69	0.819	NS
<i>PUMA98</i>	11	176 - 208	0.929	0.831	NS

*** significant deviation from Hardy-Weinberg Equilibrium at the 0.1% level.

APPENDIX B: FIGURES

Figure 1. Purple Martin migration flyway zones and approximate arrival date to breeding grounds. This map is courtesy of the Purple Martin Conservation Society (2007).

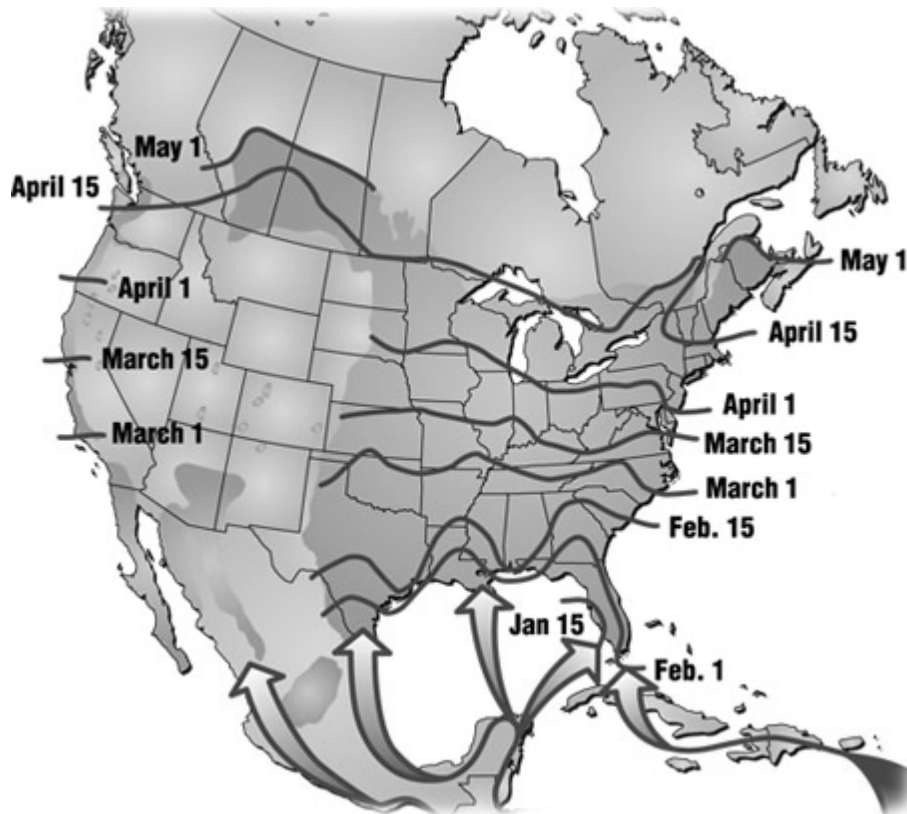


Figure 2. Frequency of extra-pair copulations by social males and females in a colony of Purple Martins in Severna Park, Maryland in 1993. Eighteen of 20 males sought EPCs that led to EPFs while 15 of 21 females sought EPCs that led to EPFs.

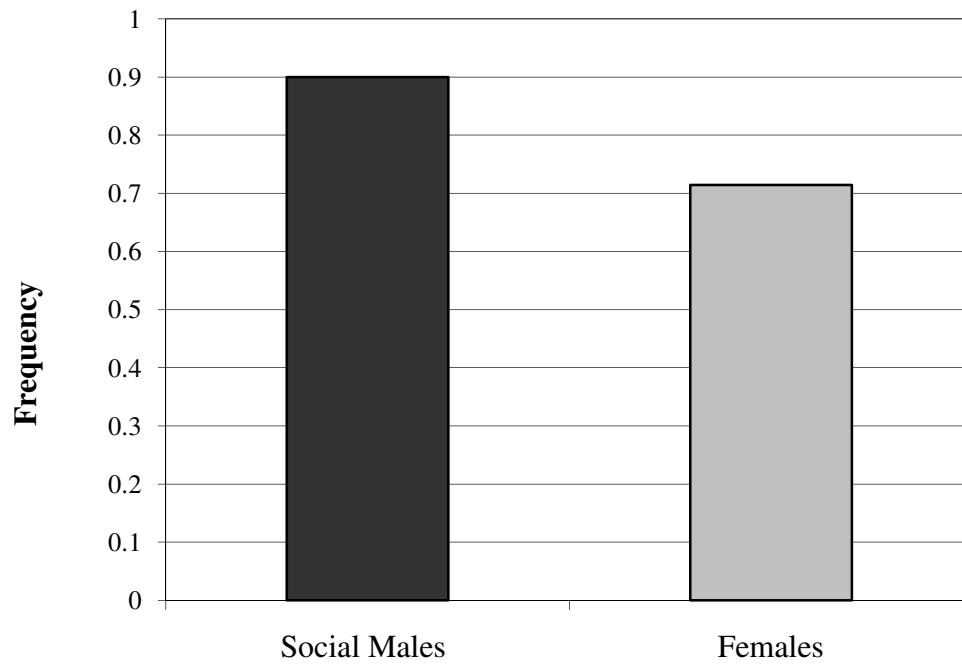


Figure 3. Numbers of offspring sired by, and proportions of, extra-pair offspring by adult (dark columns) and subadult (light columns) males from a colony of Purple Martins in Severna Park, Maryland in 1993.

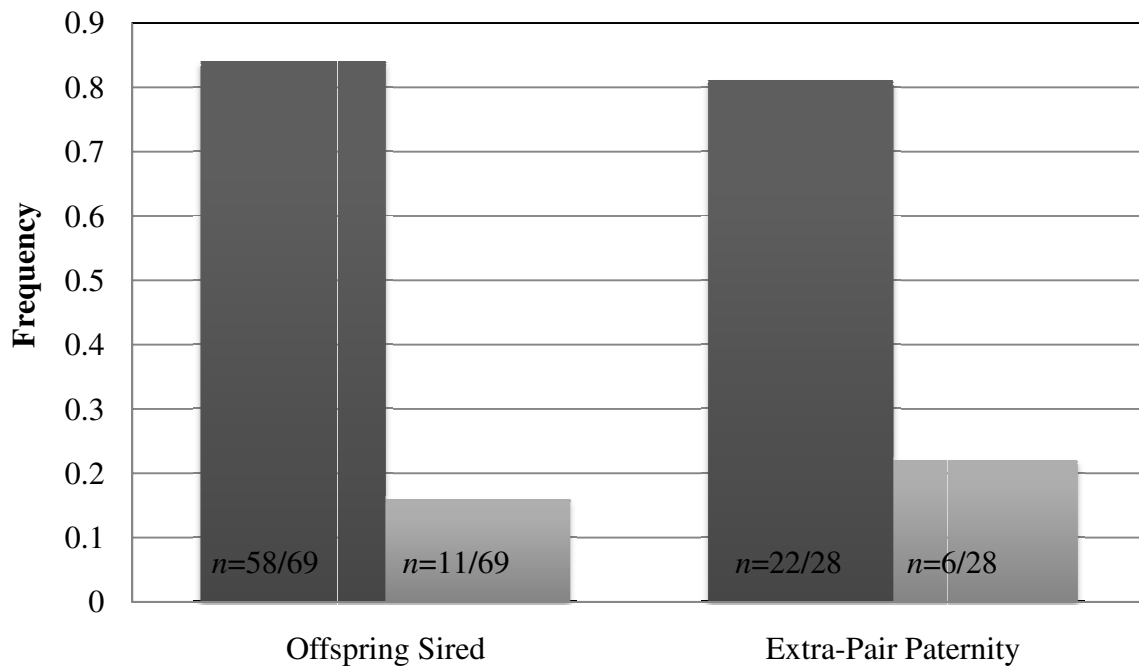


Figure 4. Mean relatedness values of a colony of Purple Martins in Severna Park, Maryland in 1993: ASY and SY males ($\mu=-0.0166$), whole population ($\mu=-0.0312$), females ($\mu=-0.0245$), ASY males only ($\mu=-0.166$) and SY males only ($\mu=-0.0825$).

*Denotes outliers, or extreme values

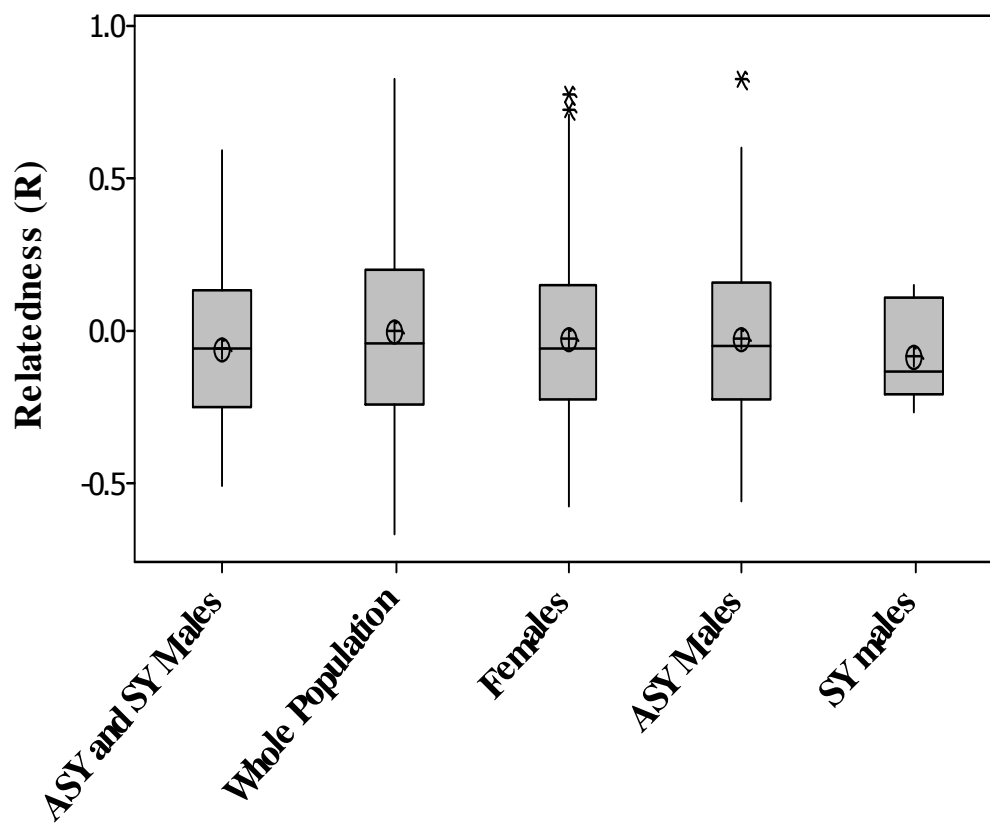


Figure 5. From a colony of Purple Martins in Severna Park, Maryland in 1993, relatedness between 15 females with mixed broods and their social mates (squares) compared to their genetic mate(s) (circles). Cases where the social mate is also a genetic mate, values are plotted twice (circle in a square).

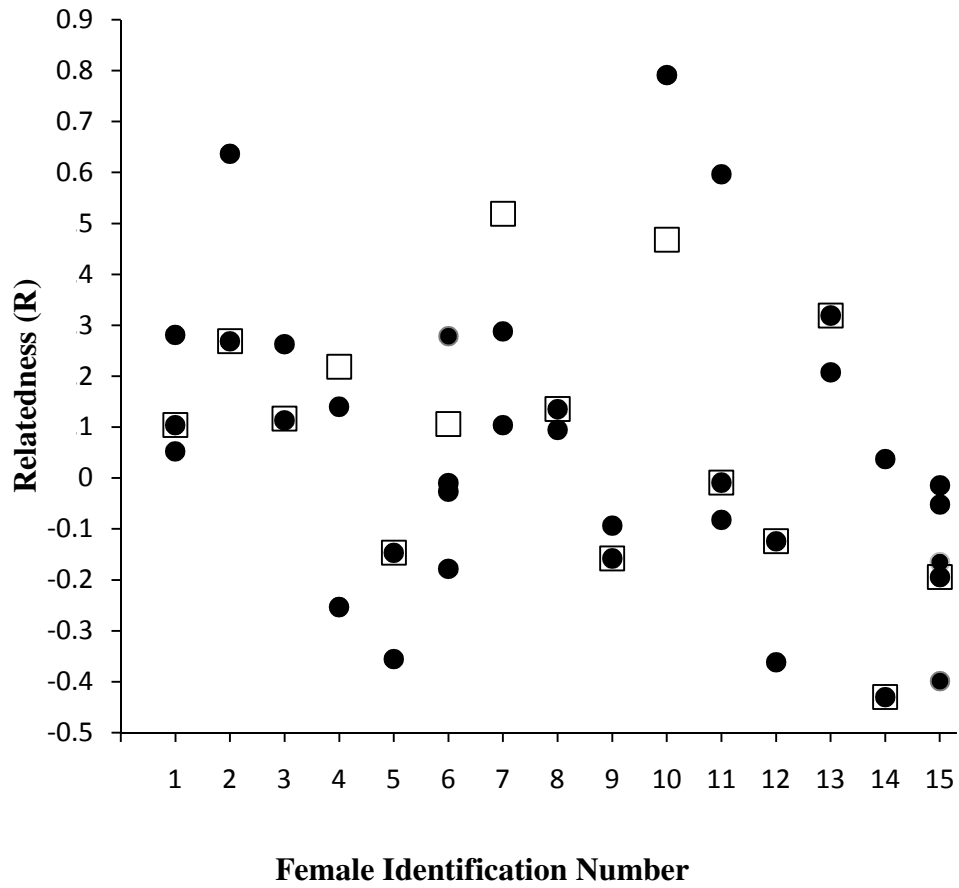


Figure 6. Mean relatedness values of females with mixed brood and their social mates versus females with mixed brood and their genetic mates from a colony of Purple Martins in Severna Park, Maryland in 1993. Means are 0.0333 and 0.0903 respectively.

*Denotes outliers, or extreme values

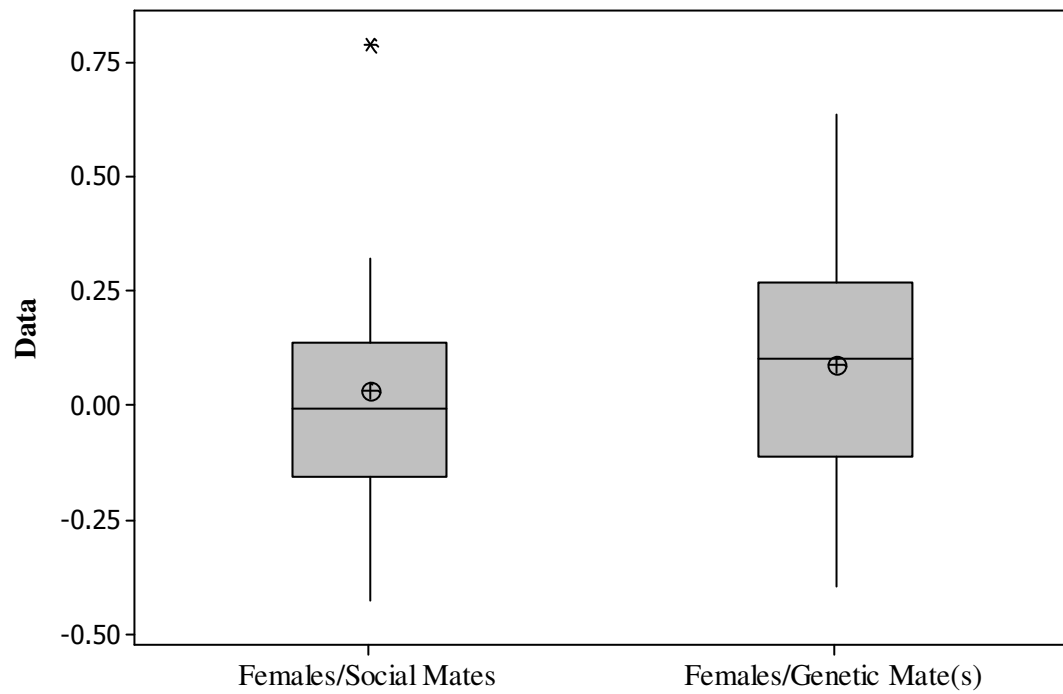


Figure 7. The number of within-pair offspring in black columns are compared to extra-pair offspring in gray columns for each of the 15 females with mixed broods. From a colony of Purple Martins in Severna Park, Maryland in 1993.

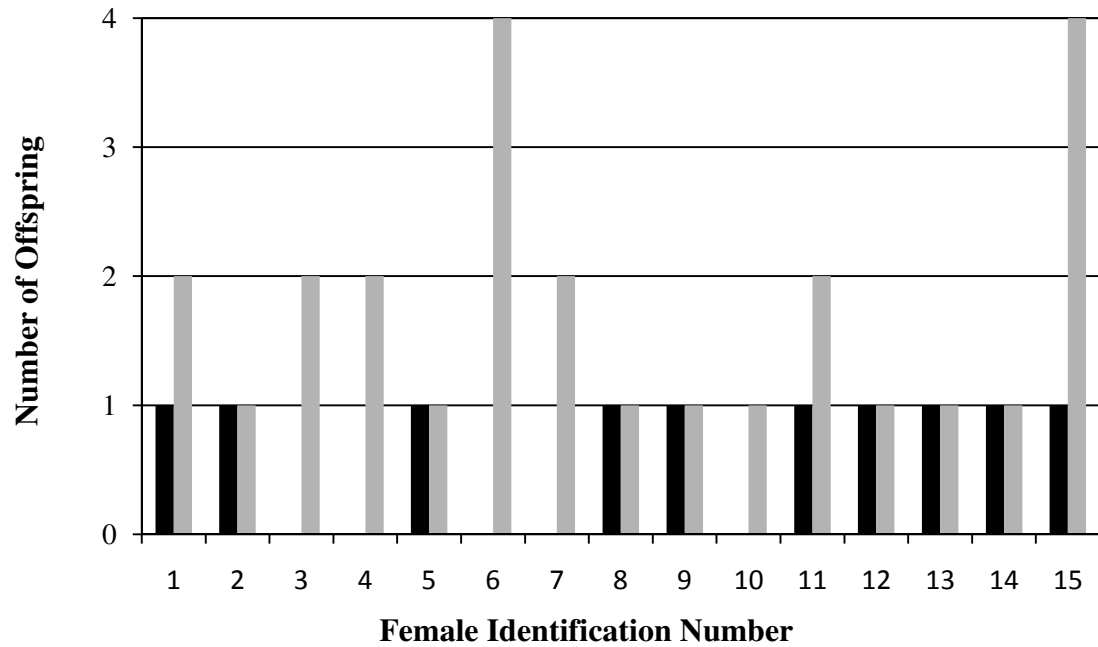
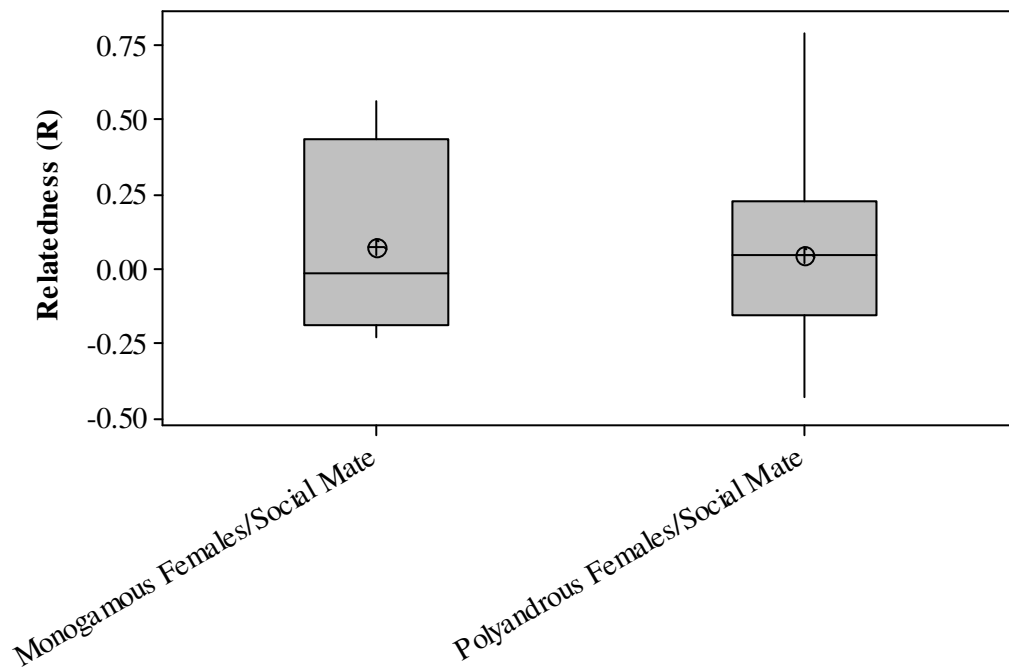


Figure 8. Mean relatedness values of monogamous females and their social mates versus polyandrous females and their social mates from a colony of Purple Martins in Severna Park, Maryland in 1993. Means were 0.076 and 0.046, respectively.

*Denotes outliers, or extreme values



APPENDIX C: PATERNITY ASSIGNMENT

C1. Paternity assignment of 69 offspring from 21 nests (alternating in shade) from a colony of Purple Martins in Severna Park, Maryland in 1993. LOD scores of known mother, her offspring and probable sires. Trio LOD score $\leq 70\%$ CL (“-“), between 70% CL and 95% CL (“+”), $\geq 95\%$ CL (“*”).

Offspring ID	Mother ID	Sire	Trio LOD score	TRIO Confidence	Offspring ID	Mother ID	Sire	Trio LOD score	TRIO Confidence	Offspring ID	Mother ID	Sire	Trio LOD score	TRIO Confidence
O1		F66	2.0164	-	O21		F107	2.7529	+	O124		F93	1.8333	-
O3	16	F66	3.3458	+	O22		F9280	2.1804	-	O125	20	F93	1.8713	-
O4		F66	2.7518	+	O23	6	F97	2.8132	+	O126		F93	1.7871	-
O139		F68	2.739	+	O24		F107	2.8446	+	O127		F93	3.6873	+
O140		F66	2.0141	-	O25		F89	2.2578	-	O128		F97	2.7677	+
O141	1	F89	3.2899	+	O26		F88	3.4402	+	O129	21	F97	4.2422	+
O142		F68	3.4351	+	O28	7	F88	3.4402	+	O131		F97	4.2359	+
O55		F70	3.1552	+	O29		F93	2.3842	-	O148		F99	3.1359	+
O56	2	F70	3.1541	+	O5		F82	4.6688	+	O149	10	F73	2.393	-
O57		F108	1.8605	-	O6		F73	2.0556	-	O150		F88	2.0993	-
O121		F89	2.7242	+	O7	8	F82	4.6752	+	O143	11	F68	1.7983	-
O122	3	F89	2.7242	+	O8		F82	4.6688	+	O146		F93	2.2274	-
O123		F79	2.6722	+	O9		F82	2.6071	+	O31	12	F104	3.8959	+
O132	4	F97	3.6017	+	O15		F91	3.0861	+	O34		F89	2.7306	+
O134		F88	3.5978	+	O16	9	F9280	6.0241	*	O59		F107	2.1382	-
O135		F66	2.7072	+	O19		F9280	6.0241	*	O60	13	F70	-2.8116	-
O136	5	F75	4.4855	+	O20		F9280	6.0385	*	O61		F107	-2.6651	-
O138		F66	2.7072	+	O52		F88	1.835	-	O37	14	F108	3.9607	+
O10		F66	2.6646	+	O53	18	F88	3.4336	+	O38		F70	2.5094	-
O11		F66	2.6646	+	O54		F88	2.0152	-	O39		F114	3.2331	+
O12	17	F66	3.995	+	O117	19	F70	2.9892	+	O40		F70	1.3725	-
O13		F66	1.5776	-	O118		F70	2.9892	+	O41	15	F70	2.2466	-
O14		F66	3.4007	+						O42		F66	2.65	+
										O43		F107	2.8273	+